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Srikumar P. Chellappan *Editor*

Chromatin Protocols

Third Edition

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Chromatin Protocols

Third Edition

Edited by

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H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL, USA

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Cover illustration: Primary rat cortical neurons undergoing apoptosis stained with P-Ser10 Histone H3 and MAP2 antibodies; nuclei were visualized using Hoechst staining. *Protocol described in Chapter 13.*

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Preface

Spectacular advancements have been made in our knowledge of chromatin structure and function in recent years. The recent development of several novel technologies to interrogate various biological processes has impacted the field of chromatin biology in a remarkable fashion. Thus, the ability to sequence large stretches of DNA, the enhanced capabilities to study protein-DNA interactions at very high resolutions, novel techniques to study histone modifications and other epigenetic changes have all shed new light on our understanding of chromatin structure as well as processes like transcriptional regulation, DNA replication and repair. In addition, the ability to edit genes using CRISPR/CAS technology has opened up new avenues and methodologies to study chromatin biology. It has also become increasingly clear that noncoding RNA molecules might play a vital role in the regulation of chromatin organization and function. The third edition of *Chromatin Protocols* compiles many of these techniques.

The first edition of *Chromatin Protocols* that was published in 1999 became the staple of laboratories studying chromatin structure and function. Significant advances in the field as well as development of novel techniques led to the publication of the second edition in 2009, which was received very well by the scientific community. The field has moved at a remarkable pace in the 5 years since the publication of the second edition, calling for a third edition. This edition carries over certain chapters and protocols from the previous edition; these have been updated. Many additional protocols that have been recently developed have been included in the volume. Thus, we have now included protocols for chromatin imaging at a very high resolution; determination of DNA methylation using Illumina BeadChips; identification and characterization of nonhistone chromatin proteins; fluorescent in situ hybridization on comets; an enChIP protocol using CRISPR; analysis of transcription using spFRET, to mention a few. Detailed protocols for these novel techniques, along with the protocols for established and time-tested methods for isolation of nucleosomes, analysis of histone modifications and chromatin function using ChIP assays etc., make this volume a handy source for information needed to study chromatin biology.

This volume is organized such that the initial part of this volume describes techniques related to the study of chromatin structure. Protocols for reconstitution of chromatin on solid supports for analysis, preparation of positioned mononucleosomes, techniques to study premature chromatin condensation, and the use of comparative genomic hybridization to assess genomic aberration are included here. Novel techniques for imaging chromatin using atomic force microscopy and the isolation of specific genomic regions using engineered DNA-binding molecules generated by CRISPR are included here. This section is followed by protocols to analyze DNA and histone modifications; eight different protocols are presented here. The third section includes methods to study DNA replication and repair, in the context of chromatin. Last but not the least, protocols for studying chromatin and its relation with transcriptional regulation are presented in a fourth section.

We believe that this updated edition of *Chromatin Protocols* will be as useful as the first two editions and will facilitate in-depth molecular analysis of various aspects of chromatin structure and function. This volume would not have been possible without the valuable

contributions from a truly international panel of highly talented and accomplished scientists. My sincere thanks to them for taking the time and effort to pen down the intricate details of their favorite techniques and for generously sharing them with the scientific community. I would also like to express my thanks to Dr. John Walker, the Series Editor, without whose valuable input and suggestions this volume would not have taken shape.

Tampa, FL, USA ***Srikumar P. Chellappan***

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Chapter 1

Beads-on-a-String on a Bead: Reconstitution and Analysis of Chromatin on a Solid Support

Raphael Sandaltzopoulos and Peter B. Becker

Abstract

Complex experimental strategies involving in vitro reconstituted chromatin or simple chromatin interaction studies are much facilitated by immobilizing the nucleosomal arrays to paramagnetic beads. Chromatin-containing beads can be retrieved from a reaction mix solution on a magnet fast and quantitatively, effectively separating bound, loosely attached and unbound components efficiently. This chapter details a convenient strategy for immobilization of linear plasmid DNA on streptavidin-coated beads, the reconstitution of chromatin on such beads, and some fundamental handling procedures.

Key words Biotin-streptavidin, Dynabeads, Solid phase, Chromatin assembly, Chromatin constituents

1 Introduction

The value of a solid support was recognized early in history by the ancient Greek engineer Archimedes who, amazed by the power of the leverage machines that he invented, exclaimed that he could even move the entire planet had he only a suitable solid support to rely on. In biochemistry, sophisticated multistep experimental procedures require that a substrate is purified and processed through a sequence of reactions under different optimal conditions. Solid-phase techniques are invaluable because they allow instant and quantitative purification of reaction intermediates and readjustment of new reaction conditions. Here we describe a method for chromatin reconstitution on a solid support and present how solid-phase chromatin can be analyzed or prepared as a substrate in subsequent reactions.

Chromatin reconstitution in crude extracts from *Xenopus* oocytes or eggs or *Drosophila* embryos provides a powerful means to study structure/function relationships in chromatin organization [1–5]. For many of those analyses, e.g., the evaluation of the transcriptional potential of a chromatin template, the chromatin

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must be purified from the complex reconstitution reaction. The most common method for chromatin purification is its centrifugation through a sucrose gradient. Although efficient, this method is time consuming and does not allow parallel processing of many samples and many loosely associated chromatin components that may be of pivotal importance, e.g., for chromatin dynamics, may be lost during the long centrifugation.

By contrast, the solid-phase approach enables the rapid, nondisruptive, and quantitative purification of chromatin. A linear fragment of DNA that bears the sequences of interest (i.e., enhancer/promoter and gene-coding regions) is biotinylated at one end and then immobilized on streptavidin-coated superparamagnetic beads (Subheading 3.1 and Fig. 1). The bead-coupled template is then subjected to chromatin assembly (Subheading 3.2.1). Chromatin assembled on immobilized DNA resembles that of soluble DNA with respect to the optimal reconstitution conditions, kinetics of chromatin assembly, nucleosomal repeat length, histone stoichiometry, association of histone H1, inhibition of transcription, and association of many nonhistone proteins. The immobilized chromatin can then be purified in a magnetic field, washed as desired, and then used to purify and analyze chromatin-associated proteins (Subheading 3.3.1) or to develop assays for putative chromatin-binding proteins. The rapid isolation of immobilized chromatin in a magnetic field facilitates quick buffer exchanges and the efficient removal of soluble components, such as nucleotides or unbound proteins. Therefore, multistep reconstitutions are facilitated, i.e., reactions in which the chromatin reconstitution must be separated from other steps, such as the interaction of transcription factors prior to chromatin assembly or subsequent chromatin "remodeling" reactions (for examples of applications, *see* refs. [6–14]).

We also describe how nucleosomes can be reconstituted from pure histones by a salt gradient dialysis procedure on immobilized DNA (Subheading 3.2.2).

2 Materials

2.1 Immobilization of DNA

1. Streptavidin-coated paramagnetic beads (Dynabeads M-280, Thermo Fisher Scientific, Life Technologies).
2. Magnetic particle concentrator (MPC-6, Thermo Fisher Scientific, Life Technologies).
3. 0.5 mM or 10 mM biotin-21-dUTP in 50 mM Tris–HCl, pH 7.5, (Clontech Laboratories, Inc.) or 0.4 mM biotin-14-dATP in 100 mM Tris–HCl, pH 7.5, 0.1 mM EDTA (Thermo Fisher Scientific, Life Technologies). Biotin-dCTP is also available from the same company but we have not tested it yet.
4. 10 mM α-thio-deoxyribonucleotides, pH 8.0.

5. Restriction enzymes and 10× digestion buffers (according to the supplier's recommendation).
6. Klenow (exo-) polymerase.
7. 10× polymerase buffer: 0.1 M Tris–HCl, pH 7.5, 50 mM $MgCl_2$, 75 mM DTT).
8. VentR (exo-) DNA polymerase (New England Biolabs).
9. 10 mg/ml glycogen (Roche Applied Science).
10. Ethanol.
11. Quick Spin columns TE Sephadex G-50 fine (Roche Applied Science) or ChromaSpin column 50-TE (Clontech Laboratories, Inc.) or equivalent homemade spin columns.
12. PBS-BSA-NP40: 1.7 mM KH_2PO_4, 5 mM Na_2HPO_4, 150 mM NaCl, pH 7.4, 0.05 % (w/v) BSA, 0.05 % v/v Nonidet P40 (NP40).
13. Wash and binding buffer (WB buffer): 2 M NaCl, 50 mM Tris–HCl, pH 8.0, 1 mM EDTA.
14. λDNA (Promega).
15. Kilobase binder reagent (Thermo Fisher Scientific, Life Technologies).
16. Shaker with regulated speed and temperature (e.g., thermomixer, Eppendorf).
17. TE: 10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA.
18. Rotating wheel with regulated speed.

2.2 Chromatin Reconstitution on Immobilized DNA

2.2.1 Reconstitution in Drosophila Embryo Extracts

1. Chromatin assembly extract [4].
2. 0.5 M $MgCl_2$.
3. McNAP mix [4]: 100 mM creatine phosphate, 30 mM ATP, pH 8.0, 10 mM dithiothreitol (DTT), 100 μg/ml creatine phosphokinase.
4. Extract buffer (EX): 10 mM Hepes-KOH, pH 7.6, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10 % v/v glycerol, 10 mM β-glycerophosphate, 1 mM DTT, 0.2 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride, Sigma-Aldrich).
5. Temperature-regulated chamber with integrated rotating wheel (e.g., hybridization oven).

2.2.2 Nucleosome Reconstitution by Salt Gradient Dialysis

1. Core histones.
2. Two peristaltic pumps.
3. Magnetic stirrer and stirrer bars.
4. DB-1: 2 M NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.05 % Nonidet P40, 1 mM β-mercaptoethanol (freshly added).

5. DB-2: 50 mM NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.05 % Nonidet P 40, 1 mM β-mercaptoethanol (freshly added).
6. Dialysis tubing Spectra/Por 2, MWCO 12–14,000, 2 ml/cm.

2.3 Analysis of Reconstituted Chromatin

2.3.1 Analysis of Chromatin Proteins

1. EX-S (EX, *see* Subheading 2.2.1), where S represents the concentration of KCl in mM.
2. EX-50-NP40: Extract buffer supplemented with 50 mM KCl and 0.05 % v/v Nonidet P40.
3. 4× SDS-loading buffer: 200 mM Tris–HCl, pH 6.8, 40 % (v/v) glycerol, 400 mM β-mercaptoethanol, 4 % (w/v) SDS, 0.002 % (w/v) bromophenol blue.
4. Equipment for PAGE.

2.3.2 Micrococcus Nuclease Digestion

1. Micrococcal Nuclease (Roche Applied Science), 50 U/μl in EX buffer.
2. 5× Nuclease stop mix: 2.5 % v/v sarkosyl, 100 mM EDTA, pH 8.0.
3. TE: 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.
4. 5× Orange loading buffer: 50 % v/v glycerol, 5 mM EDTA, pH 8.0, 0.3 % w/v Orange G.
5. Equipment for agarose gel electrophoresis.

3 Methods

3.1 Immobilization of DNA

3.1.1 Digestion of DNA

1. Cleave plasmid DNA with an appropriate pair of restriction enzymes (example is given for ClaI and EcoRI; *see* **Note 1** and Fig. 1) as follows: Mix 40 μl of supercoiled plasmid (1 μg/μl = 40 μg), 5 μl 10× digestion buffer, 5 μl Cla I (10 μ/μl). Incubate for 3 h at 37 °C.
2. Assure complete linearization by analyzing 0.2 μl of the digest by electrophoresis on an 0.8 % agarose gel and staining with ethidium bromide (*see* **Note 2**).
3. Add 50 μl H_2O, 11 μl 10× digestion buffer, and 10 μl EcoRI (10 μ/μl). Adjust the final volume to 160 μl with H_2O. Incubate for 3 h at 37 °C.
4. Precipitate DNA: Add 16 μl 3 M sodium acetate, pH 5.3. Mix. Add 480 μl ethanol, and mix. Incubate for 10 min on ice.
5. Spin 15 min at top speed in a tabletop centrifuge. Discard supernatant.
6. Wash pellet with 800 μl 80 % ethanol. Dry pellet for 2 min in speed vac without heating.
7. Dissolve pellet thoroughly in 40 μl TE.

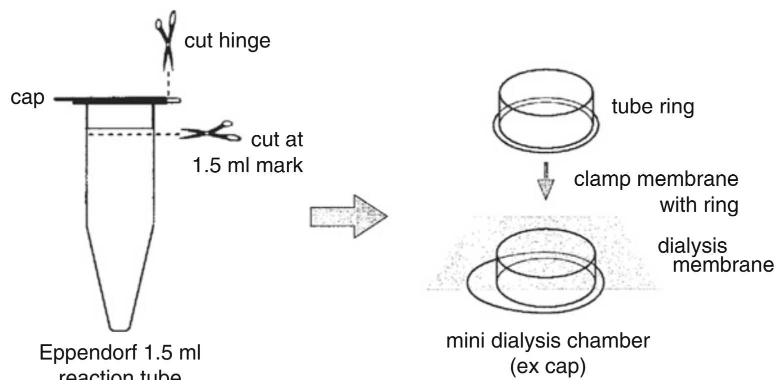

Fig. 1 DNA immobilization strategies (*see* **Note 1**)

3.1.2 Biotinylation of DNA

1. Add 7.5 μl 0.4 mM biotin-14-dATP, 1.2 μl each of 10 mM α-thio-dTTP, 10 mM α-thio-dCTP and 10 mM α-thio-dGTP (*see* **Note 3**), 6.0 μl 10× polymerase buffer, and 3.5 μl of 5 U/μl Klenow (exo-).
2. Incubate for 1 h at 37 °C (*see* **Note 4**).

3.1.3 Removal of Free Biotin (See **Note 5***)*

1. Resuspend the matrix of a Quick spin sephadex G-50 TE spin column. Uncap the top and then the bottom of the column.
2. Place in a reaction tube provided (without lid) and let drain in a vertical position (about 5 min).
3. Empty the reaction tube and put the column (together with the reaction tube) in a 15 ml Falcon tube.
4. Spin for 1 min at 1,100 × *g*.
5. Discard flow-through and spin at 1,100 × *g* for 2 min.
6. Replace the collection tube by a fresh one. Apply the biotinylation reaction slowly at the center of the resin without touching the resin.
7. Spin for 2 min at 1,100 × *g* and collect flow-through. The volume of your sample should stay constant (approx. 60 μl). Measure optical density at 260 nm to define DNA concentration in order to account for the losses during gel filtration. Usually the losses are between 10 and 30 %.
8. Add 200 μl 2 × WB buffer and 140 μl H_2O. This is the coupling mix that is ready to be added to the beads. The final NaCl concentration must be 2 M. Save 1 μl for testing immobilization efficiency.

3.1.4 Coupling of DNA to Dynabeads

1. Resuspend beads well.
2. Remove appropriate amount of bead suspension from the vial. 1 mg of beads (100 μl) is required for the immobilization of 1 pmol of DNA (*see* **Note 6**). For example, since 1 pmol of a

5 Kb DNA fragment is 3.3 μg, 1,210 μl (40/3.3 times 100) of bead suspension is needed to immobilize 40 μg of fragment.

3. Place tube on MPC (magnetic particle concentrator) for 1 min.
4. Discard supernatant.
5. Wash beads in 300 μl PBS-BSA-NP40.
6. Wash beads twice with 300 μl WB.
7. Resuspend beads in coupling mix (*see* Subheading 3.1.3, **step 8**).
8. Rotate at room temperature for at least 3 h or overnight.
9. Concentrate beads and remove supernatant.
10. Check 10 μl of supernatant (equivalent to originally 300 ng) on 0.8 % agarose gel alongside the uncoupled aliquot (*see* **Note 7**).
11. Resuspend DNA beads in WB buffer at a concentration of 30 ng of immobilized DNA/μl of buffer and store at 4 °C. (Under these conditions they can be stored for several months.)

3.1.5 Efficient Immobilization of Very Long DNA (See Note 6)

1. Mix: 300 μl λ DNA (100 μg), 40 μl 10×Vent polymerase buffer, 8 μl 10 mM α-thio dGTP, 8 μl α-thio 10 mM dCTP, 8 μl 10 mM α-thio dATP, 4 μl 10 mM biotin-21-dUTP, 5 μl Vent (exo-) DNA polymerase (2 U/μl), and 27 μl H_2O (total volume is 400 μl).
2. Incubate for 30 min at 76 °C.
3. Add 40 μl 3 M sodium acetate, pH 5.3 and mix gently.
4. Add 1,100 μl absolute ethanol. Mix and incubate for 5 min on ice.
5. Spin for 10 min at top speed in a tabletop centrifuge.
6. Wash pellet twice with 70 % ethanol.
7. Dry and resuspend in 300 μl H_2O (approximately 1 pmol/100 μl) (*see* **Note 8**).
8. Add an equal volume of 2×WB buffer and transfer to equilibrated beads (**steps 1–7** of Subheading 3.1.4). Then add 1/4 of this volume of Dynabeads kilobaseBINDER kit (*see* **Note 9**). Mix gently.
9. Rotate at room temperature overnight.
10. Check immobilization efficiency and store DNA beads as in Subheading 3.1.4, **steps 9–11** (*see* **Note 8**).

3.2 Chromatin Reconstitution on Immobilized DNA

3.2.1 Chromatin Reconstitution Using Drosophila Embryo Extracts

1. Resuspend stock of immobilized template. Pipette out appropriate amount of bead-DNA. 900 ng of DNA is sufficient for an MNase assay or analysis of bound histone by silver staining.
2. Concentrate on the MPC. Remove supernatant and wash once with 300 μl of PBS-BSA-NP40.
3. Wash again with 300 μl EX-NP40.

4. Prepare chromatin assembly reaction by mixing 70 μl chromatin assembly extract, 12 μl McNAP, and 38 μl EX buffer for each 900 ng of DNA (*see* **Note 10**).
5. Concentrate bead-DNA, remove supernatant, and resuspend beads in complete chromatin assembly reaction.
6. Transfer to 250 μl micro test tubes (*see* **Note 11**).
7. Rotate at 26 °C for up to 6 h, the rotation axis being perpendicular to the longitudinal axis of the tube.
8. Check occasionally for aggregation of beads. If necessary disperse clumps by gently tapping the tube. Some clumping may occur during the first 1–2 h of the assembly reaction. If beads are redispersed once, they usually do not clump again.

3.2.2 Chromatin Reconstitution Using Purified Histones

1. During a salt gradient dialysis reconstitution, histones and DNA are first dialyzed into high-salt buffer DB-1 (*see* **Note 12**). During overnight dialysis the salt concentration is reduced by diluting the dialysis buffer with low-salt buffer while keeping the volume of the dialysis constant.
2. Set up a beaker with 600 ml DB-1 buffer at 4 °C and prepare a larger container with 3 L DB-2. Use two peristaltic pumps and appropriate tubing to pump DB-2 at a rate of 3 ml/min into the dialysis beaker containing DB-1 while at the same time pumping the equivalent volume out of the diluted dialysis buffer into a waste container. This setup assures that the volume of the dialysis reaction remains constant while the salt concentration is reduced.
3. Prepare the samples. Mix 60 μl 5 *M* NaC1, 82.25 μl TE, 3.75 μl 20 mg/ml BSA, and 4 μl (0.375 mg/ml) purified core histones (*see* **Note 13**).
4. Prepare the mini-dialysis chamber (Fig. 2). Detach the cap of a 1.5 ml reaction tube by cutting the connecting hinge with a pair of scissors. Cut the remaining tube at the 1.5 ml mark. The cutoff ring will serve as membrane clamp. Cut dialysis membrane (12–14,000 MWCO) to 2 cm × 3 cm pieces. Equilibrate membrane pieces to DB-1 for 30 min.
5. Pipette 50 μl of bead-DNA suspension (30 ng DNA/ml) into a reaction tube. Concentrate the beads on an MPC. Discard supernatant and wash once with 200 ml PBS-BSA-NP40. Wash again with 200 μl of WB buffer (*see* Subheading 2.1). Concentrate beads, discard supernatant, and resuspend beads into the reaction mix prepared in **step 2**.
6. Transfer the suspension into an empty, inverted Eppendorf tube cap. Place a piece of dialysis membrane on top and clamp membrane with the tube ring (*see* Fig. 2). A reaction volume of 150 μl should essentially fill the cap. Avoid trapping air in the cap which will interfere with dialysis.

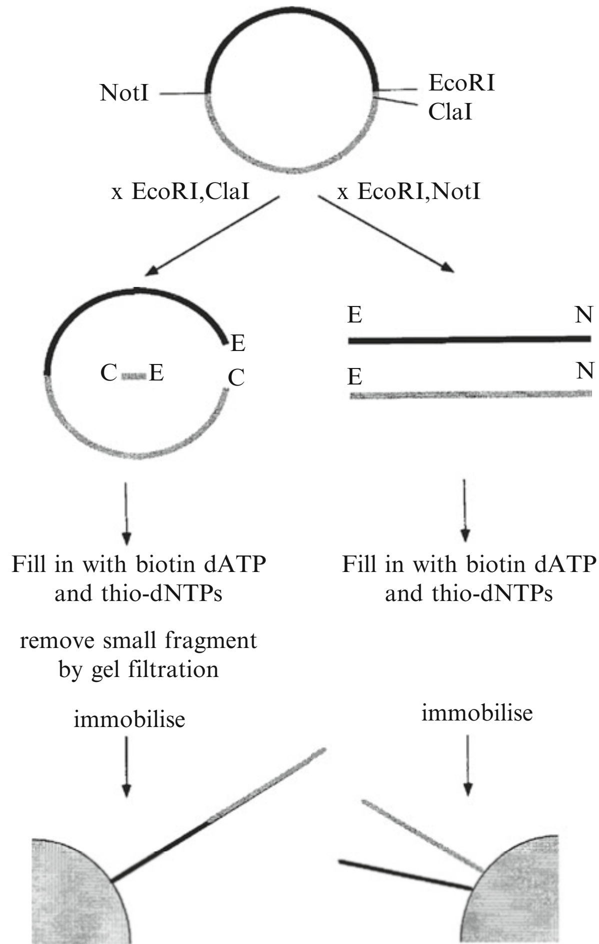

Fig. 2 The preparation of a mini-dialysis chamber (*see* Subheading 3.2.2). The entire dialysis chamber containing the nucleosome reconstitution is thrown into the beaker with dialysis buffer. The tumbling of the chamber due to the vigorous stirring will assure that the beads remain suspended during the 16-h dialysis. We acknowledge the help of Udo Ringeisen in preparing this figure

7. Throw the closed dialysis cap(s) into the dialysis container and start stirring very fast. Vigorous stirring is essential for maintaining beads in suspension.
8. Dialyze for 45 min before you turn on the pumps to dilute the salt concentration. Dialyze until most of DB-2 has been pumped into the dialysis container (15–16 h).
9. Recover bead suspension by puncturing the membrane with a pipette tip. Transfer to a fresh reaction tube and process as desired.

3.3 Analysis of Reconstituted Chromatin

Reconstituted, immobilized chromatin can be purified from the reaction mix and analyzed in various ways. Chromatin proteins can be separated by SDS-PAGE and visualized by Western blotting,

silver staining, or even Coomassie staining, depending on the scale of the reaction. The quality of the reconstituted chromatin can be tested by visualization of the correct histone stoichiometry and a regular nucleosomal array by micrococcal nuclease digestion. Chromatin association of proteins of interest can be tested and the correct stoichiometry of core histones verified.

3.3.1 Analysis of Chromatin Proteins (See Note 14)

1. If a smaller tube was used for chromatin assembly reaction transfer all liquid to Eppendorf tubes that fit into the MPC. The small tube may be rinsed with 100 μl EX-50-NP40 to ensure complete recovery. Concentrate on an MPC for 1 min (not longer!) and remove supernatant completely. Be careful not to touch the pellet with the tip. This may lead to losses.
2. Wash twice with 200 μl EX-50-NP40. Resuspend well each time by gently tapping the tubes. Do not pipette to resuspend. Remove all supernatant each time. If droplets are dispersed on the tube walls spin for 15 s at 1,000 rpm in a benchtop minifuge if necessary.
3. Suspend beads in 7.5 μl of EX-Y1-NP40 for elution (*see* **Note 15**). Concentrate beads and save supernatant. Repeat and pool supernatant for PAGE (total volume 15 μl).
4. Wash beads with 200 μl of EX-Y2-NP40. Discard wash.
5. Proceed to the next salt concentration. Each elution is done by extracting twice in 7.5 μl (save for gel) and a large 200 μl wash (for completeness).
6. Place all samples for PAGE on the MPC for 2–3 min to remove any trapped beads. Recover supernatant into new tubes containing 5 μl 4× SDS loading buffer.
7. Resuspend beads in 20 μl 1× SDS loading buffer. Incubate for 10 min at 37 °C. Do not boil (*see* **Note 16**). Concentrate the beads and save supernatant. This sample represents the proteins that are not eluted even with the most stringent wash applied.
8. Denature all samples for 5 min at 95 °C, and separate by 15 % SDS-PAGE.
9. Stain gel with silver or transfer to membrane for Western blotting.

3.3.2 Micrococcal Nuclease Treatment (See Note 17)

1. Assemble 900 ng of immobilized DNA into chromatin as described in Subheading 3.2.1. Concentrate chromatin on MPC and remove supernatant.
2. Wash chromatin twice with 100 μl of EX-Y-NP40 (*see* **Note 15**).
3. Wash beads with 50 μl EX-50-NP40.
4. Resuspend in 120 μl EX-50-NP40 containing 5 mM $MgCl_2$, prewarmed at 26 °C.

5. Add 180 μl of MNase premix (168 μl EX-50, 9 μl CaCl2, 3 μl MNase (5 μ/μl)) prewarmed at 26 °C (*see* **Note 18**).
6. After 30 s, 1 min, and 8 min recover 100 μl into a tube containing 25 μl of nuclease stop mix and vortex briefly.
7. When all samples are processed, add 1 μl RNase (10 mg/ml) and incubate for 5 min at 37 °C.
8. Add 2 μl 20 % SDS and 5 μl proteinase K (10 mg/ml) and digest overnight at 37 °C.
9. Concentrate beads on MPC and recover supernatant.
10. Add 90 μl 7.5 M ammonium acetate, pH 5.3 and 0.5 μl glycogen 20 mg/ml. Mix and add 2 volumes ethanol.
11. Leave on ice for 5 min and spin for 15 min at top speed in a benchtop centrifuge at 4 °C.
12. Wash pellet carefully with 800 μl of 75 % ethanol and air dry on the bench. Do not dry pellet in the speed vac as this may cause DNA denaturation!
13. Take pellet up in 8 μl TE and add 2 μl Orange loading buffer (5×).
14. Electrophorese on a 1.3 % agarose gel in Tris-glycine buffer ([4]; *see* **Note 19**).

4 Notes

1. In order to immobilize a plasmid two restriction enzymes must be selected as follows (*see* also Fig. 1): The plasmid must be linearized with an enzyme leaving a 5′ overhang that can be filled in with biotin-21-dUTP or biotin-14-dATP with Klenow polymerase. In order to prevent the coupling via both ends (which may result in the shearing of the DNA) the linearized DNA must be restricted with a second enzyme leaving a site where no biotin will be incorporated during the fill-in reaction (e.g., blunt ends, 3′ overhangs, or 5′ overhangs with GC-rich sequences). If the secondary cut results in two large fragments, a mixture of both fragments will be immobilized. If the secondary enzyme is chosen such that one large and one very small fragment is produced, this fragment may be removed during the subsequent gel filtration step (Fig. 1). Ideally, the biotinylated residue should not be the last nucleotide to be incorporated during the fill-in reaction so that it can be protected against exonuclease activity by sealing the ends with α-thio-dNTPs (*see* **Note 3**). Some enzymes that we have used to create an end suitable for biotinylation are EcoRI, SpeI, AflII, HindIII, and SalI. NotI and ClaI can be used for the other end. These enzymes produce 5′ overhang sequences

lacking A or T residues which are not filled in with biotin-14-dATP or biotin-21-dUTP.

2. Incomplete restriction enzyme digestion may lead to low coupling efficiency. We routinely check completeness of digestion at each step. Therefore, even when two compatible restriction enzymes are utilized, we prefer to perform the digestions in two steps rather than in one step, in order to monitor digestion efficiency. Digest first with the enzyme that creates the end that will not be biotinylated and assure complete linearization.
3. In order to protect the ends from exonuclease invasion that may occur in some experimental systems, we use α-thio-dNTPs in addition to the biotinylated dNTP to fill in the ends which increase the half-life of the ends in crude exonuclease-containing extracts considerably. Ideally, the biotinylated dNTPs should be shielded by 1–2 α-thio-dNTPs. In principle, other modified nucleotides (e.g., aminoallyl-dNTPs) which are easier to find could also be used instead of α-thio-dNTPs but we have not compared their efficiency.
4. Poor filling in by Klenow DNA polymerase affects immobilization. Avoid using ammonium acetate for DNA precipitation as it may inhibit the polymerase. Klenow Exo is better suited for this application than ordinary Klenow DNA polymerase.
5. Incomplete removal of unincorporated biotin is a common reason for inefficient coupling. Biotin reacts with streptavidin readily and may outcompete the immobilization of DNA. Spin columns from different suppliers have diverse specifications which should be followed precisely.
6. Coupling efficiency drops drastically with increasing length of DNA to be immobilized. For some applications [6] long chromatin templates may be particularly useful. We describe here a protocol for efficient immobilization of λ DNA (50 kb) using the Dynabeads kilobaseBINDER reagent from Thermo Fisher Scientific, Life Technologies. Approximately 1 pmol of λ DNA can be immobilized per 100 μl of Dynabeads.
7. If coupling was efficient the supernatant from the coupling reaction should be free of DNA (missing band test). In the case of incomplete immobilization, comparison of band intensities serves to accurately estimate the percentage of immobilized template. Efficiencies higher than 95 % are routinely obtained.
8. It is essential to dissolve the pellet completely at this step. Do not vortex to avoid shear. Allow a long time, if possible overnight, to dissolve DNA pellet. In general, minimize manipulations such as extensive pipetting that may shear the concentrated, viscous lambda DNA. We cut the end of the pipette tips with

scissors to widen the tip opening. Avoid pipetting up and down in order to resuspend λDNA after its precipitation.

9. See the manufacturer's instructions for up-to-date effective concentration (Thermo Fisher Scientific, Life Technologies, Catalog Number 60101).

10. The amount of chromatin assembly extract to be added has to be determined empirically on soluble plasmid DNA. For each amount of extract used, chromatin assembly efficiency is monitored by MNase digestion and agarose gel electrophoresis in order to define the optimum [4]. In general 50–90 μl of extract is required for 900 ng of template in a 120 μl reaction. Once the optimal conditions are determined scaling up or down is feasible. If a small amount of immobilized DNA is to be assembled into chromatin it is advisable to fill the reaction up with soluble carrier DNA to keep reaction volume conveniently high rather than scaling down.

11. Reaction tubes of different sizes are used in order to match the volume of chromatin assembly reactions. If there is too much empty space in the tube, the reaction mixture spreads all over the surface of the rotating tube. When possible scale up the chromatin assembly reaction to fill up most of the tube. A small air bubble trapped in the tube will help to maintain the beads dispersed in suspension. Since the magnetic field is much stronger close to the base of the tube, we avoid using relatively big volumes (greater than 600–700 μl) per tube as this would increase the duration of the concentration (in a viscous milieu this can lead to incomplete recovery). Thus when it is necessary to concentrate a greater volume (e.g., when conditioning a great volume of bead suspension for coupling reaction) split the reaction into aliquots and concentrate them successively. After the first aliquot of beads is concentrated and the supernatant discarded, the second aliquot is added to the tube and so on.

    If the reaction volume is very low, use small (250 μl), elongated tubes. In this case apply the reaction mixture to the bottom of the tube avoiding contact with its walls. The droplet of the reaction mixture will remain at the bottom of the tube due to surface tension.

12. The nucleosome assembly by salt dialysis is a modification of the one described by Neugebauer and Hörz [15]. For further descriptions of salt gradient dialysis procedures *see* refs. [16, 17]. Here we concentrate on those modifications to the procedure required when working with immobilized DNA.

13. A ratio of purified core histones to DNA of 1:1 reproducibly results in efficient nucleosome assembly. However, an empirical titration of core histones using soluble DNA may be required. As an internal control in the assembly reactions a short,

radioactively labeled and gel-purified PCR fragment may be added in the same dialysis chamber with the immobilized DNA. This will serve to determine the efficiency of nucleosome assembly by a bandshift assay. Complete nucleosome assembly results in a shift of the probe from free to mononucleosome band.

14. A background of proteins sticking nonspecifically to the bead matrix itself is anticipated. Consider the following parameters to optimize the signal-to-noise ratio. First, maximize the amount of DNA per bead by adding an excess of biotinylated DNA in the coupling reaction. Second, preadsorb the beads by washing them a couple of times in a buffer containing 0.01 % w/v BSA and 0.05 % v/v Nonidet P 40. This decreases background and also enables easier handling of the beads by reducing their stickiness. Third, different suppliers provide beads with different matrix characteristics. In our experience, Dynabeads gave a low background when used with *Drosophila* embryo extracts.
15. You have the option to elute proteins sequentially with washes of increasing salt to determine how tightly a protein interacts with chromatin. In general, a buffer containing 400 mM KCl strips off most of the chromatin-associated proteins while core histones require high salt (2 M) for their elution. In the following protocol substitute salt concentration (Y1 = salt 1 in mM) in the buffers according to your application.
16. Many proteins that interact with the bead matrix per se are not eluted in SDS loading buffer unless the beads are boiled. By contrast, chromatin proteins (including histones) are stripped from DNA without boiling. Therefore it is very important to omit boiling of the beads.
17. MNase digestion can be performed with or without prior purification of the template. Here we describe a protocol for MNase digestion of purified chromatin that has been washed. In the case of nuclease treatment without isolation of the DNA from the assembly reaction, approximately ten times more MNase units are required. Conversely, the more stringent the washings of chromatin the less the nuclease is needed.
18. Upon addition of the MNase mix, pipette up and down a couple of times to suspend beads. During longer incubation times resuspend beads once by tapping the tube. Alternatively use the Eppendorf Thermomixer at setting 10.
19. The appearance of the characteristic, ladderlike pattern of DNA fragments generated by MNase analysis and subsequent agarose gel electrophoresis is slightly compromised because only those fragments that are cleaved off the beads (by a double-stranded cut) are recovered for electrophoresis.

Since underdigestion may result in only very little DNA on the gel, fine-tuning of the MNase digestion may be required.

The nucleosome repeat length of immobilized chromatin assembled in *Drosophila* extracts is a bit shorter compared to chromatin assembled on plasmid template under identical conditions. This difference is not due to the immobilization, but rather reflects a difference between linear and supercoiled DNA [18].

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Chapter 2

Preparation and Analysis of Positioned Mononucleosomes

Olga I. Kulaeva and Vasily M. Studitsky

Abstract

Short DNA fragments containing single nucleosomes have been extensively employed as simple model experimental systems for analysis of many intranuclear processes, including binding of proteins to nucleosomes, covalent histone modifications, transcription, DNA repair, and ATP-dependent chromatin remodeling. Here we describe several recently developed procedures for obtaining and analysis of mononucleosomes assembled on 200–350-bp DNA fragments.

Key words Nucleosomes, Chromatin, Assembly, Methods

1 Introduction

Various processes in eukaryotic nuclei (such as DNA replication, repair, recombination, and transcription) occur on DNA organized in chromatin. The minimal structural unit of chromatin is the nucleosome core: 147-bp DNA organized in 1 3/4 superhelical coils on the surface of the histone octamer [1]. Short (150–350 bp) DNA fragments containing single-nucleosome cores have been extensively employed recently for analysis of many intranuclear processes, including binding of regulatory proteins to nucleosomes [2], histone modifications [3, 4], transcription of nucleosomal templates [5–8], DNA repair in chromatin [9, 10], ATP-dependent chromatin remodeling [11, 12], and analysis of histone chaperones [13] and nucleosome structure and thermodynamics [14–17]. In many cases such simple model templates faithfully recapitulate important aspects of these processes [2, 5, 6, 9, 12, 13]. At the same time, these experimental systems have the following advantages as compared with more complex polynucleosomal templates: (a) Structure of single positioned nucleosomes and changes in the nucleosome structure during various processes of DNA metabolism can be analyzed with a high resolution. (b) Electrophoretic mobility of mononucleosomes formed on a short DNA fragment (190–350 bp) in a native gel strongly depends on nucleosome

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position relative to DNA ends [18, 19] and on its histone composition [20, 21]. Therefore positioning and histone composition of mononucleosomes that are often changed during various processes can be easily monitored by analysis in a native gel. (c) Since the initial sample often contains only one positioned nucleosome, its fate during various processes can be determined with certainty. (d) The mobility of mononucleosomes in a native gel is typically changed upon binding of various protein complexes to the templates. Furthermore, conformationally different complexes having the same protein/DNA stoichiometry often have different mobilities in the gel [21]. Thus analysis of protein binding to nucleosomes and protein-induced changes in the conformation of the complexes are relatively straightforward using mononucleosomal templates.

The fate of histones during progression of various processive enzymes (DNA and RNA polymerases) has been extensively studied using various mononucleosomal templates. In some cases nucleosomes remain associated with DNA during progression of the enzymes. Thus on moderately Pol II-transcribed genes, extensive transcription-dependent exchange of H2A/H2B, but not H3/H4, histones was detected [22–27], suggesting that histones H3/H4 never leave DNA during transcription. The underlying Pol II-type mechanism of transcription through chromatin has been recapitulated in vitro and is conserved from yeast to human [28]. It is characterized by displacement of a single H2A/H2B dimer [29, 30], matching the apparent effect of Pol II passage in vivo [24]. Remarkably, the subnucleosome (DNA-bound histone hexamer (hexasome) formed upon release of H2A/H2B dimer from the octamer) survives Pol II passage and remains at the original position on DNA [29].

Survival of the subnucleosomes at the original position on DNA suggests an interesting possibility that at least histones H3/H4 never leave the template during Pol II transcription. This possibility is consistent with the lack of exchange of H3/H4 histones in vivo [22–27] and with the DNA looping mechanism of Pol II transcription through chromatin observed in vitro [31–33]. Indeed, histone survival during Pol II transcription in vitro is accompanied by formation of a small transient DNA loop (Ø-loop) on the surface of histone octamer including molecule of transcribing Pol II [33]. During formation of the Ø-loop, the recovery of DNA-histone interactions behind Pol II is tightly coupled with their disruption ahead of the enzyme. This coupling is a distinct feature of the Pol II-type mechanism that allows recovery of H3/H4 histones bound at the original position on DNA during transcription [32, 33].

To evaluate whether histones H3/H4 never leave the template during Pol II transcription, the following approach has been utilized (Fig. 1) [34]. If the histone octamer was transiently and fully displaced from DNA, the octamer would rebind to DNA released

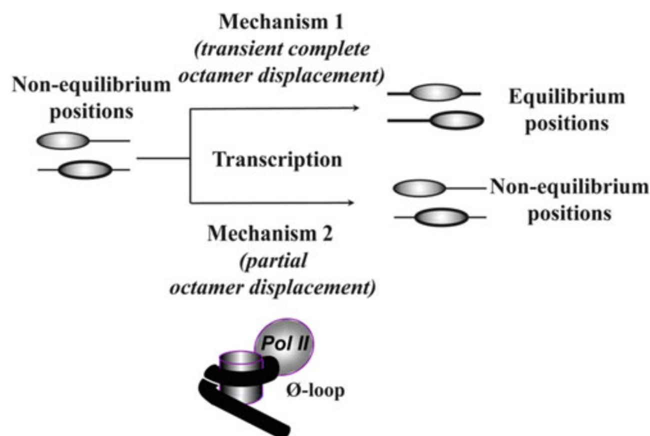

Fig. 1 Two possible mechanisms of nucleosome survival during transcription by Pol II. After Pol II transcription (or progression of other processive enzymes) through chromatin, histones often remain bound to the DNA. Such nucleosome survival could be accompanied either by complete transient displacement (mechanism 1) or only partial displacement of histone octamer from DNA (mechanism 2). According to the mechanism 1 octamer rebinds to DNA released behind the progressing enzyme; in the other case progression of the enzyme is accompanied by formation of an intranucleosomal loop (a small Ø-loop formed during Pol II transcription is shown). As a result, after the enzyme progresses through chromatin, nucleosome positions are either re-equilibrated (mechanism 1), or the original, nonequilibrium nucleosome positions are preserved (mechanism 2)

behind the progressing Pol II and nucleosome positions would be re-equilibrated during transcription (mechanism 1, Fig. 1) [34]. If the original nucleosome positions are nonequilibrium, transcription would result in a change in nucleosome positions. Alternatively, if H3/H4 never leave DNA during transcription, nucleosomes would remain at their original positions after transcription, even if these are not the preferential (equilibrated) positions on DNA before transcription (mechanism 2, Fig. 1). Since histones remain associated with DNA during progression of various processive enzymes including ATP-dependent chromatin remodelers and DNA polymerases (*see* ref. [35] for review), these approaches are likely to be useful beyond the chromatin transcription field.

Below we describe procedures for obtaining and analysis of mononucleosomes in both equilibrated and nonequilibrium positions on 200–350-bp DNA fragments.

2 Materials

2.1 Materials

1. Dialysis membranes (Spectra/Por; molecular weight cutoff of 8,000 and 12,000–14,000).
2. G-25 Quick spin columns (Boehringer Mannheim).
3. Siliconized Eppendorf tubes (PGC Scientific).

4. 3MM chromatography paper (Whatman).
5. QIAquick Gel Extraction kit (Qiagen).
6. Chicken blood (Truslow Farms).
7. CM C-25 Sephadex.
8. Centiprep-30.

2.2 Enzymes

1. T4 polynucleotide kinase (New England Biolabs, NEB).
2. Restriction enzymes (NEB).
3. Klenow fragment of *E. coli* DNA polymerase I (NEB).
4. Taq DNA polymerase (NEB).
5. T4 DNA ligase (NEB).

2.3 Reagents

1. Ethidium bromide (10 mg/ml stock).
2. Butyl alcohol (J.T. Baker).
3. Ethanol (Pharmco).
4. Equilibrated phenol (Sigma).
5. Chloroform (J.T. Baker).
6. β-Mercaptoethanol (Sigma).
7. Protease- and nuclease-free BSA (Sigma).
8. Glycerol (J.T. Baker).
9. NP-40 (Calbiochem).
10. Sodium dodecyl sulfate (GibcoBRL).
11. γ^{32}P-ATP (3,000 or 6,000 Ci/mmol; Perkin Elmer).
12. Acrylamide; *N,N′*-methylene-*bis*-acrylamide (Bis) (Bio-Rad).
13. Glycogen, 10 mg/ml solution (Boehringer Mannheim).
14. Bromophenol Blue dye (Bio-Rad).
15. Xylene Cyanol (Aldrich Chem. Co).
16. Single Stranded Salmon Testes DNA (Sigma).

2.4 Buffers and Solutions

1. PBS buffer: Phosphate-buffered saline (Gibco BRL).
2. Buffer A: 15 mM Tris–HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM β-mercaptoethanol, 0.34 M sucrose, 0.1 mM PMSF.
3. Buffer B: 10 mM Tris–HCl (pH 7.5), 350 mM NaCl, 0.5 mM EDTA, 0.1 mM PMSF.
4. NLB (nuclei lysis buffer): 0.25 mM EDTA, 0.1 mM PMSF.
5. TAE buffer: 0.04 M Tris-acetate, pH 8.0, 1 mM EDTA.
6. HE buffer: 10 mM Na-HEPES, pH 8.0, 1 mM EDTA.
7. CRB 1–4 (core reconstitution buffers): All four buffers contain HE, 5 mM mM β-mercaptoethanol, 0.1 % NP-40, and NaCl at

the following concentrations: Buffer 1—1 M; 2—0.8 M; 3—0.6 M; 4—0.01 M.

8. SP6 buffer: 45 mM Na-HEPES, pH 8.0, 6 mM $MgCl_2$, 2 mM spermidine and 2 mg/ml BSA, 10 mM β-mercaptoethanol.
9. 1× TB (transcription buffer): 20 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, and indicated concentration of KCl, mM.
10. 1× RLB (RNA loading buffer): 95 % Formamide, 10 mM EDTA, 0.1 % SDS, and 0.01 % of each brome phenol and xylene cyanol dyes.
11. 4× Chromatin loading buffer: 100 mM Tris–HCl (pH 7.5), 40 mM EDTA, 40 % sucrose, and 1 mg/ml of salmon testes DNA.

3 Methods

3.1 Protein Purification

We purified hexahistidine-tagged yeast Pol II using published protocols [7] and H2A/H2B and H3/H4 histone pairs were isolated from adult chicken blood by chromatography on hydroxyapatite [36, 37].

3.2 Preparation of Donor Chromatin from Chicken Erythrocytes

The protocol described below is a modified version of the method published earlier [6, 39] (*see* **Note 1**).

3.2.1 Red Cell Isolation

1. Collect red cells from 200 ml chicken blood by centrifugation at 1,800 × *g* for 10 min at 4 °C.
2. Carefully remove white cells forming from the top of the pellet.
3. Resuspend cells in 48 ml PBS buffer.
4. Collect red cells by centrifugation at 3,000 × *g* for 5 min at 4 °C.
5. Repeat **steps 3** and **4** two more times.

3.2.2 Nuclei Isolation

1. Conduct all manipulations at 4 °C with precooled buffers, unless indicated otherwise.
2. Resuspend cells in PBS buffer (half of the volume of the pellet).
3. Add 10 volumes of buffer A supplemented with 0.5 % NP-40 and mix by inversion.
4. Collect nuclei by centrifugation at 12,000 × *g* for 10 min at 4 °C.
5. Resuspend nuclei in 200 ml of buffer A (no NP-40).
6. Collect nuclei by centrifugation at 12,000 × *g* for 10 min at 4 °C.

7. Repeat **steps 3** and **4** several times until red color disappears.
8. Resuspend purified nuclei in a small volume (50–100 ml) of buffer A.
9. Resuspend ~1 μl of nuclei in 0.9 ml of HE buffer, add 0.1 ml of 10 % SDS, and measure A_{260}. Concentration of nuclei should be ~200–400 A_{260}/ml.

3.2.3 Chromatin Preparation

1. Adjust nuclei concentration to 100 A_{260}/ml.
2. For analytical digestion with micrococcal nuclease (MNase) warm 1 ml of nuclei to 37 °C.
3. Add $MgCl_2$ and $CaCl_2$ to 1 mM final concentration.
4. Add 1 μl of MNase to 10 U/ml final concentration and incubate at 37 °C.
5. Remove 0.1-ml aliquots after 1, 2, 4, 8, 15, 30, and 60 min and stop the digestion by adding EDTA to 10 mM and SDS to 1 % final concentrations.
6. Extract the samples once with one volume of 1:1 (v/v) phenol:chloroform.
7. Precipitate DNA with 3 volumes of ethanol, wash with 70 % (v/v) ethanol, dry, and dissolve in HE buffer.
8. Analyze DNA in 1 % agarose gel and identify the digestion point where the sizes of DNA fragments are 3–20 kb (Fig. 2, 15-min digestion point was selected).
9. For preparative digestion with MNase warm the nuclei in a 500 ml conical glass flask to 37 °C.

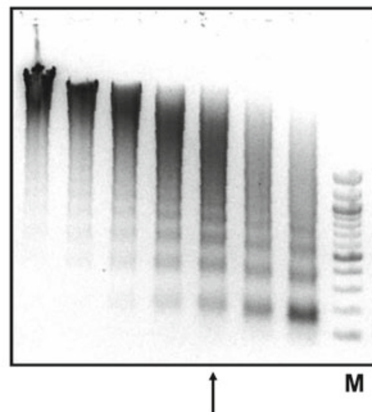

Fig. 2 Time course of digestion of chicken erythrocyte nuclei with micrococcal nuclease (MNase). Nuclei were digested for various time intervals (1, 2, 4, 8, 15, 30, or 60 min), DNA purified, and analyzed in 1 % agarose gel (ethidium bromide stain). 15-min digestion point was selected for preparative digestion of the nuclei (indicated by *arrow*). M—100-bp DNA ladder (New England Biolabs)

10. Add $MgCl_2$ and $CaCl_2$ to 1 mM final concentration, and MNase to 10 U/ml final concentration, and incubate at 37 °C for the required time.
11. Stop the digestion by adding EDTA to 10 mM final concentration.
12. Collect nuclei by centrifugation at 12,000 × *g* for 10 min at 4 °C.
13. Estimate the volume of the pellet and resuspend the nuclei in equal volume of NLB buffer (nuclei should become semitransparent).
14. Collect nuclear debris by centrifugation at 12,000 × *g* for 10 min at 4 °C.
15. Remove the supernatant containing soluble chromatin (it should be slightly opaque and gray). Discard the pellet.
16. Resuspend an aliquot of chromatin in 0.9 ml of HE buffer, add 0.1 ml of 10 % SDS, and measure A_{260}. Concentration of chromatin should be ~50–80 A_{260}/ml.

3.2.4 Removal of Linker Histones H1/H5 from Chromatin

1. Adjust concentration of chromatin to 50–100 A_{260}/ml.
2. Presoak CM C-25 Sephadex (Pharmacia, 36 mg of resin per mg of chromatin) in buffer B for 1 h.
3. Slowly add 2 M NaCl to chromatin to 0.35 M final concentration (*see* **Note 2**).
4. Add 1/3 of presoaked CM C-25 Sephadex to chromatin and slowly stir for 2 h at 4 °C.
5. Collect the resin by centrifugation at 12,000 × *g* for 10 min at 4 °C. Remove supernatant containing soluble chromatin.
6. Repeat **steps 4** and **5** two more times.
7. Analyze protein composition of −H1/5 chromatin by SDS-PAGE (Fig. 3). Dissolve ~10 μg of histones in 10–60 μl of Laemmli loading buffer. Electrophorese histones in an 18 % (acrylamide:bis = (30:0.15)) Laemmli gel (17 × 17 × 0.15 cm) for 5–6 h at 32 mA. Stain the gel with Coomassie Blue.
8. Concentrate −H1 chromatin to ~100 A_{260}/ml (5 mg/ml) on Centriprep-30 by centrifugation at 1,300 × *g* for required time at 4 °C.
9. Dialyze −H1/5 chromatin overnight against buffer B and store at −70 °C (*see* **Note 3**).

3.3 Preparation of DNA Fragment for Reconstitution

The protocol described below is a modified version of the method published earlier [7].

1. The 204-bp DNA fragment was obtained by PCR amplification using pVT1 5S-containing plasmid as was described previously [38].

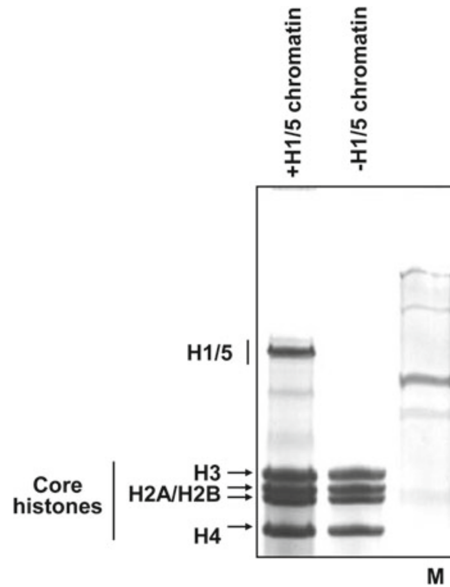

Fig. 3 Analysis of protein composition of histone H1/5-containing and H1/5-depleted chicken erythrocyte chromatin. Histones were resolved by 18 % Laemmli SDS-PAGE. Positions of histones on the gel are indicated. M—10–250 kDa protein markers (BioRad)

2. 5′-End-label the PCR primers with γ [^{32}P] ATP (6,000 Ci/mmol) using T4 polynucleotide kinase according to the manufacturer's protocol.
3. PCR-amplify DNA fragments in 500 μl volume (5 × 100 ml reactions) using Taq DNA polymerase [7].
4. Resolve the obtained DNA fragments in a 1.5 % (w/v) agarose gel containing 0.5 μg/ml ethidium bromide and TAE buffer at 4–6 V/cm for 1.5–3 h, depending on the resolution required for clear band separation.
5. Using a long-wavelength UV lamp (to reduce nicking of DNA), identify and excise the required band(s).
6. Purify the fragment using the QIAquick Gel Extraction kit.
7. Extract the samples once with one volume of 1:1 (v/v) phenol:chloroform.
8. Precipitate DNA with 3 volumes of ethanol, wash with 70 % (v/v) ethanol, dry, and dissolve in 100 μl of HE buffer.
9. Determine DNA concentration by measuring the A_{260} (using A_{260} = 20 for 1 mg/ml DNA) and store at −20 °C.
10. 204-bp PCR fragment was digested with *Tsp*R1 restriction enzyme for 3 h at 65 °C.
11. Resolve the obtained DNA fragments in a 1.5 % (w/v) agarose gel containing 0.5 μg/ml ethidium bromide, 4 M urea, and TAE buffer at 4–6 V/cm for 1.5–3 h.

12. Using a long-wavelength UV lamp (to reduce nicking of DNA), identify and excise the required band(s).
13. Purify the fragment using QIAquick Gel Extraction kit (Qiagen).
14. Extract the sample once with one volume of 1:1 (v/v) phenol:chloroform.
15. Precipitate DNA with 3 volumes of ethanol, wash with 70 % (v/v) ethanol, dry, and dissolve in 100 μl of HE buffer.
16. Use the 204-bp fragment for reconstitution or ligation with 50-bp promoter fragment for 3 h at 15 °C using T4 DNA ligase to obtain the 254-bp DNA fragment (Fig. 4) according to the manufacturer's protocol.
17. The ligated products were gel-purified as a template for reconstitution with donor –H1 chromatin.

3.4 Reconstitution of Mononucleosome Cores Using Donor –H1 Chromatin Before or After Ligation

Reconstitution was performed using donor –H1 chromatin or purified histones (*see* ref. [6] for detail). Nucleosome assembly was performed on the 204-bp DNA fragment and then ligated to the 50-bp promoter-containing fragment, or on the 254-bp DNA fragment that already contains the promoter (Fig. 4 [34]).

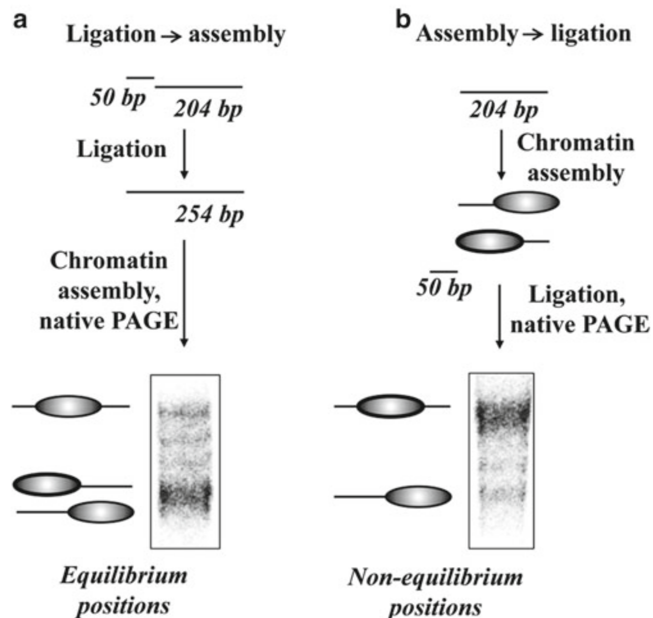

Fig. 4 Experimental approach for obtaining equilibrated and nonequilibrium sets of mononucleosomes. Nucleosome assembly on the 204-bp DNA fragment was conducted either after (**a**) or before (**b**) ligation to the promoter-containing 50-bp fragment. Positions of nucleosomes are equilibrated during but not after assembly. Therefore in both cases nucleosome positions are equilibrated during the process of assembly, but in (**b**) ligation to the promoter-containing DNA fragment to the assembled nucleosomes produces templates containing nucleosomes occupying nonequilibrium positions before transcription by Pol II

1. Cool 500 ml each of CRB1 to CRB4 buffers to 4 °C.
2. Mix one to three micrograms of the DNA fragment with long –H1/H5 donor chromatin at a ratio of 1:60 (w:w) in 0.04–0.1 ml of CRB1 buffer (*see* **Note 4**).
3. Dialyze successively against CRB2 and CRB3, each for 2 h at 4 °C. Then dialyze the sample against CRB4 for 3 h or overnight.
4. Transfer the reconstitute to a siliconized Eppendorf tube and store at 4 °C (do not freeze).
5. Ligate nucleosomes assembled on 204-bp fragment with 50-bp promoter fragment [17] for 3 h at 15 °C using T4 DNA ligase according to the manufacturer's protocol.
6. Check the samples by analysis by native PAGE (*see* below).

3.5 Mapping the Positions of Nucleosome Cores by Native PAGE

1. Preparation of the sample to load to native gel: the concentration of the reconstituted nucleosomes is determined by the specific activity of the DNA.
2. Loading buffer is added providing a final concentration of 10 % sucrose and 50 lg/ml sheared herring testes DNA (Intergen, Purchase, NY).
3. The templates are resolved by native gel electrophoresis (4.5 % acrylamide (39:1), 5 % glycerol, 20 mM Na–HEPES, pH 8, and 0.1 mM EDTA) at 100 V for 4 h at 4 °C as described [38].
4. Quantitation is performed using a Cyclone Phosphor System (Packard, Meriden, CT).

Nucleosome mobility in the gel is dictated by nucleosome positioning on the 254-bp DNA fragment. Nucleosomes occupy different positions when assembly was conducted before or after DNA ligation (Fig. 4), suggesting that nucleosome occupied non-equilibrium positions when the assembly was conducted before ligation [34]. Since these nonequilibrium nucleosome positions are preserved after Pol II transcription [29], the data suggest that histones never leave DNA during this process.

4 Notes

1. Donor chromatin isolated from chicken erythrocytes contains mostly unmodified core histones [39]. The main advantage of using this source of histones is the minimal presence of proteases in chicken erythrocytes.
2. Chromatin becomes insoluble at 150 mM NaCl and then becomes soluble again when the concentration of NaCl reaches 350 mM.

3. Donor –H1 chromatin could be further purified by chromatography on Sephacryl S-400HR (Pharmacia) [40].
4. The ratio of DNA to donor chromatin may need to be adjusted because different chromatin preparations produce slightly different results.

Acknowledgements

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Chapter 3

Chromatin Imaging with Time-Lapse Atomic Force Microscopy

Yuri L. Lyubchenko and Luda S. Shlyakhtenko

Abstract

Time-lapse atomic force microscopy (AFM) is widely used for direct visualization of the nanoscale dynamics of various biological systems. The advent of high-speed AFM instrumentation made it possible to image the dynamics of proteins and protein-DNA complexes within millisecond time range. This chapter describes protocols for studies of structure and dynamics of nucleosomes with time-lapse AFM including the high-speed AFM instrument. The necessary specifics for the preparation of chromatin samples for imaging with AFM including the protocols for the surface preparation are provided.

Key words Chromatin dynamics, Nucleosome dynamics, Atomic force microscopy, AFM, Time-lapse AFM, Single-molecule techniques

1 Introduction

Chromatin dynamics is needed for executing all genetic function by the cell such as DNA replication and transcription. A fundamental unit of chromatin, the nucleosome core particle (NCP), is very compact and considered stable. A regular NCP includes 147 bp of DNA duplex that is tightly wrapped around an octameric core, comprising histones H2A, H2B, H3, and H4. The NCP structure is stabilized by electrostatic interactions, specific hydrogen bonds, and salt bridges, identified in the high-resolution crystal structure of NCPs [1]. The high stability of the NCPs leads to the question of how the DNA within the nucleosome can be accessed by regulatory proteins and polymerases for transcription and DNA replication. Chromatin remodeling proteins are capable of the DNA dissociation from the histone core [2–5] suggesting that nucleosome dynamics should be involved into the nucleosome dissociation process. Indeed, studies performed in the past decade with the use of various techniques, including single-molecule approaches, showed that the NCP is not a static structure. Rather, DNA can spontaneously and transiently dissociate, and single-molecule fluorescence and time-resolved

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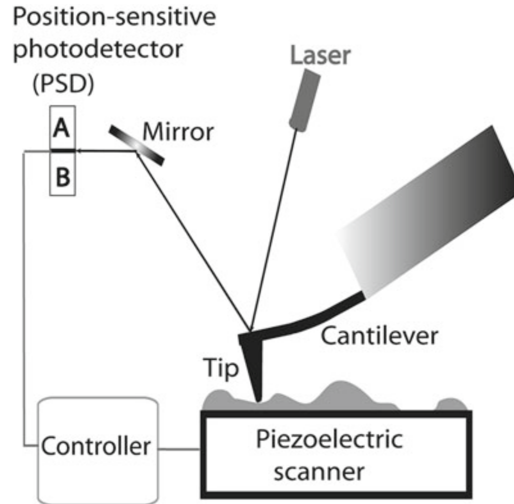


Fig. 1 Schematic explaining the principles of AFM. The position of the tip relative to the sample is controlled by a piezoelectric scanner. The vertical displacement of the tip during scanning is detected using the optical lever principle, in which the position of the light spot on the PSD is measured. Figure was reproduced from [23] with permission. Copyright (2011) Elsevier

techniques revealed that nucleosomes undergo local dissociation of DNA in the absence of remodeling proteins [6–10] and this process occurs on the sub-second timescale [8]. Atomic force microscopy (AFM) was instrumental in direct visualization of the nucleosome dynamics with the nanometer-range spatial resolution [11, 12]. Implementation of high-speed AFM (HS-AFM; (reviewed in refs. 13–15) capable of the nanometer resolution on the millisecond timescale made it possible to identify various pathways of the NCP dynamics [16, 17]. The AFM methodologies enabling direct visualization the chromatin dynamics using regular and high-speed time-lapse AFM modes are outlined in this chapter.

A schematic illustrating the principles of the AFM operation is shown in Fig. 1. A sharp stylus (AFM tip shown as a triangle) reads the sample topography (shown as a bumpy profile) while it moves over sample in a raster pattern termed scanning. The tip cantilever works as a spring pressing the tip against the sample during scanning. The vertical movement of the tip is detected by the optical lever principle in which the tip displacement is measured by the changing of the laser spot location on the position-sensitive photodetector (PSD). Note that no special contrasting sample is needed for AFM imaging. Additionally, scanning can be performed in any media at ambient conditions, including physiological conditions enabling direct visualization of the sample dynamics as described in this chapter. AFM instruments include a number of important features that enable the production of

high-resolution images. First, the position of the sample relative to the tip is controlled by the scanner with an accuracy of less than 1 nm. Second, the tip can be atomically sharp. Third, the displacement of the tip relative to the surface is determined with subnanometer accuracy. All these features are critical for the use of AFM for biomedical studies including the chromatin dynamics.

2 Materials

Prepare all solutions using deionized water; Aquamax Water System (Aquamax Laboratory, Van Nuys, CA) produces low-conductivity water (18.2 MΩ) with a required quality. Use analytical grade reagents when preparing the solutions.

1. A vacuum cabinet or desiccator for storing samples. A Gravity Convention Utility Oven (VWR) is recommended.
2. Plastic tubes, 15 mL.
3. Eppendorf tubes, 1.5 mL.
4. Plastic cuvettes.
5. Scissors.
6. Razor blades.
7. 2 L glass desiccators and vacuum line (50 mmHg is sufficient).
8. Pipettes with plastic tips for rinsing the samples.
9. Tweezers.
10. Gas tank with clean argon gas. Nitrogen gas can be used as well.
11. Mica substrate: Any type of commercially available mica sheets (green or ruby mica) can be used. Asheville-Schoonmaker Mica Co (Newport News, VA) supplies thick and large (more than 5 × 7 cm) sheets (Grade 1) suitable for making substrates of different thickness and size.
12. Deionized water filtered through 0.2 μm filter for mica functionalization and AFM sample preparation.
13. Apparatus for protein gel electrophoresis.
14. AFM instruments.

3 Methods

3.1 Preparation of Nucleosomal DNA

The 601 Widom sequence was used as a template for the nucleosome assembly which has the very high affinity for binding of the histone core compared to other sequences [18].

1. Use plasmid pGEM3Z-601 that contains Widom-601 motif and generate DNA by PCR.
2. Run the PCR reaction (33 cycles of 94 °C/30 s, 54 °C/30 s, 72 °C/30 s) in buffer containing 2.5 mM $MgCl_2$, 0.15 mM dNTPs, and 0.016 U/μL of Taq DNA polymerase with the following primers: forward primer 5′-GEMf CGGCCAG TGAATTGTAATACG-3′; reverse primer GEMr 5′-CGGGA TCCTAATGACCAAGG-3′. These primers produce 353 bp DNA template that was used in the AFM time-lapse experiments in [12, 17, 19].

3.2 Histone Octamer Assembly and Purification

Histone octamers were assembled according to the protocol described in [20]. Commercially available histones H2A, H2B, H3, and H4 (New England Biolab, Ipswich, MA) are suitable.

1. Take 80 μL of 1 mg/mL solutions of each histone (H2A, H2B, H3, H4), load them on Microcon centrifugal filter devices (MWCO 3,000, Millipore, Billerica, MA), and spin at 12,700 × *g* for 35 min. Make 15 μL total volume in the recovery spin.
2. Add 100 μL of unfolding buffer (UB) containing 6 M guanidine chloride, 20 mM Tris–HCl, pH 7.5, and 5 mM DTT.
3. Use UV spectrophotometer (280 nm) to measure the histone concentration with a buffer as a reference. 1 mg/mL is the desired histone concentration.
4. Combine the histones in equal molar amounts.
5. Dialyze the histone mixture at 4 °C using Slide-A-Lyzer dialysis cassette with molecular weight cutoff of 7,000 (Pierce) against three changes of 250 mL of refolding buffer containing 2 M NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM Na-EDTA, and 5 mM 2-ME. Overnight dialysis at 4 °C is sufficient for the assembly process. Measure the protein concentration with UV spectrophotometer.
6. Concentrate the sample with Microcon centrifugal filter devices (MWCO 10,000) in ~20 μL volume. A typical concentration run is for 10 min at 12,700 × *g* followed by the recovery spin.
7. Perform gel permeation chromatography to separate fully assembled histone octamers from tetramers, dimers, and monomers.
8. To accomplish this step, the sample is loaded on Superdex 200 PG 3.2/30 column (GE Healthcare), which was pre-equilibrated with RB at 4 °C and fractions are collected. Figure 2 shows one of such chromatograms.
9. Analyze selected fractions around the major peak 1 and peak 2 by 15 % PAGE-SDS gel electrophoresis. The running acrylamide gel contained 15 % acrylamide, 0.375 M Tris, 0.1 %

Fig. 2 Size-exclusion chromatography of the octamer sample reconstitution. Three major peaks analyzed with gel electrophoresis are indicated

SDS, 0.08 % ammonium persulfate, and 0.05 % TEMED. The stacking gel is recommended to use for improvement of the resolution of the protein bands. It contained 6 % acrylamide, 0.125 M Tris, 0.1 % SDS, 0.08 % APS, and 0.05 % TEMED. Figure 3 shows the results of such an analysis. The gel was stained using Coomassie Blue. Lanes containing octamer (left fractions of first peak), tetramer (fractions of the corresponding to the right segment of peak 1), and dimers appearing in peak 2 are indicated.

10. Fractions containing histones H2A, H2B, H3, and H4 in equal ratios were pooled and concentrated using Microcon concentration device MCWO 10,000. This preparation can be stored in 50 % (v/v) glycerol solution at −20 °C.

3.3 Nucleosome Reconstruction

1. Mix in equimolar concentrations histone octamer sample prepared as described above with the DNA template containing the Widom 601 sequence in the refolding buffer containing 2 M NaCl and keep for 30 min at room temperature.

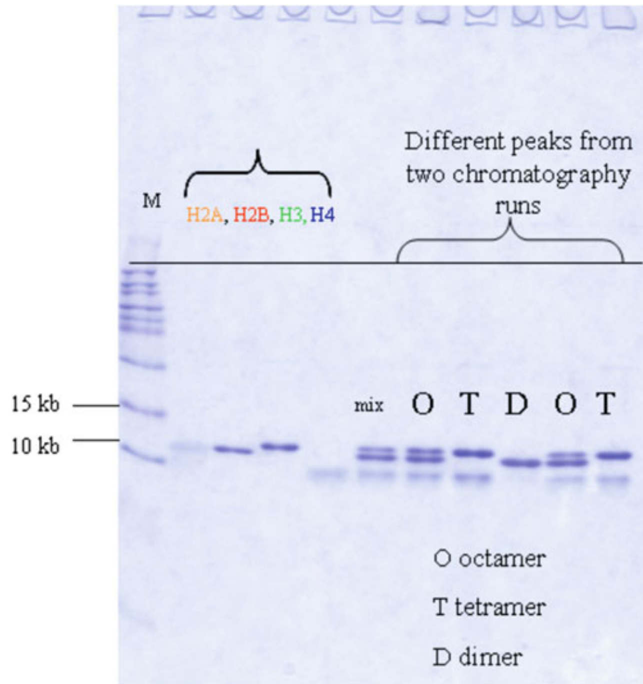


Fig. 3 SDS-PAGE gel electrophoresis of various fraction of the chromatography column. M—protein markers; H2A, H2B, H3, and H4 are the lanes at which pure histone fractions were run. Mix—equimolar mixture of all histones. O, T, and D correspond to the chromatography fractions containing octamers (left half of peak 1), tetramers (right half of peak 1), and dimers (peak 2)

2. Dialyze the mixture into buffers of decreasing ionic strength using Slide-A-Lyzer Mini Dialysis Unit MWCO 3500 (Pierce). A dilution series was prepared using 10 mM Tris–HCl with NaCl concentrations 1 M, 0.67 M, and 0.5 M. Keep diluted samples at 4 °C for 1 h before dialysis against one change of volume of 0.2 M NaCl overnight.
3. Concentrate nucleosomes to obtain ~300 nM using Microcon centrifugal filter device, MWCO 10,000 (7,000 × *g* for 10 min at 4 °C), and dialyze against one change of 200 mL of the buffer containing 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA for 3 h at 4 °C. Take UV spectrum to measure the DNA concentration and run AFM to characterize the sample (*see* protocol below). Figure 4 shows AFM images of assembled nucleosomes. The sample at such conditions remains stable for several months.

3.4 Sample Preparation for AFM Imaging in Air

The sections below describe the procedure for the preparation of samples of nucleosomes for AFM imaging. This protocol utilizes the methodology of mica functionalization with 1-(3-aminopropyl) silatrane (APS-mica) that provides very reproducible and reliable results. The APS synthesis protocol is described in [21].

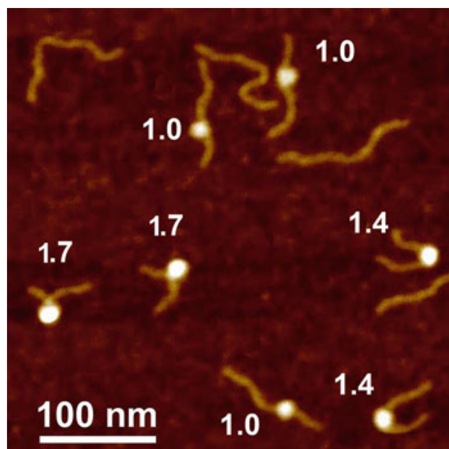


Fig. 4 AFM images of reconstituted mononucleosome sample. The figures near each nucleosome correspond to the number of DNA turns calculated as described in the text. Figure was reproduced from [12] with permission. Copyright (2009) American Chemical Society

3.4.1 Preparation of the APS-Mica Substrates

1. Prepare a 50 mM APS stock solution in water and store it in refrigerator. The stock solution can be kept for more than a year at 4 °C.
2. Prepare the APS working solution from the stock in a 1:300 ratio in water by diluting 45 μL of the stock in 15 mL H_2O to make the working APS solution for mica modification; it can be stored at 4 °C for several days.
3. Cut both sides of the mica sheets to make strips of the needed size (typically 1 cm × 3 cm) and cleave the strips with a razor blade, or scotch tape to make them as thin as ~0.1 mm. Do not touch the cleaved mica surface.
4. Place the mica strips in appropriate plastic tubes (*see* **Note 1** for more details).
5. Pour the working APS solution to cover the mica strip completely.
6. Leave the tubes/cuvettes on the bench for 30 min. After 30 min discard the APS solution.
7. Rinse both sides of the mica with deionized water and completely dry both sides of the mica strips under argon flow.
8. The strips are ready for the sample preparation. Additional storage in a vacuum for 1–2 h is recommended when the environment is humid. Dry APS mica strips can be stored under Ar gas in the clean dry cuvette or vacuum desiccator for 2 weeks without losing the DNA binding activity. *See* **Notes 2** and **3** for the APS mica storage and the shelf life.

3.4.2 Preparation of the Nucleosome Samples for Imaging of Dried Samples in Air

1. Prepare the solution of the nucleosome in a buffer containing 10 mM HEPES (pH 7.5) and 4 mM $MgCl_2$. The nucleosome concentration between 1 nM and 0.3 nM is optimal.
2. Cut the APS mica substrates to a desired size (1 × 1 cm squares for the Multi Mode AFM instrument (Bruker-Nano, Santa Barbara, CA) are optimal) and place 5–10 μL of the nucleosome solution in the middle of the substrate for 2 min.
3. Rinse the sample thoroughly with deionized water (1–3 mL per sample) to remove all buffer components. 5 mL or 10 mL plastic syringes are useful for rinsing. Attach an appropriate plastic tip instead of a metal needle.
4. Dry the sample with clean argon gas. Additional drying of samples for an hour or two prior to imaging is recommended to ensure low tip adhesion. The samples can be stored in vacuum cabinets or desiccators filled with argon. The samples, as prepared, can be imaged many times provided that after imaging they are stored as described. Their shelf life is several months [21].

3.5 AFM Imaging

This section describes the AFM imaging procedures for imaging dried and wet samples.

3.5.1 AFM Imaging in Air

For imaging in air, any type of tip with a spring constant of approximately 40 N/m and a resonant frequency between 300 and 340 kHz can be used. For example, Olympus silicon probes (Asylum Research, Santa Barbara, CA) with a spring constant of 40 N/m and a 300 kHz resonant frequency in air work reliably in the tapping/oscillating mode for imaging in air. Probes with similar characteristics are currently manufactured by a large number of other vendors.

1. Mount the sample prepared at **step 4** above onto the AFM stage using a double-stick tape.
2. Tune the AFM probe to find the resonance frequency corresponding to the AFM probe. Adjust the drive amplitude. For the Multi Mode AFM, 6–8 mV is typical. Set the image size to 100 × 100 nm and start approaching the surface.
3. Gradually reduce the set point until the surface of the sample is clearly seen. Increase the scan size and acquire the images.
4. Typical AFM images of mononucleosome sample obtained with the use of the functionalized procedures are shown in Fig. 4. Nucleosomes appear as white blobs with DNA filaments of different sizes on the flanks. The flank sizes correspond to the asymmetric position of 601 motif within the DNA template [12]. Note a number of features of the APS-mica procedure enabling the quantitative analysis of the samples [12]. First, the background is smooth, enabling unambiguous visualization

of DNA. Second, the concentration of sample was adjusted in such a way that the molecules are spread over the surface with no overlap. Third, no glutaraldehyde fixation was used for obtaining these images.

3.5.2 Imaging in Aqueous Solutions

For imaging in liquid a regular AFM, Si_3N_4, 100 μm long probes (SNL, Bruker-Nano/Veeco, Santa Barbara, CA) with a spring constant of approximately 0.06 N/m and a resonance frequency around 7–10 kHz are recommended. AFM probes with similar characteristics from other vendors are available. The protocols described below assume the use of Multi Mode AFM (Bruker-Nano/Veeco), but they can be adapted to any type of AFM. Protocol for imaging with HS AFM is given in a separate chapter.

1. Mount the tip on the tip holder.
2. Place the stage with the attached APS-mica substrate on the instrument stage. It is a scanner for the MM AFM instrument. Mica pieces 1 × 1 cm work well for MM AFM. Double-stick tape can be used to attach the modified mica substrate to the metal discs. Do not glue APS-mica as the glue vapors react with the mica surface deteriorating the surface.
3. Use the video camera to find the tip and approach the surface manually, leaving a 500–100 μm gap between the tip and the surface.
4. Place a droplet of the prepared sample solution and readjust the spot position. The spot changes due to the difference in the refractive indexes of air and water. For MM AFM, 50 μL of the solution is sufficient to fill the gap. Note that due to the elevated hydrophobicity of APS-mica compared to bare mica, the spot does not spread far; therefore, no O-ring is required to keep the solution in place.
5. Find a resonance peak. Typically it is quite a broad peak, around 7–10 kHz, for the Multi Mode AFM instrument. Follow the recommendations given in the instrument manual on how to find the peak in fluid.
6. Start the computer-controlled approach. Operate with the set point voltage and drive amplitude parameters to improve the quality of images. Minimize the drive amplitude. The number varies from tip to tip, but the free amplitude as low as 10 nm or less and a scanning rate of approximately 2 Hz provide good-quality pictures.
7. The image acquisition starts with the area 1–2 μm, select the area of interest with ~500 nm, and start time-dependent acquisition. Scan rate 1–2 Hz is typical for MM AFM with 512 × 512 data acquisition density.

3.5.3 Imaging in Aqueous Solutions with High-Speed AFM

This section describes the protocol for the preparation of nucleosome samples for imaging with high-speed AFM (HS AFM) instrument designed by T. Ando [22]. This paper also provides a protocol for the instrument operation. The instrument is designed for mica disks as small as 1 mm and 1.5 mm available from RIBM (Tsukuba, Japan).

1. Glue the glass rod to the scanner stage with a nail polish and leave it for 15 min to dry.
2. Glue mica disk to the glass rod with silylated urethane resin (Konishi bond ultra versatile SU, available on Amazon). Very small amount of the glue is needed. Use plastic pipette tip to place a small droplet of the glue to the glass rod. Press the mica firmly with a clean pipette tip and let the glue to harden for two hours.
3. Cleave mica by removing the top layers using a scotch tape. Check the scotch tape and the mica surface for a good cleavage; use magnifying glass for better inspection of the cleavage.
4. Apply 2 μL of freshly prepared APS solution in DI water (167 μM) to a freshly cleaved mica, place a plastic cap with a wet filter paper disk inside on the top of the scanner, and allow the modification for 30 min. A wet paper disk creates a humid atmosphere avoiding the evaporation of APS solution. Photographs in Fig. 5 show the scanner with the mica mounted on the sample holder and the plastic cap with a wet paper disk inside (a). Photo in (b) shows the sample holder covered with the cap. While the modification process is going, mount the cantilever, so you will be able to start with imaging as the sample is prepared.
5. Place a cantilever in the cantilever holder using a microscope. BL-AC10DS-A2 cantilevers (Olympus, Japan) modified by the electron beam deposition method and sharpened by using

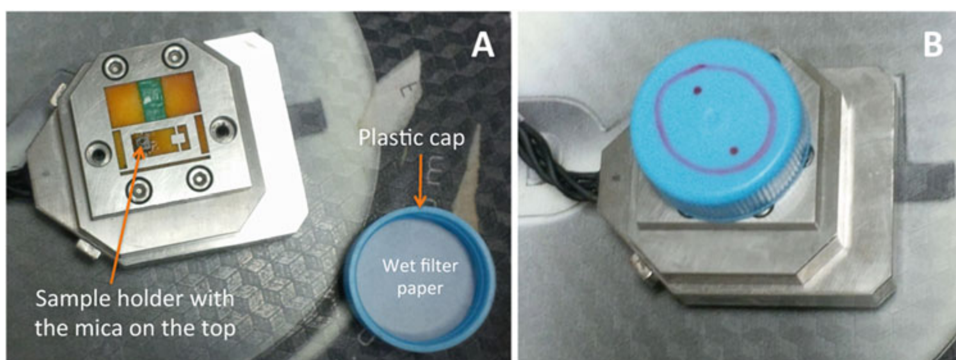


Fig. 5 Photographs of the scanner with the sample holder and the cap (**a**); the same assembly with the cap covered the sample holder (**b**)

plasma etcher as described in [22] are recommended. Place a cantilever using a Teflon-coated tweezer. Avoid any static discharge, which can also damage the tip. Tighten the screws half way. Tap a little to make sure that the cantilever is sitting in the groove. Fully tighten the screws without using extra force.

6. Place the cantilever holder on the AFM stage. The tip faces up.
7. Rinse the cantilever holder chamber with 120 μL DI water three to four times. Then rinse with the buffer two times. Finally fill the chamber with 110 μL of imaging buffer so that the tip is covered with buffer. Such procedure allows to get the tip fully immersed into the buffer avoiding potential spill to the scanner. Follow the protocol described in [22] for additional information on the instrument operation.
8. Adjust the position of the cantilever so that the laser hits it.
9. When the APS functionalization step is completed, rinse the mica with 40 μL of DI water total by applying ~4 μL for each wash. At the end of fifth wash, place a water droplet and let it sit for 5 min to desorb any leftover nonspecifically bound APS. Then complete the rest of the wash (sixth to tenth). Kim wipes are used to remove water. Caution: Touch the side and not the center of the mica and try to be quick between wash steps to avoid surface drying.
10. Rinse the APS mica five times with 3 μL of imaging buffer containing 10 mM HEPES (pH 7.5) and 4 mM $MgCl_2$ 5 prior to depositing the nucleosome sample.
11. Apply 1.5 μL of freshly prepared nucleosome solution in the imaging buffer. The nucleosome concentration between 0.5 nM and 1 nM provides a good surface coverage.
12. Place the scanner with the sample on the top of the tip holder, so the mica with the sample deposited is faced down, and start the imaging. Follow the instruction described in [22]. A brief description is given below.
13. Auto-approach and use the set point A_s close to the free oscillation amplitude A_0. $A_s = 0.95\ A_0$ is the goal, although operating at even 83 % of the free amplitude can be used with precautions. The set point will be adjusted during the imaging. The cantilever amplitude is another parameter that should be kept at small as possible as the energy provided by the tip to the sample depends on the square of the amplitude value [17]. The instrument operates stably at amplitudes as low as 1 nm [22]. A typical area of 150 × 150 nm contains several nucleosomal particles, the dynamics of which can be analyzed. Fig. 6a shows the set of images taken from [17]. Imaging rate can be as fast as 10–15 frames per second; however even smaller rate, ~300 ms per frame, was sufficient to uncover such details as transient unlooping of nucleosomes and such rare events as transient nucleosome translocations [17].

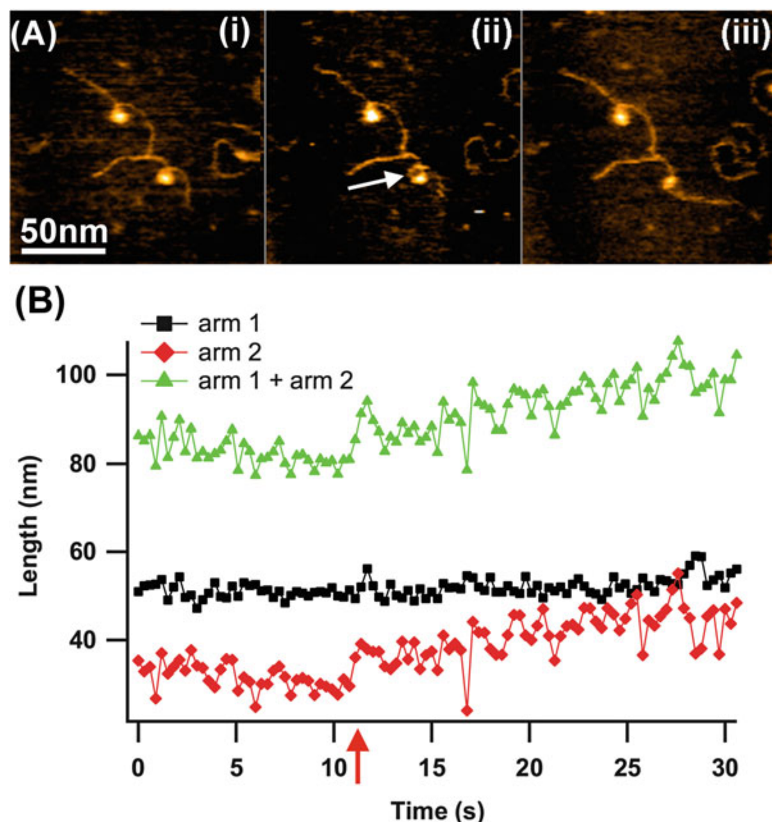
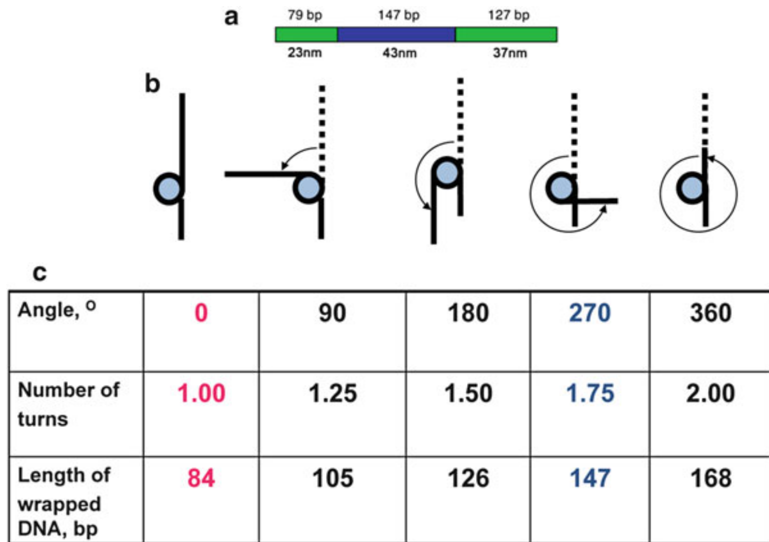


Fig. 6 Loop formation and unfolding. (**a**) A set of images corresponding to 8.7 s (i), 14.7 s (ii), and 17.1 s (iii). In the image (ii), the position of the DNA dissociation and unlooping events are indicated with *white arrow*. (**b**) The length measurements for the looping and the loop unfolding process. The lengths of the left (arm 1) and right (arm 2) arms and the total DNA length are shown with *black squares*, *red diamonds*, and *green triangles*, respectively. In the graph (**b**), the *dashed lines* correspond to the image-acquired times shown in (**a**). The scan rate was one frame per 301 ms. Figure was reproduced from [17] with permission. Copyright (2011) American Chemical Society

3.6 Nucleosome Parameters Calculated from AFM Images

Schematics in Fig. 7 explain how the structural parameters of nucleosomes can be obtained from the AFM images.

The DNA template (353 bp; panel A) contains the sequence with a high specificity for nucleosome binding (147 bp, Widom 601 motif; blue color) located at different distances from the ends. Such an asymmetry was beneficial for structural analysis of nucleosome allowing us to distinguish the dynamics of the left and right flanks during the time-lapse imaging. DNA wraps around the histone core with 1 and ¾ turns to make a crystallographic structure [1]. However the dynamics of this structure can lead to nucleosomes with variable number of DNA turns around the histone octamer and schematically this dynamics is shown in Fig. 7b.



Angle, °	0	90	180	270	360
Number of turns	1.00	1.25	1.50	1.75	2.00
Length of wrapped DNA, bp	84	105	126	147	168

Fig. 7 Schematic for calculation of nucleosome parameters from the AFM images. (**a**) DNA construct used; the nucleosome-specific Widom 601 segment is shown in *blue color*. (**b**) The arrangement of the nucleosome DNA flanks depending on the number of DNA turns within the nucleosome. Table **c** shows the lengths of wrapped DNA depending on the number of the turns and angle between the flanks

The starting position in this set is the nucleosome with one turn around the core. Rotation of the arm by a quarter of turn leads to the nucleosome with 90° angle between the flanks. Further wrapping produces the structure with the parallel orientation of the flanks and additional one-quarter wrapping step leads to the crystallographic structure with 1.75 DNA turns with 147 bp around the core. One more rotation leads to the structure that is geometrically similar to the starting one, but the lengths of flanks are twice shorter. Table c summarizes the structural parameters of the structures described above.

3.6.1 Measuring of the Number of DNA Turns on AFM Images

Two major parameters measured in assigning the number of turns on the AFM images of nucleosomes are the lengths of the free DNA flanks and angles between the flanks. DNA contributes substantially to the nucleosome volume; therefore this parameter can also be used for additional validation of the DNA wrapping values.

1. Select a nucleosome and measure the lengths of both free DNA flanks. Use the table in Fig. 7c to calculate the number of turns.
2. Measure the angles between flanks. Select DNA segments ~10 nm from the attachment point and draw a line. DNA persistence length is ~45 nm, so a 10 nm DNA segment is typically straight.

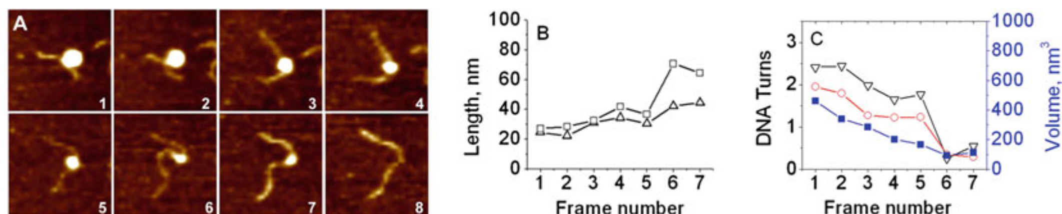


Fig. 8 Time-lapse AFM visualization of the nucleosome unwrapping process. (**a**) Consecutive AFM images of nucleosomes taken during continuous scanning in the buffer. Each frame size is 200 nm. (**b**) Dependence of arm lengths on the frame number. (**c**) The dependence of number of DNA turns around the core calculated from arm lengths (*black*) and from the angle between DNA arms (*red*) on the frame number. The dependence of nucleosome volume on the frame number is shown in *blue* (right *Y*-axis). Each frame takes about 170 s to scan. Figure was reproduced from [12] with permission. Copyright (2009) American Chemical Society

3. The volume measurements: These are not direct measurements of the number of turns due to the tip convolution effect and the model used for the volume measurements. For the volume measurements, the blob was approximated as a segment of a sphere with a diameter measured at half-maximal height of the protein [12]. *See* **Note 4** for additional information. Numbers in Fig. 4 were obtained using the procedure described above.

3.6.2 Analysis of the Nucleosome Dynamics with Time-Lapse AFM Images

1. Acquire a necessary set of frames for the data analysis.
2. Select the areas containing the nucleosome of interest on all frames and analyze each nucleosome for each frame. It is instructive to make the frame assembly for each nucleosome as it is shown in Fig. 8a.
3. Measure the lengths of the flanks for each frame and plot the flank length values against time. *See* Fig. 8b for the reference.
4. Calculate the number of turns based on the length measurements by calculating the length of DNA incorporated into the nucleosome after subtracting the lengths of flanks from the free DNA size (*see* **Note 4**).
5. Measure the inter flank angles and calculate the number of the DNA turns using the table in Fig. 7c.
6. Measure the volumes and make the estimates for the nucleosome size based on these measurements as described in **Note 5**.
7. Generate the plot on the time dependence of the nucleosome dynamics as shown in Fig. 8c (*see* **Note 6**).

3.6.3 Analysis of the Nucleosome Dynamics with HS AFM

The analysis is essentially similar to the one described above and is performed over considerably larger dataset. The length and angle measurements are typically sufficient for the analysis.

1. Select the nucleosome of interest in the dataset and assemble a subset of images to identify the major effect.
2. Perform the length measurements and plot both measured values depending on time.

3. Identify the major events for the analysis. Figure 6 illustrates the unwrapping of the nucleosome accompanied by the formation of a small loop that transiently associates with the core. Note that only one flank changes during this process. Another flank remains unchanged.

4 Notes

1. Depending on the size of the mica strip, the plastic disposable 3 mL cuvettes or plastic 15 mL tubes are suitable for these purposes.
2. As prepared, the APS mica sheets can be stored dry (plastic tubes or cuvettes) in the argon atmosphere for at least a week.
3. Nitrogen gas can be used but it is recommended to use Ar gas as it is heavy and does evaporate from the tube.
4. The length measurements can be performed in nanometers. These values can be converted in the number of base pairs. The conversion coefficient can be obtained from the length measurements of free DNA. Generate the histogram from multiple measurements (100 molecules is sufficient) and divide 353 bp by the mean length number. For APS mica procedure this value is very close to 0.34 nm for B-DNA base pair spacing.
5. The volume measurements can be used, but these are not direct measurements of the number of turns due to the tip convolution effect and the model used for the volume measurements. We recommend to make a calibration plot using the volume measurements for a set of nucleosomes with the number of turns unambiguously determined by the angle and length measurements. Make a graph as a dependence of the volume on the number of the DNA turns. Use the same model of nucleosome for all measurements. The effect of the tip convolution effect can be incorporated by the measurements of the DNA heights and width. Similar parameters justify the measurements.
6. The estimates of the DNA turns obtained by different methods should not exactly be the same, but typically they are close. Each type of measurements has limitation. The length measurements are limited by the identification of the DNA detaching point on the nucleosome image. The nucleosome size on the AFM images is enlarged due to the tip convolution effect and therefore the measured DNA length is shorter as it should be. Therefore, the length of wrapped DNA is increased leading to the elevated values for the DNA turns as it is seen in Fig. 8c. However, the dependence of the values over time should be similar and this is illustrated in Fig. 8c.

Acknowledgements

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Chapter 4

Isolation of Specific Genomic Regions and Identification of Associated Molecules by Engineered DNA-Binding Molecule-Mediated Chromatin Immunoprecipitation (enChIP) Using CRISPR

Toshitsugu Fujita and Hodaka Fujii

Abstract

Isolation of specific genomic regions retaining molecular interactions is necessary for their biochemical analysis. Here, we describe engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using the CRISPR system, for purification of specific genomic regions retaining molecular interactions. In this form of enChIP, specific genomic regions are immunoprecipitated with antibody against a tag(s), which is fused to a catalytically inactive form of Cas9 (dCas9), which is co-expressed with a guide RNA (gRNA) and recognizes endogenous DNA sequence in the genomic regions of interest. enChIP combined with mass spectrometry (enChIP-MS), next-generation sequencing (enChIP-Seq), and RNA-Seq (enChIP-RNA-Seq) can identify proteins, other genomic regions, and RNA, respectively, that interact with the target genomic region.

Key words enChIP, CRISPR, dCas9, gRNA, Mass spectrometry, Locus-specific chromatin immunoprecipitation

1 Introduction

Function of eukaryotic genome is mediated by molecular complexes in the context of chromatin [1]. Elucidation of molecular mechanisms of genome functions requires identification of components mediating the genome function. To purify specific genomic regions retaining molecular interactions in vivo, we recently developed a novel method, engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP), for purification of specific genomic regions [2, 3]. In enChIP, a tagged engineered DNA-binding molecule recognizing an endogenous target DNA sequence such as a zinc-finger protein [4], a transcription activator-like (TAL) protein [5], and the CRISPR (clustered regularly interspaced short palindromic repeats) system consisting of a catalytically inactive form

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of Cas9 endonuclease (dCas9) and small guide RNA (gRNA) [6] is expressed into the cell to be analyzed. Subsequently, the target genomic region is subjected to affinity purification such as immunoprecipitation with an antibody against the tag.

Here, we describe enChIP using the CRISPR system, for purification of specific genomic regions retaining molecular interactions (Fig. 1).

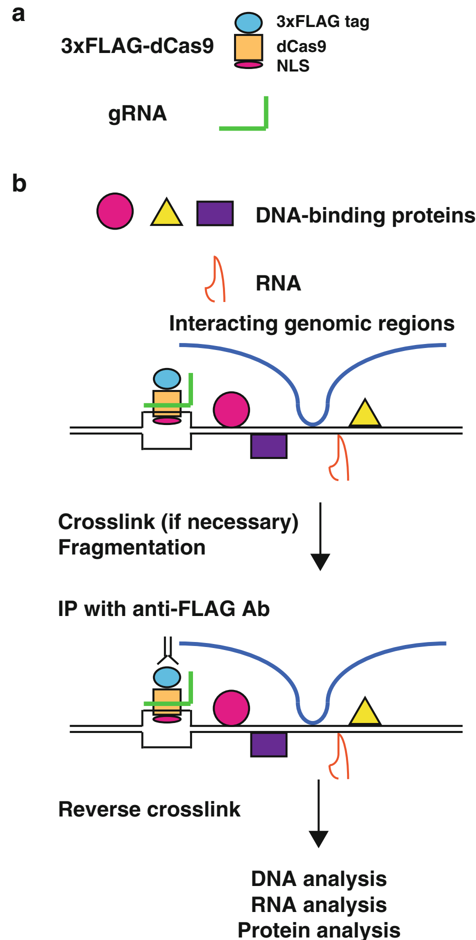

Fig. 1 Overview of engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using the CRISPR system consisting of dCas9 and gRNA. (**a**) The system is composed of a fusion protein, 3xFLAG-dCas9, consisting of 3xFLAG-tag, dCas9, and the nuclear localization signal (NLS) of SV40 T-antigen, and a guide RNA (gRNA). (**b**) 3xFLAG-dCas9 and gRNA are expressed in appropriate cells. The cells are cross-linked, if necessary, lysed, and fragmented by sonication or other methods. The complexes are immunoprecipitated with anti-FLAG Ab, and cross-link is reversed when cross-linkers are used. Molecules (DNA, RNA, proteins, and others) associated with the target genomic region are isolated and characterized

2 Materials

2.1 Construction of gRNA Expression Vectors and Generation of Cells for enChIP Analysis

1. 3xFLAG-dCas9/pCMV-7.1 (catalog number 47948) (Addgene, Cambridge, MA, USA).
2. gRNA Cloning vector (catalog number 41824) (Addgene).
3. Phusion polymerase (catalog number M0530) (New England Biolabs Inc., Ipswich, MA, USA).
4. Gibson Assembly Master Mix (catalog number E2611) (New England Biolabs Inc.).
5. Anti-FLAG M2 antibody (catalog number F1804) (Sigma-Aldrich, St. Louis, MO, USA).
6. Fluorescein isothiocyanate (FITC)-conjugated anti-FLAG M2 antibody (catalog number F4049) (Sigma-Aldrich).

2.2 Formaldehyde Cross-Linking of Cells

1. 37 % Formaldehyde.
2. 1.25 M Glycine solution: Dissolve 18.8 g of glycine in 200 ml water.

2.3 Preparation of Chromatin

1. Cell lysis buffer (CLB): 10 mM Tris (pH 8.0), 1 mM EDTA, 0.5 % IGEPAL CA-630, 1× protease inhibitors (Complete, Mini, EDTA-free (catalog number 04 693 159 001) (Roche Diagnostics GmbH, Mannheim, Germany), 1 tablet/10 ml). Protease inhibitors should be added immediately before use for all solutions.
2. Nuclear lysis buffer (NLB): 10 mM Tris (pH 8.0), 1 mM EDTA, 0.5 M NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.5 % lauroylsarcosine, 1× protease inhibitors.
3. Modified lysis buffer 3 (MLB3): 10 mM Tris (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 0.1 % sodium deoxycholate, 0.1 % SDS, 1× protease inhibitors.

2.4 enChIP

1. Dynabeads-Protein G (catalog number DB10004) (Life Technologies, Carlsbad, CA, USA).
2. Normal mouse IgG (catalog number sc-2025) (Santa Cruz Biotechnology, Dallas, TX, USA).
3. Phosphate buffered saline (PBS) (pH 7.4).
4. PBS-T: PBS (pH 7.4), 0.01 % Tween-20.
5. BSA fraction V (7.5 %) (catalog number 15260) (Life Technologies).
6. PBS-T-BSA: PBS (pH 7.4), 0.01 % Tween-20, 0.1 % BSA.
7. Low-salt buffer (LSB): 20 mM Tris (pH 8.0), 2 mM EDTA, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS.
8. High-salt buffer (HSB): 20 mM Tris (pH 8.0), 2 mM EDTA, 500 mM NaCl, 1 % Triton X-100, 0.1 % SDS.

9. LiCl buffer: 10 mM Tris (pH 8.0), 1 mM EDTA, 0.25 M LiCl, 0.5 % IGEPAL CA-630, 0.5 % sodium deoxycholate.
10. TBS-IGEPAL CA-630: 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1 % IGEPAL CA-630.
11. 3xFLAG peptide (catalog number: F4799) (Sigma-Aldrich).
12. Elution buffer: 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1 % IGEPAL CA-630, 500 μg/ml 3xFLAG peptide.
13. 2-propanol.
14. 3 M sodium acetate buffer solution (pH 5.2).
15. 20 mg/ml glycogen.
16. 70 % ethanol.
17. 2× sample buffer: 125 mM Tris (pH 6.8), 10 % 2-mercaptoethanol, 4 % SDS, 10 % sucrose, 0.004 % bromophenol blue.
18. 4–20 % Mini-PROTEAN TGX Gel, 10 well, 50 μl (catalog number 456-1094) (Bio-Rad).
19. Running buffer solution for SDS-PAGE, Tris-Glycine.
20. ChIP DNA clean & concentrator (catalog number D5205) (Zymo Research, Irvine, CA, USA).
21. RNasin Plus RNase Inhibitor (catalog number N2611) (Promega, Madison, WI, USA).
22. Isogen II (catalog number 311-07361) (Nippon Gene, Tokyo, Japan).

3 Methods

3.1 Design of gRNA and Construction of gRNA Expression Vectors

Construction of gRNA expression vectors is done basically according to the protocol described in Mali et al. [7] with some modifications.

1. Using the **CRISPRdirect** web tool (http://crispr.dbcls.jp), find candidate gRNA target sequences in the genomic region of interest. The output window shows 23 bp genomic sites of the form 5′-N_{20}NGG-3′ within your target region. These sites may reside on the + or – strand (*see* **Note 1**).

5′-NNNNN NNNNN NNNNN NNNNN NGG-3′
PAM

2. Synthesize gBlock as follows. Appropriate sites of restriction enzymes should be added for subcloning purpose. TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCA GTCGACTGGATCCGGTACCAAGGTCGGGCA GGAAGAGGGCCTATTTCCCATGATTCCTTCATATTT GCATATACGATACAAGGCTGTTAGAGAGATAATT

AGAATTAATTTGACTGTAAACACAAAGATATTAGT ACAAAATACGTGACGTAGAAAGTAATAATTTCTTG GGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATG GACTATCATATGCTTACCGTAACTTGAAAGTATTT CGATTTCTTGGCTTTATATATCTTGTGGAAAGGA CGAAACACCGNNNNNNNNNNNNNNNNNNNGTT TTAGAGCTAGAAATAGCAAGTTAAAATAAGGC TAGTCCGTTATCAACTTGAAAAAGTGGCACCG AGTCGGTGCTTTTTTT

3. Alternatively, incorporate 20 bp of the selected target sequence 5′ to PAM: 5′-NNNNN NNNNN NNNNN NNNNN NGG-3′ into two 61-mer oligonucleotides as indicated below. The region of N_{20} in Insert_F corresponds to the abovementioned 20-mer. The region of N_{20} in Insert_R corresponds to reverse complements of the 20-mer.

   Insert_F: 5′-TTTCTTGGCTTTATATATCTTGTGGAAAGG ACGAAACACC*G<u>NNNNNNNNNNNNNNNN NNNN</u>*-3′

   Insert_R: 5′-GACTAGCCTTATTTTAACTTGCTATTTCTA GCTCTAAAAC*<u>NNNNNNNNNNNNNNNNN NNN</u>C*-3′

4. Anneal the two oligos and extend these to make a 101 bp double-strand DNA fragment using Phusion polymerase.
5. Linearize the gRNA cloning vector with *Afl* II and incorporate the 101 bp DNA fragment into the linearized vector using Gibson assembly Master Mix.

3.2 Generation of Cells for enChIP Analysis

1. Express 3xFLAG-dCas9 and gRNA into cells to be analyzed. If transfection efficiency is high, transient transfection can be used. Alternatively, stable transformant clones can be established by co-transfection of 3xFLAG-dCas9/pCMV-7.1 and the gRNA expression vector together with suitable selection marker genes. Sequential transfection of 3xFLAG-dCas9/pCMV-7.1 and the gRNA expression vector would be preferable because different gRNA can be expressed in the clones expressing 3xFLAG-dCas9 (*see* **Note 2**).
2. Confirm expression of 3xFLAG-dCas9 by immunoblot analysis using anti-FLAG Ab. Expression of 3xFLAG-dCas9 can also be detected by intracellular staining using FITC-conjugated anti-FLAG Ab and flow-cytometric analysis (*see* **Note 3**).
3. Confirm expression of gRNA by RT-PCR.

3.3 Formaldehyde Cross-Linking of Cells

1. Culture target cells (*see* **Note 4**). Use 2×10^7 cells × 2 (total 4×10^7 cells) (e.g., Ba/F3) for chromatin preparation. Collect 2×10^7 cells in a 50 ml centrifuge tube × 2. (If necessary, increase cell number).

2. Suspend cell pellets (2×10^7 cells) in 30 ml of medium and add 810 μl of 37 % formaldehyde to 1 % final concentration in the cell suspension. Incubate at 37 °C for 5–10 min (usually 5 min).
3. Stop cross-linking by adding 3 ml of 1.25 M Glycine solution to 127 mM final concentration. Incubate at room temperature for 10 min.
4. Collect cells by centrifugation ($200 \times g$, 4 °C for 5 min).
5. Wash the pellet with PBS twice (30 ml × 2). Collect the pellet (cells). The cells can be stored at −80 °C.

3.4 Preparation of Chromatin (per 2×10^7 Cells)

1. Suspend the fixed cells in 10 ml of CLB. Incubate on ice for 10 min.
2. Centrifuge at $830 \times g$ at 4 °C for 8 min. Discard carefully the supernatant.
3. Suspend the pellet in 10 ml of NLB. Incubate on ice for 10 min. Vortex every 2–3 min.
4. Centrifuge at $830 \times g$ at 4 °C for 8 min. Discard carefully the supernatant.
5. Suspend the pellet in 10 ml of PBS. Centrifuge at $830 \times g$ at 4 °C for 10 min. Collect the pellet as the chromatin fraction. The chromatin fraction can be stored at −80 °C after immediate freezing in liquid nitrogen.

3.5 Sonication of Chromatin (per 2×10^7 Cells)

1. Suspend the collected chromatin fraction in 800 μl of MLB3. Transfer the suspension into a 1.5 ml microtube.
2. Sonicate the chromatin by using Ultrasonic disruptor UD-201 (TOMY SEIKO, Japan). Keep the position of the tip of the sonication bar approximately 0.5 cm away from the tube bottom to avoid foaming. Conditions are as follows:
   - Output: 3; Duty: 100 % (continuous); Time: Free
   - 10–18 cycles of sonication for 10 s and cooling on ice for 20 s
   - 2 min on ice after 5–6 cycles to avoid excessive heating
3. Centrifuge at $16{,}000 \times g$ at 4 °C for 10 min. Collect the supernatant (800 μl). The supernatant can be stored at −80 °C after immediate freezing in liquid nitrogen.

3.6 Evaluation of Fragmentation of Chromatin

1. Suspend 10 μl of the fragmented chromatin in 85 μl of distilled water.
2. Add 4 μl of 5 M NaCl. Incubate at 65 °C overnight.
3. Add 1 μl of 10 mg/ml RNase A. Incubate at 37 °C for 45 min.
4. Prepare 1 % agarose gel without staining dye.
5. Add 2 μl of 0.5 M EDTA (pH 8.0), 4 μl of 1 M Tris (pH 6.8), and 1 μl of 20 mg/ml Proteinase K. Incubate at 45 °C for 1.5 h.

6. Use 10 μl for electrophoresis in 1 % agarose gel without staining dye. Run the gel at 100 V for 30 min.
7. Stain the gel with staining dye (e.g., ethidium bromide) for 0.5–1 h.
8. The remaining samples can be subjected to purification of DNA (*see* Subheading 3.10). The purified DNA can be used as input DNA to calculate yield of enChIP procedure.

3.7 Preparation of Dynabeads Conjugated with Antibody (Anti-FLAG Antibody or Normal Mouse IgG) (per 2×10^7 Cells)

1. Transfer 300 μl of Dynabeads-Protein G in a 2 ml tube.
2. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.
3. Suspend the pellet in 1 ml of PBS-T. Put the tube on a magnet stand and wait for 2 min. Discard the supernatant by pipetting.
4. Repeat the step (Subheading 3.7, **step 3**).
5. Suspend the pellet in 1.5 ml of PBS-T-BSA.
6. Add 30 μg of antibody (anti-FLAG antibody or normal mouse IgG). Rotate at 4 °C overnight.
7. Spin down briefly. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.
8. Suspend the pellet in 1.5 ml of PBS-T. Invert several times and spin down briefly. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.
9. Repeat the step (Subheading 3.7, **step 8**), twice. The Dynabeads are ready for the next step.

3.8 Chromatin Immunoprecipitation

1. Transfer 1.6 ml of the fragmented chromatin, which corresponds to chromatin extracted from 4×10^7 cells, into a 2 ml tube.
2. Add 400 μl of 5 % Triton X-100 (in MLB3) to 1 % final concentration.
3. Transfer all (2 ml) of the chromatin solution into the tube, in which the Dynabeads conjugated with normal mouse IgG were prepared. Rotate at 4 °C for 1 h.
4. Put the tube on a magnet stand and wait for 3 min.
5. Transfer the supernatant into the tube, in which the Dynabeads conjugated with anti-FLAG antibody were prepared. Rotate at 4 °C overnight.
6. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.
7. Wash one and two: Add 1.8 ml of LSB. Rotate at 4 °C for 5 min. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant by pipetting. Repeat again (total two times).
8. Wash three and four: Repeat the step (Subheading 3.8, **step 7**) with HSB (two times).

9. Wash five and six: Repeat the step (Subheading 3.8, **step 7**) with LiCl buffer (two times).
10. Add 1.8 ml of TBS-IGEPAL CA-630. Rotate at 4 °C for 5 min. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.
11. Elution: Add 200 μl of elution buffer. Incubate at 37 °C for 20 min. Put the tube on a magnet stand and wait for 3 min.
12. Transfer the supernatant (200 μl) into the 1.5 ml microtube (*see* **Note 5**). The eluate can be used for purification of proteins (Subheading 3.9), DNA (Subheading 3.10), or RNA (Subheading 3.11).

3.9 SDS-PAGE, Staining, Mass Spectrometric Analysis

1. Mix eluate (200 μl) with 500 μl of 2-propanol, 25 μl of 3 M sodium acetate buffer solution (pH 5.2) and 5 μl of 20 mg/ml glycogen. Precipitate proteins at −20 °C overnight.
2. Centrifuge at 16,000 × *g* at 4 °C for 30 min. Discard the supernatant.
3. Rinse with 1 ml of 70 % ethanol. Centrifuge at 1,600 × *g* for 10 min at 4 °C. Discard the supernatant completely by pipetting.
4. Add 40 μl of 2× sample buffer. Vortex for 5 min to completely solve the precipitant. Incubate at 100 °C for 30 min (protein denaturing and reverse-cross-linking).
5. SDS-PAGE. Run the eluate on polyacrylamide gel until the dye reaches 1 cm from the well.
6. Coomassie Brilliant Blue (CBB) staining or silver staining.
7. Cut the gel into 5 pieces × 2 mm. An example of gel image is shown in Fig. 2 in the ref. [2].
8. In gel digestion and mass analysis. Our current system is:

   A nanoLC-MS/MS system, composed of LTQ Orbitrap Velos (Thermo Fisher Scientific) coupled with nanoLC (Advance, Michrom BioResources) and HTC-PAL autosampler (CTC Analytics).

3.10 Purification of DNA

1. Add 8 μl of 5 M NaCl and 0.4 μl of 0.5 M EDTA to the eluate (200 μl) (Subheading 3.8, **step 12**). Incubate at 65 °C, overnight.
2. Add 3 μl of 10 mg/ml RNase A. Incubate at 37 °C for 1 h.
3. Add 10 μl of 10 % SDS and 5 μl of 20 mg/ml Proteinase K per 200 μl of eluate. Incubate at 45 °C for 2 h.
4. Purify DNA using ChIP DNA Clean & Concentrator kit (Zymo Research, D5205).
5. The purified DNA is ready for downstream analyses such as PCR, microarray analysis, and next-generation sequencing.

3.11 Purification of RNA

1. For purification of RNA, the enChIP procedure is performed as described above with 5 U/ml of rRNasin Plus in all the buffer solution except for MLB3 in which 40 U/ml of rRNasin Plus is added.
2. Add 8 μl of 5 M NaCl and 0.4 μl of 0.5 M EDTA to the eluate (200 μl) (Subheading 3.8, **step 12**). Incubate at 65 °C, overnight.
3. Add 10 μl of 10 % SDS and 5 μl of 20 mg/ml Proteinase K per 200 μl of eluate. Incubate at 45 °C for 2 h.
4. Purify RNA with 1 ml of Isogen II.
5. The purified RNA is ready for downstream analyses such as RT-PCR, microarray analysis, and next-generation sequencing (RNA-Seq).

4 Notes

1. Binding of the CRISPR complex may affect chromatin structure such as nucleosome positioning and abrogate normal genome activities such as gene expression. Although the effects of binding need to be tested empirically for each locus, the author's group has guidelines to avoid potential aberrant effects caused by binding of the CRISPR complex. (a) For analysis of promoter regions near transcription start sites (TSSs), the gRNA binding site should be several hundred base 5′ to the TSS so that the binding would not inhibit transcription or disrupt nucleosome positioning. In contrast, for identification of binding molecules of genomic regions with distinct boundaries such as enhancer or silencer, the binding site of gRNA can directly be juxtaposed to the regions because of less probability of inhibition of their function. (b) The binding site should not be conserved among species because conserved regions are often binding sites of conserved binding molecules. In this regard, however, since multiple gRNA can be generated easily, a trial-and-error approach can work.
2. Retroviral systems are available through Addgene.
3. The protocol of intracellular staining and flow-cytometric detection of the FLAG epitope can be downloaded from our laboratory website:
   http://www.biken.osaka-u.ac.jp/lab/microimm/fujii/top/index_e.html
4. For SILAC (stable isotope labeling using amino acids in cell culture) experiments, culture a set of comparison group cells in light medium and heavy medium. SILAC media can be purchased from Thermo Fisher Scientific and other vendors.
5. Elution step can be repeated to increase the yield.

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Chapter 5

Drug-Induced Premature Chromosome Condensation (PCC) Protocols: Cytogenetic Approaches in Mitotic Chromosome and Interphase Chromatin

Eisuke Gotoh

Abstract

Chromosome analysis is a fundamental technique which is used in wide areas of cytogenetic study including karyotyping species, hereditary diseases diagnosis, or chromosome biology study. Chromosomes are usually prepared from mitotic cells arrested by colcemid block protocol. However, obtaining mitotic chromosomes is often hampered under several circumstances. As a result, cytogenetic analysis will be sometimes difficult or even impossible in such cases. Premature chromosome condensation (PCC) (*see* **Note 1**) is an alternative method that has proved to be a unique and useful way in chromosome analysis. Former, PCC has been achieved following cell fusion method (cell-fusion PCC) mediated either by fusogenic viruses (e.g., Sendai virus) or cell fusion chemicals (e.g., polyethylene glycol), but the cell fusion PCC has several drawbacks. The novel drug-induced PCC using protein phosphatase inhibitors was introduced about 20 years ago. This method is much simpler and easier even than the conventional mitotic chromosome preparation protocol use with colcemid block and furthermore obtained PCC index (equivalent to mitotic index for metaphase chromosome) is usually much higher than colcemid block method. Moreover, this method allows the interphase chromatin to be condensed to visualize like mitotic chromosomes. Therefore drug-induced PCC has opened the way for chromosome analysis not only in metaphase chromosomes but also in interphase chromatin. The drug-induced PCC has thus proven the usefulness in cytogenetics and other cell biology fields. For this second edition version, updated modifications/changes are supplemented in Subheadings 2, 3, and 4, and a new section describing the application of PCC in chromosome science fields is added with citation of updated references.

Key words Cytogenetic study, Premature chromosome condensation (PCC), Prematurely condensed chromosomes (PCCs), Protein phosphatase inhibitors, Calyculin A, Okadaic acid, Mitotic index, PCC index

1 Introduction

Analyzing the chromosomes is widely performed in various areas in cytogenetic fields, such as heredity genetics, mutagenic studies, and radiation biology. Conventional chromosome analysis has been achieved by obtaining mitotic phase arrested chromosomes (mitosis) utilized with colcemid block method. The established

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colcemid block protocol is very popular and widely used; however, the artisans have frequently faced the problems that it has been often difficult or even impossible to obtain a sufficient number of mitotic chromosomes from the cells under certain circumstances: cells growing at a slow rate or even arresting, some kinds of tumor cells or highly damaged cells after exposure of high doses of cell-damaging agents such as ionizing irradiation [1], because these cells arrest cell cycle and do not reach mitosis. An alternative useful method is premature chromosome condensation (PCC) method [2, 3] that facilitates the visualization of interphase chromatin as condensed form of chromosomes [3–7]. Therefore, a lot of literature using this method has been published in cytogenetic studies, particularly in radiation biology and DNA replication/repair analysis [8–12]. PCC has been usually achieved by fusing the recipient interphase cells with mitotic donor cells utilized with either fusogenic virus (i.e., Sendai virus) or polyethylene glycol (cell-fusion-mediated PCC or fusion-PCC) [2, 13]. However, fusion-PCC has several disadvantages: (1) the fusion efficiency is highly dependent on virus activity, and on polyethylene glycol (PEG) quality or cell viability, and the resulting PCC index can be often very low; (2) fusion-PCC protocol is time consuming and technically demanding; and (3) the obtained chromosomes are mixture of mitotic inducer cells and recipient interphase cells, and hence differential replication staining of mitotic cells is often required for distinguishing recipient cells from donor cells [1, 8]. Thus, fusion-PCC has been utilized in the limited number of laboratories, although it has been recognized as a quite valuable tool for chromosome analysis [3, 9–11, 14–19]. PCC induction by chemical agents (drug-induced PCC) was therefore desired and a number of approaches have been carried out [20–22]. The first successive drug-induced PCC was introduced about 10 years ago; calyculin A or okadaic acid, specific inhibitors of protein serine/threonine phosphatase, directly induces premature chromosome condensation in mammalian cells in any phase of cell cycle [23]. Drug-induced PCC has several advantages over colcemid block or fusion-PCC: (1) It is very easy to achieve by simply substituting the use of colcemid to calyculin A or okadaic acid in known chromosome preparation protocol [24]. (2) Approximately only 30-min incubation of calyculin A induces a sufficient number of chromosomes, that is much shorter than colcemid protocol (usually 2–4 h). Incubation time can be much shortened to 5–10 min owing to the experiment design [7, 25]. (3) PCC index is usually much higher (>20 %) than mitotic index (usually 1–2 %, but will be often much lower) that facilitates the observation and analysis of chromosome much easier [1]. Therefore, drug-induced PCC has been becoming popular and many articles using the technique have been published [7, 26–40]. In addition, the drug-induced PCC has an outstanding merit that allows the interphase chromatin (i.e., G1-, S-, and

G2-phase chromatin) to be visualized as condensed form of chromosome structure [1, 23]. Hence, the drug-induced PCC method will be a useful way being not only an alternative method for mitotic chromosome analysis but also a new analytical tool for interphase chromatin cytogenetics [1]. In this new edition chapter, the more detailed protocol of drug-induced PCC is described updated from the previous edition [41]. The protocol is very simple as easily recognized by the unskilled person in chromosome preparation and it is hoped that the drug-induced PCC will be used in wider ranges in cytogenetics and related fields.

2 Materials

1. Calyculin A ($C_{50}H_{81}N_4O_{15}P = 1{,}009.17$, Wako Chemicals cat. No. 038-14453 10 μg, cat. No. 032-14451 100 μg) (*see* **Note 2**). Figure 1a shows chemical structure for calyculin A.
   - (a) Reconstitution of calyculin A for 100 μM stock solution.

     For 100 μg of calyculin A (Wako Chemicals cat. No. 032-14451) lyophilized in a vial supplied from the supplier, dissolve with 1 mL of solvent (100 % DMSO or 100 % ethanol, as calyculin A does not dissolve in water) which will make approximately 100 μM stock solution (M.W. of calyculin A is 1009.17). Aliquot in 100 μL in a microcentrifuge tube and stored at –20 °C and can be stable for at least 3 months (*see* **Notes 3** and **4**).
2. 0.075 M KCl.
   - (a) 7.45 g of KCl (M.W. 74.55): Dissolve in 100 mL MilliQ water to make a 1 M KCl stock solution.
   - (b) Take 7.5 mL of the 1 M stock solution and make it up to 100 mL with MilliQ water to obtain a 0.075 M KCl working solution.
3. Carnoy's fixative (methanol:glacial acetic acid = 3 parts:1 part).
   - (a) Take three parts of methanol and one part of glacial acetic acid (for example 150 mL of methanol and 50 mL of acetic acid for making 200 mL fixative) and mix together well. The temperature of mixture will be increased according to exothermic reaction.
   - (b) Preferable to prepare just before use; however the fixative can be stored at –20 °C for a month. After stored in longer time, esterification will proceed resulting in production of methyl acetate and water. Consequently, fixative smells some ester fragrance, and when dropped on a glass slide, fixative does not spread well circularly and water will be remained after evaporation. In this case, new fixative should be prepared; otherwise chromosome does not spread well.

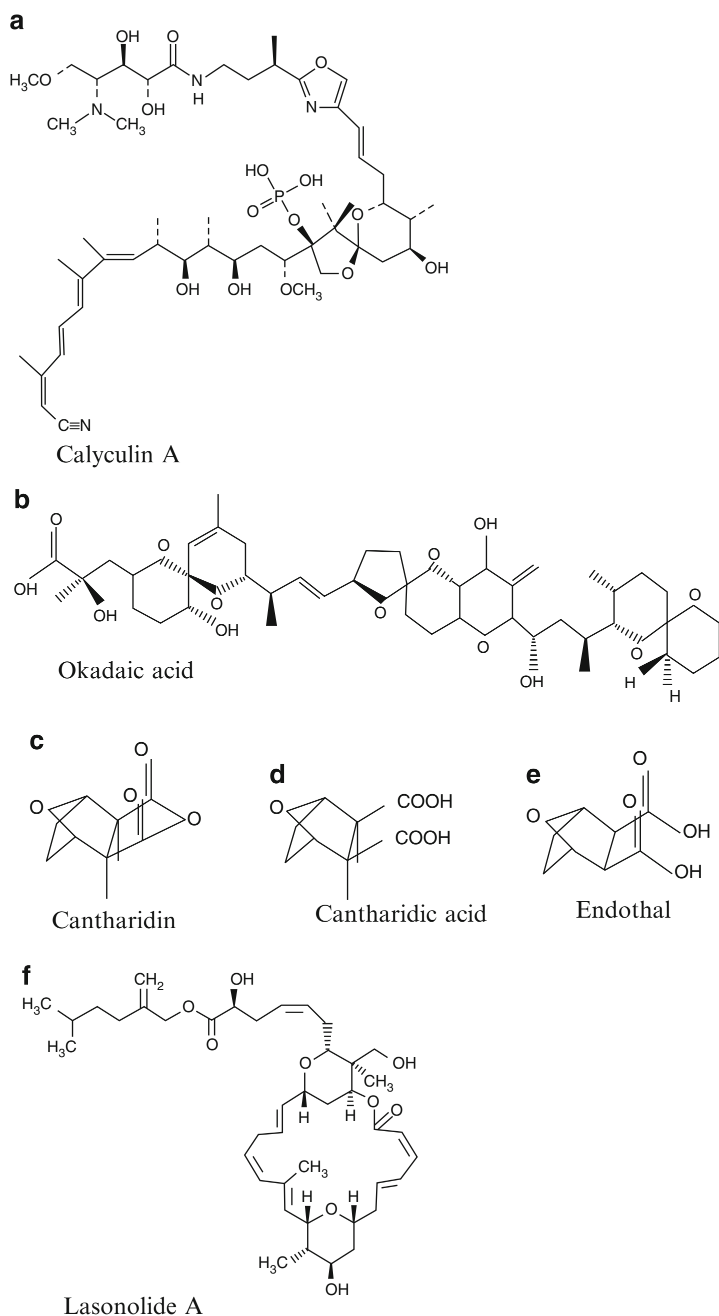

Fig. 1 Chemical structures of several major protein phosphatase inhibitors. (**a**) Calyculin A. *Source*: marine sponge (*Discodermia Calyx*), Chemical structure: $C_{50}H_{81}N_4O_{15}P$, Molecular Weight (M.W.): 1,009.18 (**b**) Okadaic acid. *Source*: marine sponge (*Halichondria Okadai* (Kadota)), $C_{44}H_{68}O_{13}$, M.W. 805.2 (**c**) Cantharidin. *Source:* Blister Beetle (*Meloe corvinus*), $C_{10}H_{12}O_4$, M.W. 196.2 (**d**) Cantharidic acid. $C_{10}H_{14}O_5$, M.W. 214.2 (**e**) Endothal. $C_8H_{10}O_5$, M.W. 186.2 (**f**) Lasonolide A. *Source*: marine sponge (*Forcepia* sp.), $C_{41}H_{60}O_9$, M.W. 696.90

4. 10 mM Phosphate buffer (pH 6.8 at 25 °C) for Giemsa staining.
   (a) Solution A: Dissolve 136.09 g KH_2PO_4 with 1 L MilliQ water to make 1 M stock solution.
   (b) Solution B: Dissolve 174.18 g K_2HPO_4 with MilliQ water to make it up to 1 L for 1 M stock solution.
   (c) Mix 50.3 mL solution A with 49.7 mL solution B and make it up to 1 L with lliQ water to make 10 mM working solution. If desired, adjust the pH before the final mess up to 1 L.
   (d) Gurr Buffer tablet (one tablet for 1 L solution, pH 6.8, available from Invitrogen, cat. No. 10582013) or phosphate buffer powder (for 1 L solution, 1/15 M, pH 6.8, available from Wako Chemicals, cat. No. 163-16471) are for example also used well for a convenient substitution for phosphate buffer for Giemsa staining.
5. Giemsa dye solution (Merck KGA cat. No. 1.09204.0100).
   Dilute with 10 mM phosphate buffer (pH 6.8 at 25 °C) or Gurr buffer to make 5 % (4–6 %) solution (freshly prepare just before use) (*see* **Note 9**).
6. Glass slides and cover slips.
   Glass slides are soaked in methanol until use (*see* **Note 10**). Wipe out methanol with KIMWIPES (KIMTECH SCIENCE).

3 Methods

3.1 Drug-Induced Premature Chromosome Condensation for Adherent Cells

3.1.1 Cell Culture, Premature Chromosome Condensation, and Cell Harvest

To achieve a high PCC index, cells should be plated on culture dishes 1–2 days before experiment, as the cells reach in 70–80 % confluent and growing exponentially at the experiment (*see* **Note 5**). One 35 mm culture dish of cells is usually sufficient to obtain a substantial number of PCCs for analyzing the one data point of the experiment. Add 1/2,000 culture medium volume of 100 μM calyculin A (50 nM final concentration) to the medium, and then incubate for another 30 min at 37 °C (*see* **Note 6**). After incubation, most cells turn round shape and loosely attach on flask. The cells are gently pipetted with medium using a 1,000 mL Pipetman with a blue tip, and cells easily detach and suspend in the medium. It is no use of trypsinization or no use of cell scraper for detaching the cells as usual done in colcemid block protocol, because the cells become round and attach very loosely on the dishes after calyculin A exposure. If the cells do not become round and detach from the dish, it suggests loss of calyculin activity. In such case, try again using new aliquot calyculin A. Move the cell-suspended medium into the centrifuge tube, centrifuge at 250×*g* for 5 min, and discard the supernatant by inverting the tube (*see* **Note 7**).

3.1.2 Chromosome Preparation

Loosen the cell pellet by tapping and shaking the tube and add gently prewarmed (37 °C) 1.5 mL of 0.075 M KCl to suspend the cells for hypotonic treatment (*see* **Note 8**).

Incubate at 37 °C for 20 min. Fix cells by slowly adding the same volume of cold Carnoy's fixative to the medium and mix well gently. Centrifuge at 250 × *g* for 5 min, discard the supernatant, loose the cell pellet with small amount of remained fixatives, add the fixative, and centrifuge again. Repeat three to four times these steps for complete fixation. After fixation, cells were finally suspended in adequate volume of fixative (200–300 μL for 35 mm dish culture of cells). Chromosome suspension (~15 μL using a Pipetman with a yellow tip) was dropped on a glass slide and air-dried. To make sure that the cells are certainly condensed prematurely, observe the chromosome spreads under a microscope in low magnification before staining. As usual, many chromosome spreads will be seen like fireworks in the sky, and 10–20 % PCC index will be achieved. Stain with 5 % Giemsa dye solution, cover by a cover slip, and mount with sealant for microscopic observation (*see* **Note 9**). Usually, one sample slide may give a substantial number of PCCs for karyotyping or chromosome aberration analysis. Unstained chromosome specimens will be stored in a desiccated container and will be subjected to several special banding procedures (G-, Q-, or R-banding), in situ hybridization including FISH, or immunostaining.

3.2 Drug-Induced PCC for Suspension Cells

3.2.1 Cell Culture, Premature Chromosome Condensation, and Cell Harvest

The protocol for suspension cells such as peripheral blood lymphocytes is basically the same as for adherent cells. For suspension cells, okadaic acid (Fig. 1b) also works well as calyculin A (*see* **Note 4**). Figure 1 shows the chemical structure of okadaic acid.

Adjust appropriate cell concentration of suspension cells (i.e., 10^5–10^6 cells/mL, depends on cell types used for experiment) as the cells maintain keep on exponentially growing at the time of PCC induction (*see* **Note 5**). Prior to the PCC induction, it is convenient to transfer the cell suspension in a round-bottomed centrifuge tube (*see* **Note 7**). One mL of suspended cell culture is usually sufficient to obtain a substantial number of PCCs for analyzing the one data point. Add 50 nM final concentration of calyculin A to the culture medium, and process as same as the protocol for attached cells.

3.2.2 Chromosome Preparation

After 30-min incubation with calyculin A, harvest the cells by centrifuging and subject to hypotonic treatment, cell fixation, and spreading on a glass slide as same as adherent cells (*see* Subheading 3.1.2).

3.3 Application of PCC Technique for Various Cytogenetic Research

PCC is a very useful tool for cytogenetic fields. Chromosomes are usually prepared from mitotic arrest cells using colcemid block method which simply inhibits assembly of spindle body at mitotic phase. In contrast, PCC forces the chromosomes to be condensed "prematurely" not only in mitosis but also in interphase nuclei of

any cell cycle (G1-, S-, G2-, M-phase) [23]; hence interphase nuclei are allowed to be visualized as condensed chromosome form like mitotic chromosomes. Due to this unique aspect, PCC has been used for analyzing various nuclear events that proceed during interphase, such as chromosome breakage and repair after exposure of ionizing irradiation or mutagens using either fusion-mediated PCC [8, 16, 42–45] or chemical-mediated PCC [26, 27].

Since the first report of PCC phenomenon (fusion-PCC) [2, 13], PCC technique has been applied in particular in radiation biology, because chromosomes are easily damaged following ionizing irradiation, and analyzing of chromosome damage followed by repair has been widely performed using PCC-fusion method [8–10, 17]. One of the most successful applications of PCC technique is on radiation biodosimetry, in particular for estimating the irradiation dose after accidental exposure of large-dose irradiation [27, 35]. Estimation of body-absorbed dose by means of scoring chromosomal aberrations is widely used as a standard biodosimetry. Prepare and score chromosomes of peripheral blood lymphocytes, and use with colcemid block protocol which is a simple and popularly established method (cytogenetic biodosimetry, see reviews by Lloyd [46] or International Atomic Energy Agency (IAEA) 1986 [47]. However, after human body is irradiated with large doses (i.e., greater than 10 Gy γ-ray equivalent dose for whole body), the cells in the peripheral blood arrest at G2 or G1 phase and do not enter mitosis or even undergo mitotic cell death or apoptosis. As a consequence, it is usually impossible to obtain chromosome from severely damaged cells for cytogenetic analysis, which has limited the application of the conventional colcemid block method for cytogenetic biodosimetry to estimate radiation doses above 10 Gy. This dose limitation was first overcome using drug-induced PCC technique [23], and showed that the maximum estimable dose was 40 Gy of γ-rays [27]. Since then, many studies have been reported about developing the biodosimetry for high-dose irradiation accidents [36, 48–55]. PCC technique, either fusion-induced PCC or drug-induced PCC, has been qualified as standard methodologies for the cytogenetic biodosimetry following irradiation accidents [40, 56].

New approaches for chromosome studies such as DNA replication or chromosome condensation studies have been challenged utilizing PCC technique. For example, it was classically thought that chromosomes condense during mitotic phase. However, recent findings revealed that chromosome condensation does not proceed solely during mitosis, but chromosome condensation is tightly coupled with DNA replication or chromosome repair process [57–60]. However, visualizing chromosome condensation dynamics coupled with DNA replication is somehow limited, because it must require observing chromosomes in S-phase. However, chromosomes are usually invisible in S-phase as they are de-condensed in interphase nuclei.

Visualizing dynamics of chromosome condensation has been accomplished using drug-induced PCC technique [7], which showed that chromosome structure formation proceeding during S-phase is very tightly coupled with DNA replication.

Molecules (proteins/enzymes: such as condensin and cohesin, SMC proteins) involved in chromosome condensation mechanism have been recently clarified [61]. Involvement of condensin II in sister chromatid separation during the S-phase has also been elucidated by means of drug-induced PCC [62]. SMC5/6 complex plays a role in chromosome assembly as they regulate DNA replication progression during interphase [63].

The precise mechanism of chromosome condensation is still almost unclear and therefore precise mechanism of PCC induced by protein phosphatase is also still mostly unclear. PCC is partly the similar but not completely the same phenomenon as normal chromosome condensation [64–66], but elucidating the PCC phenomenon will provide a new insight into understanding the mechanism of eukaryote chromosome condensation [67]. Accumulated evidence has revealed several molecules that might be playing key roles in PCC induction. Maturation/mitosis-promoting factor (MPF) is a key enzyme that induces PCC or GVBD in somatic cell or oocyte, respectively. MPF is now known as the complex of p34cdk2/cyclinB [68–70] and is playing a central role in cell cycle regulation and cell growth control. Activation of p34cdk2/cyclinB complex (phosphorylated form) starts at mitotic stage and accumulated activated p34cdk2/cyclinB complex promotes chromosome condensation [71]. Activation of p34cdk2/cyclinB is negatively regulated by cdc25 and cdc25 is sensitive to type 1 and type 2A protein phosphatases such as okadaic acid and calyculin A [72–74]. These inhibitors may influence the activity of cdc25 and p34cdk2/cyclinB [73, 74]. Activated p34cdk2/cyclinB complex may finally promote premature chromosome condensation [1, 24] (*see* **Note 11**).

A number of molecules which may involve in the mitotic events have been recently identified such as condensin (chromosome condensation), cohesion (chromosome cohesion of replicated chromosomes) [61], or aurora kinases in centromere function [75–77]. These molecules cooperatively regulate the condensation/compaction and formation of eukaryote chromosomes, but the detail mechanism is still unknown and will be elucidated in the future.

4 Notes

As easily recognized, the drug-induced PCC is very simple way whereby replace colcemid with calyculin A in established chromosome preparation protocol. However, there are some keys or tips for achieving high PCC index and good-quality PCCs. Following

are the additional remarks or methodological subtleties that I have experienced through my study.

1. PCC usually means the phenomenon or method, and the condensed interphase chromatin is called as prematurely condensed chromosomes (PCCs). They give the similar abbreviations that are usually used interchangeable.
2. Wako Chemicals is in Japan (Osaka, Japan). Calyculin A is now available from many other chemical suppliers over the world (Subheading 2).
3. It is hard to see and weigh out the lyophilized powder in the vial. So, put off the rubber plug very gently so as not to blow out the lyophilized powder. Pour the solvent (ethanol or DMSO) into the vial using a Pipetman, gently slant and swirl the vial to dissolve the whole chemicals, and transfer the solution to the microcentrifuge tube (e.g., Eppendorf tube). Sometimes, lyophilized powder attaches on the rubber seal. In such cases, pour small amount of solvent to lyophilized powder on rubber seal using Pipetman with a yellow tip, gently pipetting several times to resolve them, then back the solution into the vial and adjust the final volume with additional solvent. If dissolved in ethanol, the screwed lid with a rubber seal-type tube is preferable (Subheading 2); otherwise ethanol will escape in vapor after repeated use and the concentration of calyculin A will change. Accordingly, DMSO is more preferable for dissolving calyculin A because DMSO is a very less evaporative solvent.
4. Okadaic acid (Fig. 1a), its salts or derivatives, or other inhibitors cantharidin, cantharidic acid, or endothal (Fig. 1c–e, respectively) can be also used as PCC inducers [23, 26, 27], but these work well only in suspension-type cells (for example peripheral blood lymphocytes) but not in adherent cells. The reason is unclear and presumably due to the different inhibitory power against protein phosphatase, different cell permeability, or cell sensitivities to these chemicals. Therefore, the use of calyculin A is recommended for PCC induction both in suspension and adherent types of cell. If okadaic acid is used in suspension cells, 100 nM final concentration rather than 50 nM will work better (Subheadings 3.1.1 and 3.2.1).
5. The PCC efficiency is highly dependent on cell types, cell-growing condition, dose, or incubation time of calyculin A. Usually, 50 nM final concentration of calyculin A works very well in many kinds of cells and a sufficient number of PCCs will be obtained following 30 min of incubation. However, some cells show the resistance to calyculin A but others are hypersensitive to calyculin A. In such cases, start the experiment at first with 50 nM concentration and for 30-min incubation time, and then increase or decrease the dose and/or the incubation time depending on the cell response and resulting PCC index. Keep in mind

that the cells should be growing exponentially at the induction of PCC; otherwise PCC index will decrease. In the case of adherent types of cells, cells should be replated 1–2 days before the experiment to reach 70–80 % confluency at the time of PCC induction. Avoid replating the cells at the day of the experiment because the cells arrest cell cycle after plating. The higher the dose and the longer the incubation time, the higher the PCC index but the resulting PCCs will become hyper-condensed. Therefore, the appropriate dose and incubation time should be taken on balance depending on the experiment design. PCC already starts 5 min after beginning calyculin exposure, and 10 min of incubation will induce a substantial number of PCCs [7, 25, 31] (Subheadings 3.1.2 and 3.2.2), but incubation for 30 min is usually taken for obtaining sufficient number of chromosomes.

6. Usually, calyculin A solely works efficiently and gives a high PCC index. Combinational use of colcemid with calyculin A will be sometimes effective in obtaining the higher PCC index [29].

7. A round-bottomed centrifuge tube (for example Becton & Dickinson 5 mL polystyrene centrifuge tube with snap cap, cat. No. 352058) is preferable for chromosome preparation than the conical bottomed tube, so the cells are less packed after centrifugation so as to avoid the cell aggregation (Subheadings 3.1.1 and 3.2.1).

8. 0.075 M KCl is usually used for hypotonic treatment for chromosome preparation. However, if cells do not swell well use with 0.075 M KCl; alternative hypotonic buffers such as 0.45 % sodium citrate and 0.075 M KCl (1:1 vol/vol) [78] or 1 % sodium citrate with 2 % human serum albumin [79] are recommended to use.

9. The tint of Giemsa dye staining varies as the pH of phosphate buffer shifts; color turns from reddish purple (pH 6.8) to bluish purple (pH 7.2). Which pH of phosphate buffer to be used depends on the individual favorite, but reddish purple color seems to be better for observation and taking images under a microscope (Subheadings 3.1.2 and 3.2.2).

10. Formerly, glass slides should be pretreated with methanol or ethanol; glass slides would be dirty with spilled oil during cutting of the glass. However, the glass slides recently provided by makers are very clean and not dirty with oil. So it is usually of no problem to use the glass slide as it is (Subheading 2, **item 4**).

11. Calyculin A, okadaic acid, or other phosphatase inhibitors such as cantharidin, cantharidic acid, or endothal are able to induce PCC predominantly in G2- and S-phases but only few in G1- and G0-phases [1]. Presumably drug-induced PCC exploits the activation of intracellular MPF by phosphatase inhibitors, but the amount of intracellular maturation promoting factor (MPF,

also known as p34cdc2/cyclinB complex) [68–70, 80, 81] is very low in G1- or G0-phase [1, 82]. In the cases that PCC analysis is requested in G0- or G1-phase of cells, the conventional fusion-PCC is still worthwhile to do. Recently new protein phosphatase inhibitor lasonolide A (Fig. 1f) is shown to be possible inducer of PCC even in G0 cells [83]. In their report, lasonolide A seems to be much useful agent for PCC inducer in particular in G0 cells, but the chemical is not commercially available at the present time and the drug is only available in limited laboratories [83], so far as I know. So the usefulness of lasonolide A still remains to be verified in the future study. Another approach of drug-induced PCC in G0-phase peripheral blood lymphocytes was reported by combination use of adenosine triphosphate (ATP) and p34cdc2/cyclinB with okadaic acid [84]. The protocol of fusion-PCC or okadaic acid:p34cdc2/cyclinB:ATP-induced PCC has been described elsewhere [2, 11, 13, 48, 84].

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Chapter 6

Analysis of Genomic Aberrations Using Comparative Genomic Hybridization of Metaphase Chromosomes

Melanie A. Carless

Abstract

Comparative genomic hybridization (CGH) allows the global screening of copy number aberrations within a sample. Specifically, large (>20 mb) deletions and amplifications are detected, based on utilization of test and reference (karyotypically normal) DNA. These samples are whole-genome amplified by DOP-PCR and then differentially labeled with fluorophores via nick translation. Test and reference samples are competitively hybridized to normal metaphase chromosomes. The relative amount of each DNA that binds to a chromosomal locus is indicative of the abundance of that DNA. Thus, if a chromosomal region is amplified, the test DNA will out-compete the reference DNA for binding and fluorescence will indicate amplification. Conversely, if a region is deleted, more reference DNA will bind and fluorescence will indicate a deletion. The following chapter outlines the protocols used for CGH analysis of metaphase chromosomes. These protocols include metaphase chromosome slide preparation, DNA extraction (from blood, cell lines, and microdissected formalin-fixed paraffin-embedded tissue), DOP-PCR, nick translation, in situ hybridization, and fluorescence microscopy and image analysis.

Key words Comparative genomic hybridization (CGH), Microdissection, Degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR), Nick translation, Fluorescence microscopy, Copy number aberrations (CNAs)

1 Introduction

1.1 Comparative Genomic Hybridization

Comparative genomic hybridization is a molecular cytogenetic technique that enables the global screening of DNA sequence copy number aberrations (CNAs) within a sample in a single experiment. Pioneered by Kallioniemi and colleagues, CGH is used primarily to investigate tumors and detects large copy number changes such as amplifications and deletions that are typically greater than 20 mb in size [1, 2]. The principle of this technique is that genomic regions of deletion or amplification are likely to harbor tumor suppressor genes and oncogenes, respectively, which may contribute to a malignant phenotype. Other applications of CGH include prenatal, postnatal, and pre-implantation screening (for detection of trisomies, monosomies, etc) and detection of

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aberrations related to mental retardation [3]. A more recent application of array-based CGH has allowed increased mapping resolution for the detection of locus-by-locus DNA copy number changes [4]. This chapter will deal with methodologies for "chromosome CGH"; detailed methodologies for array CGH have been reviewed elsewhere [5–9] and include information on amplification, nick translation, hybridization, imaging, and analysis.

CGH analysis involves DNA extraction from both test (usually tumor) and reference (karyotypically normal) samples, followed by universal amplification of the DNA [2]. Amplified products then undergo nick translation whereby the test and reference DNA are differentially labeled with fluorescent dyes and are hybridized to normal metaphase chromosomes in the presence of COT-1 blocking DNA [2, 10]. The amounts of test and reference DNA that are bound at a chromosomal locus are dependent on the relative abundance of those particular sequences within the two samples [2]. Differential fluorescent hybridization signals emitted by the metaphase spread are therefore indicative of chromosomal gains and losses of the test DNA relative to the reference DNA [10]. Ratios of the fluorescent signals can be quantified along the length of each chromosome and a copy number karyotype (ratio profile) of the test DNA can be generated [10]. CGH can be performed on fresh tissue and also formalin-fixed paraffin-embedded (FFPE) tissue, making it an ideal cytogenetic technique to study solid tumors.

The following chapter outlines various protocols used for the detection of genomic aberrations by comparative genomic hybridization. These include metaphase chromosome preparation, DNA extraction methods, degenerate oligonucleotide primed polymerase chain reaction, nick translation, hybridization, and image generation and analysis.

2 Materials

All reagents used throughout each of the CGH-related protocols should be of molecular biology grade where applicable. Where a company is preceded by "e.g.", the reagent may be purchased from other vendors, companies that are not preceded by "e.g." should be used for purchasing of reagents to ensure high-quality results. Sterile techniques should always be used in preparation of reagents to prevent contamination during the protocol. Ensure that water used is MilliQ (autoclaved) or DNase/RNase-free.

2.1 Metaphase Chromosome Preparation

*Note: You may purchase metaphase slides from Abbott Molecular or make your own using the reagents and materials listed here. We typically have higher success rates with purchased metaphase slides.

1. Sodium heparin vacutainer tube (to collect 10 ml blood), syringe, alcohol wipe (BD).

2. 75 cm^2 cell culture flasks with vented caps (e.g. Corning, Fisher Scientific).
3. 50 ml centrifuge tubes (e.g. Corning, BD, Fisher Scientific, USA Scientific).
4. 37 °C incubator with 5 % CO_2, biological safety cabinet.
5. 100 ml Lymphocyte media (store at 4 °C; warm to 37 °C prior to addition of blood).
   - 86.5 ml 1× RPMI media with L-glutamine (2 mM) (e.g. Life Technologies; store at 4 °C)
   - 2.5 ml 1 M HEPES buffer (25 mM final concentration) (e.g. Life Technologies; store at 4 °C)
   - 10 ml fetal calf serum (10 % final concentration) (e.g. Life Technologies; store in aliquots at −20 °C)
   - 1 ml 10,000 U Penicillin/10,000 μg streptomycin (the final concentration is 100 U/100 μg) (e.g. Life Technologies; store in aliquots at −20 °C)
6. Phytohemagglutinin (M-form) (PHA, concentration not given) (Life Technologies; store at −20 °C).
7. 1× Hank's buffered saline solution (HBSS) (e.g. Life Technologies).
8. Methotrexate (Sigma-Aldrich; powder, soluble in 0.1 M NaOH; toxic).
   - Prepare a stock solution using a minimum volume of 1 M NaOH and then dilute to 10^{-5} M with HBSS (store in aliquots at −20 °C in dark)
9. Thymidine (Sigma-Aldrich; powder).
   - Prepare a stock solution using a minimum volume of 1 M NaOH and then dilute to 10^{-3} M with HBSS (store at room temperature)
10. 10 μg/ml KaryoMAX-Colcemid® (in HBSS) (Life Technologies; store at 4 °C).
11. Water bath (set to 37 °C).
12. Fixative (3:1 methanol/glacial acetic acid; prepare fresh) (e.g. Sigma-Aldrich).
13. SuperFrost slides (e.g. Fisher Scientific).
14. DAPI/Vectorshield mix: add 35 μl 1 mg/ml DAPI to 965 μl Vectorshield (0.35 μg/ml final concentration) (store 4 °C in the dark up to 2 months, after this time additional DAPI can be added to the solution).
    - 1 mg/ml DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride) (Sigma-Aldrich; powder; when in liquid form store in aliquots at −20 °C in dark; irritant)
    - Vectashield (Vector Laboratories; store at 4 °C in dark; irritant)

15. 25 mm × 25 mm coverslip (e.g. Fisher Scientific).
16. Fluorescent Microscope (e.g. Olympus, Nikon, Leica).
17. Coplin staining jars (e.g. Fisher Scientific).
18. 20× SSC (e.g. Fisher Scientific, Sigma-Aldrich).
    - Or make solution: 0.3 M sodium citrate, 3 M NaCl (e.g. Sigma-Aldrich)
19. 2× SSC (dilute 100 ml of 20× SSC with 900 μl MilliQ water).
20. Denaturation solution (70 % formamide in 2× SSC, pH 7.0).
    - 35 ml formamide (e.g. Sigma-Aldrich; store at 4 °C; toxic)
    - 15 ml 2× SCC buffer

2.2 Sample Preparation and DNA Extraction

2.2.1 DNA Extraction from Blood/DNA Extraction from Cell Lines

1. Lithium heparin vacutainer tube (to collect 10 ml blood), syringe, alcohol wipe (BD, Fisher Scientific).
2. 50 ml centrifuge tubes (e.g. Corning, BD, Fisher Scientific, USA Scientific).
3. 15 ml centrifuge tubes (e.g. Corning, BD, Fisher Scientific, USA Scientific).
4. NKM buffer (140 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$) (e.g. Sigma-Aldrich).
5. RSB buffer (10 mM Tris–HCl pH 7.5, 1 mM NaCl, 3 mM $MgCl_2$) (e.g. Sigma-Aldrich).
6. Shaking water bath, set at 37 °C (e.g. Fisher Scientific, VWR).
7. Plastic transfer pipettes (e.g. Fisher Scientific).
8. Lympholysis buffer (25 mM Tris pH 7.5, 25 mM EDTA; 75 mM NaCl, 1 % SDS) (e.g. Sigma-Aldrich).
9. Proteinase K buffer (2 mg/ml proteinase K, 10 mM $CaCl_2$-10 mM Tris pH 7.5) (e.g. Sigma-Aldrich).
10. 6 M NaCl (e.g. Sigma-Aldrich).
11. Ethanol (e.g. Sigma-Aldrich, Fisher Scientific).
12. Plastic inoculating loops (e.g. Fisher Scientific).
13. Microfuge tube (e.g. Fisher Scientific, USA Scientific).
14. TE buffer (10 mM Tris, pH 7.2; 1 mM EDTA) (e.g. Sigma-Aldrich).
15. ND-1000 spectrophotometer (NanoDrop) or similar instrument for quantitation.
16. Cell line/s for assessment (e.g. MCF-7, MDA-MB-231, or HeLa; purchased from ATCC or other).
17. Complete cell culture media (e.g. 1× RPMI containing 10 % FBS and 1 % antibiotic).
    - Media (RPMI, DMEM, other—grow each cell line according to recommended cell culture conditions) (e.g. Life Technologies; store at 4 °C)

- Fetal bovine serum (FBS) (e.g. Life Technologies; store in aliquots at −20 °C)
- Penicillin/streptomycin (10,000 U/10,000 μg) (e.g. Life Technologies; store in aliquots at −20 °C)

16. 75 cm^2 Flasks (e.g. Corning, Fisher Scientific).
17. Phosphate buffered saline (PBS) (e.g. Life Technologies; store at 4 °C or room temperature).
18. 0.05 % Trypsin-EDTA (e.g. Life Technologies; store temporarily at 4 °C or at −20 °C for longer periods).
19. Inverted phase-contrast microscope (e.g. Nikon, Olympus).

2.2.2 DNA Extraction from Formalin-Fixed Paraffin-Embedded Tissue

1. Formalin-fixed paraffin-embedded tissue sections, 6–12 sections, 5 μm thick on SuperFrost slides (e.g. Fisher Scientific) (samples obtained from a pathologist).
2. Xylene (e.g. Sigma-Aldrich; toxic, possible carcinogen).
3. Ethanol (e.g. Sigma-Aldrich, Fisher Scientific; toxic).
4. Mayer's Hematoxylin (e.g. Sigma-Aldrich; toxic).
5. Eosin Y solution (e.g. Sigma-Aldrich; toxic).
6. 22 mm × 60 mm coverslips (e.g. Fisher Scientific).
7. Histomount (Life Technologies; toxic, possible carcinogen; cap after use to prevent drying).
8. Methyl green (Sigma-Aldrich; irritant).
    - Add 0.5 g of methyl green to 50 ml MilliQ water.
9. Stereomicroscope (e.g. Nikon, Fisher Scientific).
10. Scalpel blade (#11) (e.g. Fisher Scientific).
11. PCR tubes (e.g. Eppendorf, Fisher Scientific, USA Scientific).
12. 20 mg/ml proteinase K (from *Tritirachium album*; Sigma-Aldrich; toxic).
13. Proteinase K Tween Buffer (PKTB).
    - 100 μl *Taq* polymerase reaction buffer (*Taq* DNA polymerase used in Subheading 2.3, reagent 6) (Roche Applied Science; store at −20 °C)
    - 20 μl 20 mg/ml proteinase K (0.4 mg/ml final concentration) (from *Tritirachium album*; Sigma-Aldrich; toxic)
    - 5 μl Tween 20 (0.5 % final concentration) (Sigma-Aldrich; toxic)
    - 875 μl DNase/RNase-free water (e.g. Fisher Scientific, USA Scientific, Life Technologies)
14. Thermocycler (e.g. Life Technologies, or other).
15. Mineral oil (autoclaved and filtered) (e.g. Sigma-Aldrich, syringe filters available from Fisher Scientific).

2.3 Degenerate Oligonucleotide Primed Polymerase Chain Reaction (DOP-PCR)

1. PCR tubes (e.g. Fisher Scientific, USA Scientific).
2. Aerosol filter barrier tips (e.g. Fisher Scientific, USA Scientific).
3. Extracted DNA (FFPE tissue—1 μl; fresh tissue or blood—50 ng in 1 μl).
4. PCR tubes (e.g. Eppendorf, Fisher Scientific, USA Scientific).
5. 32 U/μl Thermo Sequenase DNA Polymerase (with *Thermoplasma acidophilum* Inorganic Pyrophosphatase (TAP)) (GE Healthcare Life Sciences; store at −20 °C).
   - Includes 10× reaction buffer: 260 mM Tris–HCL (pH 9.5), 65 mM $MgCl_2$
6. 5 U/μl *Taq* DNA polymerase (Roche Applied Science; store at −20 °C).
   - Includes 10× reaction buffer: 100 mM Tris–HCl (pH 8.3), 15 mM $MgCl_2$, 500 mM KCl
7. 10 μM UN-1 degenerate primer (5′-CCGACTCGAGNNN NNNATGTGG-3′) (e.g. Integrated DNA Technologies, Fisher Scientific).
8. 10 mM dNTP mix (e.g. Life Technologies).
9. UltraPure DNase/RNase-free water (e.g. Fisher Scientific, USA Scientific, Life Technologies).
10. Mineral oil (autoclaved and filtered) (e.g. Sigma-Aldrich, syringe filters available from Fisher Scientific).
11. Thermocycler (e.g. Life Technologies, or other).
12. Parafilm (e.g. Fisher Scientific).

2.4 Nick Translation

1. 10× dNTP mix
   - 10 μl each of 10 mM dATP, dCTP, dGTP (200 μM final concentration) (e.g. Promega, Life Technologies; store in aliquots at −20 °C, avoid freeze/thaw cycles)
   - 250 μl 1 M Tris, pH 7.8 (500 mM final concentration) (e.g. Sigma-Aldrich; require HCl for pH adjustment)
   - 25 μl 1 M MgCl2 (50 mM final concentration) (e.g. Sigma-Aldrich)
   - 3.5 μl 14.3 M β-mercaptoethanol (100 mM final concentration) (e.g. Sigma-Aldrich; toxic)
   - 1 μl 50 mg/ml Ultra Pure Bovine Serum Albumin (BSA, 100 μg/ml final concentration) (e.g. Life Technologies; store at −20 °C)
   - 190.5 μl UltraPure DNase/RNase-free water (e.g. Fisher Scientific, USA Scientific, Life Technologies)
2. ChromaTide® Fluoroscein-12-dUTP (FITC-dUTP) (Life Technologies; store at −20 °C in dark).

3. ChromaTide® Texas-Red®-dUTP (TR-dUTP) (Life Technologies; store at −20 °C in dark).
4. BioNick enzyme mix (Life technologies: BioNick™ Labeling System; store at −20 °C; DNase I is sensitive to physical denaturation, do not vortex).
   - Contains: 0.5 U/μl DNA Polymerase I; 0.007 U/μl DNase I; 50 mM Tris–HCl, pH 7.5; 5 mM Magnesium Chloride; 0.1 mM Phenylmethylsulfonyl Fluoride (PMSF); 50 % (v/v) Glycerol; 100 μg/ml nuclease-free BSA
5. DNA Polymerase I/DNase I (Life Technologies; store at −20 °C; DNase I is sensitive to physical denaturation, do not vortex).
   - Contains: 0.5 U/μl DNA Polymerase I; 0.4 mU/μl DNase I; 50 mM Tris–HCl, pH 7.5; 5 mM Magnesium Acetate; 1 mM 2-Mercaptoethanol; 0.1 mM PMSF; 100 μg/ml BSA; 50 % (v/v) Glycerol
6. 10 U/μl DNA Polymerase (Life Technologies; store at −20 °C).
7. UltraPure DNase/RNase-free water (e.g. Fisher Scientific, USA Scientific, Life Technologies).

2.5 Agarose Gel Electrophoresis

1. Analytical Grade Agarose (e.g. Promega, Sigma-Aldrich, Fisher Scientific).
2. 1× TAE: Add 975 ml MilliQ water to 25 ml 40× TAE (e.g. Promega).
   - 40× TAE: 1.6 M Tris-acetate, 40 mM EDTA
3. Conical flask.
4. 10 mg/ml ethidium bromide solution (e.g. Promega, Sigma-Aldrich, Fisher Scientific; mutagenic, store in dark).
5. 6× gel loading dye (e.g. Fisher Scientific).
6. 100 bp DNA molecular weight marker (containing loading dye) (e.g. NEB, Promega, Fisher Scientific).
7. Gel tray and combs (7 cm × 10 cm tray and 1 × 11 teeth comb is sufficient for ten samples, if analyzing more samples than this you may need to run a larger gel or multiple small gels) (e.g. Fisher Scientific, Bio-Rad).
8. Electrophoresis tank and power source (e.g. Fisher Scientific, Bio-Rad).
9. UV light box and camera (UV light is harmful, ensure cover/glasses/face shield is worn when using UV light box) (e.g. Fisher Scientific, Bio-Rad, Syngene).

2.6 In Situ Hybridization

1. Coplin jars (e.g. Fisher Scientific).
2. Test and reference DNA (labeled by nick translation).

3. 1 mg/ml Human Cot-1 DNA® (Life Technologies; store at −20 °C).
4. 3 M Sodium acetate, pH 5.2 (e.g. Fisher Scientific, Sigma-Aldrich; toxic irritant).
5. Ethanol (e.g. Sigma-Aldrich, Fisher Scientific; toxic).
6. 20× SSC (e.g. Sigma-Aldrich, Fisher Scientific).
   - Optional laboratory made: 0.3 M sodium citrate, 3 M NaCl (e.g. Sigma-Aldrich)
7. 2× SSC (dilute 100 ml of 20× SSC with 900 μl MilliQ water).
8. Hybridization buffer (50 % formamide, 10 % dextran sulfate, 2× SCC, pH 7.0).
   - 500 μl formamide (e.g. Fisher Scientific, Sigma-Aldrich; store at 4 °C; toxic)
   - 100 mg dextran sulfate sodium salt (e.g. Sigma-Aldrich; possibly toxic)
   - 2× SSC up to 1 ml (approximately 400 μl; ensure that 20× SCC is diluted with DNase/RNase-free water for this buffer)
9. Denaturation solution (70 % formamide in 2× SSC, pH 7.0).
   - 35 ml formamide (e.g. Fisher Scientific, Sigma-Aldrich; store at 4 °C; toxic)
   - 15 ml 2× SCC buffer
10. Metaphase slides (made in Subheading 3.1 or purchased from Abbott Molecular; store at −20 °C).
11. Diamond-tipped scribe (e.g. Fisher Scientific).
12. Slide warmer (e.g. LabScientific, Cole-Parmer, Electron Microscopy Sciences).
13. Rubber cement (e.g. Fisher Scientific, can also purchase from bike store, automotive store, office store, Amazon; should be purchased in a tube for ease of use).
14. Chamber with rack—must be a dark box or kept in the dark.
   - Containing 2× SSC-soaked paper towel on bottom for humidity control
   - e.g. Humidity Chamber Plus from Covance, Slide Humidity Incubation Box from LabScientific Supplies and Equipment; if chamber box is clear, it should be covered with foil or kept in an oven that does not allow light exposure of the slides
   - Alternatively these can be made from any box with a rack to fit slides horizontally. If the container is not completely air tight paper towels may have to be re-soaked with 2× SSC each day if evaporation occurs.

15. 37 °C oven (e.g. Fisher Scientific).
16. 3×50 ml wash solution (50 % formamide in 2× SCC).
    - 75 ml formamide (e.g. Fisher Scientific, Sigma-Aldrich; store at 4 °C)
    - 75 ml 2× SSC
17. 1×50 ml PN buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 0.1 % NP-40, pH 8.0).
    - 5 ml 1 M NaH2PO4 (monobasic) (e.g. Sigma-Aldrich; irritant, toxic)
    - 5 ml 1 M Na2HPO4 (dibasic) (e.g. Sigma-Aldrich; irritant, toxic)
    - 50 µl nonidet P40 (NP-40, Igepal®CA-630) (e.g. Sigma-Aldrich; toxic, irritant)
18. DAPI/Vectorshield mix: add 35 µl 1 mg/ml DAPI to 965 µl Vectorshield (0.35 µg/ml final concentration) (store 4 °C in the dark up to 2 months, after this time additional DAPI can be added to the solution).
    - 1 mg/ml DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride) (Sigma-Aldrich, powder form; once in liquid form store in aliquots at −20 °C in dark; irritant)
    - Vectashield (Vector Laboratories; store at 4 °C in dark; irritant)
19. 25 mm×25 mm coverslip (e.g. Fisher Scientific).

2.7 Fluorescence Microscopy and Image Analysis

1. Immersion oil (e.g. Sigma-Aldrich; irritant).
2. Fluorescent microscope with mercury burner lamp (e.g. Olympus, Leica, Nikon).
3. Fluorescent filter set (specific for fluorophores used) (e.g. ChromaTechnology, Nikon; dependent upon microscope used).
4. High performance black/white CCD camera (e.g. Cohu, Optronics).
5. Software package (e.g. Isis CGH Software from MetaSystems, PowerGene CGH Package by Perceptive Scientific Instruments Incorporated (Persci), CytoVision® software from Leica Microsystems).

3 Methods

3.1 Metaphase Chromosome Preparation

Metaphase chromosome preparation is crucial for CGH analysis; an adequate number of cells must be in metaphase and chromosomes must be well spaced (not overlapping) and of sufficient size. Metaphase chromosome spreads are obtained from short-term

cultured blood samples, whereby cell division is stimulated by phytohemagglutinin (PHA), cells are synchronized (DNA synthesis is blocked in S phase by methotrexate and released by thymidine) and mitosis is halted in metaphase by addition of colcemid [11]. This protocol typically yields an increased number of mitotic cells (in metaphase) and results in improved chromosome morphology [11]. The following protocol is modified from those presented in The AGT Cytogenetics Laboratory Manual [12]. Even when this protocol is optimized and followed, good quality metaphase spreads suitable for hybridization are not guaranteed. Alternatively, metaphase chromosome spreads can be purchased from Abbott Molecular. Although these are also not guaranteed to give good results, the slides typically provide quality metaphase spreads and are often the preferred choice.

This protocol should be carried out in a biological safety cabinet to prevent contamination. Ensure sterile cell culture techniques.

1. Collect 10 ml blood into a sodium heparin tube. Blood must come from a healthy volunteer who is karyotypically normal.
2. Add 2 ml of blood to each of four 75 cm^2 cell culture flasks containing 25 ml lymphocyte media and 750 μl PHA (concentration 3 % v/v).
3. Incubate flasks for 48 h in a 37 °C incubator with 5 % CO_2.
4. Add 275 μl HBSS containing 10^{-5} M methotrexate (10^{-7} M final concentration).
   - Alternatively, you can leave one or two of the flasks unsynchronized
5. Return flasks to incubator for a further 17 h.
6. Transfer cultures to 50 ml conical tubes and centrifuge at 800 rpm for 8 min.
7. Remove media, loosen cell pellet (by gentle tapping) and resuspend in 25 ml prewarmed (37 °C) lymphocyte media containing 250 μl 1×10^{-3} M thymidine (10^{-5} M final concentration).
8. Return cultures to the incubator for a further 5 h.
9. Add 25 μl 10 μg/ml KaryoMAX-Colcemid® (10 ng/ml final concentration) and incubate for 10–15 min at 37 °C.
   - It is advised to use different times for each of the flasks, for example 10 and 15 min each for synchronized and unsynchronized cells; this will allow you to determine the condition at which the best metaphase chromosomes are obtained.
10. Transfer cultures to 50 ml conical tubes and centrifuge at 800 rpm for 8 min.

11. Discard all but 500 µl of the media and resuspend the cell pellet in this.
12. Add 10 ml prewarmed (37 °C) 750 mM KCl solution (to swell cells) and incubate at 37 °C for 20 min (in water bath).
13. Centrifuge the tubes at 800 rpm for 5 min, discard the supernatant (except for 500 µl) and resuspend the cell pellet.
14. Add 10 ml fresh fixative (add the first 2 ml drop-wise while gently shaking the tube by hand).
15. Repeat **steps 13–14** several more times (usually 3 or 4) until the fixative becomes clear and the cell pellet is opaque in color.
16. Resuspend the pellet in 5 ml fixative (*see* **Note 1**).
17. Microscope slides should be cleaned with 100 % ethanol and dried vertically.
18. Prepare one slide for each of the four tubes: using a plastic pipette place 2 separate drops of cells/fixative on the slide (well spaced, these are the two target areas; initially, drop from a distance of about 1–2 cm above the slide) and allow the cells to air dry (about 30 s) (*see* **Note 2**).
19. Stain each target area with 20 µl DAPI/Vectashield mix (0.35 µg/ml) and visualize with fluorescence and phase contrast microscopy (under 10×, 40× and 60× objectives) (*see* **Note 2**).
20. Once the most appropriate condition has been identified, prepare all other slides like this, at least 50 slides should be prepared. Slides should be stored at room temperature for 2 weeks to age.
21. After aging, denature one of the slides in denaturation solution (in a coplin jar) for 5 min at 73 ± 1 °C, stain with DAPI (**step 19**) and view banding pattern (*see* **Note 3**). Ensure that slides are appropriate for analysis.
22. Store aged slides at −20 °C (to prevent further aging).

3.2 Sample Preparation and DNA Extraction

CGH is useful for the detection of copy number aberrations in diseased samples from any tissue source. Blood provides a good source of DNA for analysis, but often extraction of DNA from solid tissue is more difficult due to limited availability of tissue or the requirement of formalin-fixed paraffin-embedded tissue. Here are methods of DNA extraction from whole blood (required for reference DNA), cell lines (required for positive controls) and formalin-fixed paraffin-embedded tissue (test DNA).

3.2.1 DNA Extraction from Blood

Acquisition and testing of both male and female DNA is necessary for the generation of a ratio threshold and dynamic range. DNA therefore needs to be extracted from at least one male and one female (both karyotypically normal). The following protocol is a modification of that developed by Miller and colleagues [13].

This is a salting out procedure whereby red blood cells are lysed and removed using salt solutions, white blood cells are lysed, proteins are degraded and removed, and ethanol is used to precipitate the DNA.

1. Collect 5–10 ml blood into a lithium heparin tube. Blood must come from healthy volunteers (one male, one female), who are karyotypically normal.
2. Transfer the blood to a 50 ml tube and add 40 ml NKM buffer.
3. Mix vigorously, then centrifuge at 4,800 rpm for 25 min at room temperature.
4. Using a plastic pipette, carefully pipette off the supernatant (containing lysed red blood cells), making sure that the white cell layer remains intact. Tap the tube to loosen the cell pellet.
5. Add a small amount of RSB buffer, resuspend the cell pellet well and then add RSB buffer up to a final volume of 50 ml. This will lyse the remaining red cells.
6. Mix well then centrifuge at room temperature (RT), 4,000 rpm for 15 min.
7. Carefully pipette off the supernatant and tap the tube to loosen the cell pellet.
8. Repeat **steps 5**–7 once or twice until most red blood cells have been lysed and removed and the pellet appears to be white in color. Ensure that the cell pellet is resuspended well in a small amount of RSB buffer (about 500 μl).
9. Add 8 ml lympholysis buffer and 500 μl proteinase K buffer to lyse the white cells.
10. Shake vigorously at 37 °C overnight (in shaking water bath).
11. Add 4 ml of sterile saturated NaC1 (approx. 6 M), mix vigorously for 15 s.
12. Centrifuge at 2,500 rpm at 4 °C for 15 min to pellet cellular proteins.
13. Carefully decant the supernatant containing the DNA into a 15 ml centrifuge tube.
14. Centrifuge the 15 ml tube at 2,500 rpm at 4 °C for 15 min to pellet any remaining proteins.
15. Using a plastic pipette carefully transfer the supernatant to a 50 ml centrifuge tube, leaving the protein undisturbed as a pellet.
16. Measure the volume and add exactly 2 volumes of chilled absolute ethanol.
17. Gently swirl and observe the DNA strands precipitate.

18. Using a plastic loop, carefully transfer the DNA precipitate into a microfuge tube containing 500 μl DNase/RNase-free water and incubate at 37 °C 4–6 h or until DNA is dissolved.
19. Measure the concentration and quality of the DNA using a spectrophotometer (260/280 absorbance ratio should be about 1.7). The DNA quality can also be viewed via agarose gel electrophoresis (*see* Subheading 3.5). Adjust the concentration of samples to 50 ng/μl before storing.

3.2.2 DNA Extraction from Cell Lines

To ensure that your protocol is working and for quality control across experiments, DNA from cell lines can be used as a positive control in CGH. Two commonly used positive control cell lines are MCF7 and MDA-MB-231, although any cell line can be used if the copy number abnormalities are known. Most cell lines, including MCF7 and MDA-MB-231, can be purchased from the American Type Culture Collection (ATCC). Cells should be cultured in prewarmed (37 °C) media using aseptic techniques, according to the vendors' instructions.

1. In a 37 °C 5 % CO_2 incubator, grow cell lines to 80 % confluence in one or more 75 cm^2 flasks using complete media.
   - For MCF-7 and MDA-MB-231 1× RPMI media containing 10 % FBS and 1 % antibiotic can be used, although many other media formulations could be substituted. Other cell lines may require a different media.
2. Remove the media and wash the cells once with 8 ml prewarmed (37 °C) PBS.
3. Add 4 ml trypsin and put the flask in the 37 °C incubator for about 2–5 min. Remove the cells when they are rounded up and starting to lift from the flask (by viewing under a phase-contrast inverted microscope) and add 6 ml complete media.
4. Tap the flask to remove cells from the flask wall and put media (containing cells) into a 50 ml centrifuge tube.
5. Centrifuge at 1,000 rpm for 5 min to pellet the cells and remove media.
6. Extract the DNA as per instructions in Subheading 3.2.1, starting at step 9 (half volumes may be used if the pellet is small).

3.2.3 DNA Extraction from Paraffin-Embedded Tissue

Formalin-fixed paraffin-embedded tissue can be obtained from most pathologists. To define cancerous tissue (versus normal tissue), you should consult the pathologist. If contaminating normal tissue is microdissected along with the cancerous tissue the sample will be diluted and false negative results might be obtained. It is therefore very important that the samples are microdissected with care, and if possible photos should be taken before and after microdissection

for comparison. Typically 6–12 slides of 5 μm thickness are collected. Of these, 2–3 are used as “guide” slides and are stained with hematoxylin and eosin, the remaining slides are stained with methyl green for microdissection. The guide slides should be from either end of the sections and if 12 sections are used, a third guide slide from the middle of the sections should also be used. Following microdissection of the methyl green slides, DNA is extracted by digesting proteins in a proteinase K buffer. This protocol outlines staining techniques, microdissection, and DNA extraction from paraffin-embedded, formalin-fixed samples. Some investigators also have access to a microscope capable of laser capture microdissection, which can isolate individual cells for analysis.

1. De-paraffinize and stain 2–3 slides with Hematoxylin and eosin:
   - Immerse in Xylene twice (3 min each), an ethanol series (100 %, 100 %, 90 %, and 70 % for 1 min each), rinse in running water, hematoxylin (5 min), rinse in running water, eosin (2 min), rinse in running water (1 min), Xylene twice (2 min each); apply a coverslip with Histomount.
2. De-paraffinize and stain the remaining slides with methyl green:
   - Immerse in Xylene twice (3 min each), an ethanol series (100 %, 95 %, 70 %, 50 % and MilliQ water for 2 min each), 1 % methyl green (5 min), rinse in MilliQ water (1–5 min); do not apply a coverslip.
3. Using a stereomicroscope and the hematoxylin and eosin stained slides as a guide, microdissect the tumor tissue by hand from the methyl green stained slide using a scalpel blade (*see* **Note 4**).
   - If possible, take photos of the slide before and after microdissection.
   - If the tumor is small, you may want to microdissect more than one methyl green slide, however the amplification process (Subheading 3.3) will increase the amount of DNA to be hybridized even if small tumor specimens are used.
4. Place the microdissected tissue into a PCR tube containing 15–30 μl PKTB (volume dependent on the size of the tumor sample).
5. Overlay the samples with 50 μl mineral oil.
6. Incubate samples at 55 °C for 3 days, each day add 0.6 μl fresh 20 mg/ml proteinase K to each sample.
   - You may wish to vortex the samples once or twice a day to break up the tissue, however this is typically not necessary.
7. On day 3, denature the proteinase K by heating samples to 95 °C for 10 min.
8. Remove oil from the sample by rolling the entire sample on Parafilm. The oil will stick to the parafilm and the aqueous

sample will remain in droplet form that can be pipetted and placed in a microcentrifuge or PCR tube and stored at 4 °C until ready for use.

3.3 Degenerate Oligonucleotide Primed Polymerase Chain Reaction (DOP-PCR)

Universal amplification of DNA is performed using degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR). Briefly, this involves an initial low stringency pre-amplification using Thermo Sequenase DNA polymerase (5 μl volume) followed by a high stringency amplification using Taq DNA polymerase (50 μl volume). The protocol below is a modification of the protocol provided by Kuukasjärvi et al. 1997 [14]. To prevent contamination, these steps should be carried out in a laminar flow hood using aerosol filter barrier tips. As with any PCR, when making the bulk mixes you should always use DNase/RNase-free water, adding this first to the bulk mix tube and the enzyme last. Also, a negative control (containing no DNA) should be used for each experiment.

1. Aliquot 1 μl (*see* **Note 5**) of each of your extracted DNA samples into a suitable PCR tube. It is recommended to perform DOP-PCR on multiple samples at once but this should be limited to 12 or 13 at the most, reducing chances of contamination and potential enzyme degradation (due to a more limited time frame). For each DOP-PCR experiment various controls should be run in addition to the test DNA, including a negative control (no DNA template), a positive control (cell line such as MPE-600 or MCF-7, with known chromosomal abnormalities) and sufficient reference DNA for your experiments (typically, one reference PCR sample is sufficient for 2 experiments—*see* **Note 5**). Reference DNA is considered to be DNA extracted from an individual (male or female) with no cytogenetic abnormalities.
2. Prepare a pre-amplification bulk mix sufficient for all samples to be DOP-PCRed, including a little extra for pipetting inaccuracies (e.g., 13.5× bulk mix for 13 samples which will allow a negative control, positive control, six test DNA samples, and five reference DNA samples (include at least one male and one female reference DNA sample)):

Reagent	Final concentration	1× bulk mix (μl)	13.5× bulk mix (μl)
10× Thermo Sequenase buffer	1×	0.5	6.75
10 mM dNTPs	0.2 mM	0.1	1.35
10 μM UN-1 Primer	1.2 μM	0.6	8.1
32 U/μl Thermo Sequenase	2 U	0.0625	0.84
DNase/RNase-free water	–	2.3375	31.56
Final Volume		4	54

3. Add 4 μl of your pre-amplification bulk mix to each of your DNA samples.
4. Overlay each sample with about 50 μl mineral oil.
5. Place samples into the thermocycler for low stringency pre-amplification using the following cycling conditions: initial denaturation at 96 °C for 3 min; 5 cycles of 94 °C for 1 min, 25 °C for 1 min, a slow ramp to 74 °C over 3 min, 74 °C for 2 min; final extension at 74 °C for 10 min. Prior to adding the amplification mix to your samples, you should denature your samples at 95 °C for 5 min.
6. While pre-amplification thermo-cycling is occurring prepare your amplification mix for high-stringency amplification, although make sure you add the *Taq* DNA polymerase enzyme just prior to completion of the pre-amplification cycling (i.e. during the 5 min denaturation step):

Reagent	Final concentration	1× bulk mix (μl)	13.5× Bulk mix (μl)
10× Taq polymerase buffer	1×	4.5	60.75
10 mM dNTPs	0.2 mM	0.9	12.15
10 μM UN-1 Primer	1.3 μM	5.85	78.975
5 U/μl Taq DNA polymerase	2 U	0.4	5.4
DNase/RNase-free water	–	29.75	401.625
Final Volume		45	607.5

7. Add 45 μl of your amplification bulk mix to each of your DNA samples. Vortex briefly and pulse centrifuge (it is important to do this step as quickly as possible).
8. Return the PCR tubes to the thermocycler for high stringency amplification using the following cycling conditions: initial denaturation at 95 °C for 5 min; 35 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 3 min; final extension at 72 °C for 5 min.
9. Electrophorese 5 μl of each sample on a 2 % agarose gel for 45–60 min to determine if contamination has occurred and that amplified DNA is of an appropriate length (*see* **Note 6**). *See* Subheading 3.5 for agarose gel preparation. Figure 1 shows an example of typical DOP-PCR products.

3.4 Nick Translation

Comparative genomic hybridization quality is highly dependent upon the size of DNA fragments. Nick translation is the process by which DNA is digested to produce smaller fragments, while incorporating a fluorescent dye. DNase I cleaves the DNA while DNA

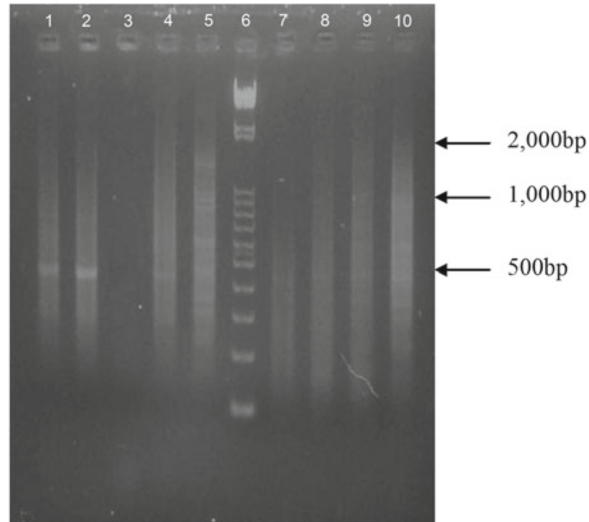

Fig. 1 Typical DOP-PCR results. Lanes 1 and 2 show a distinct band at about 400 bp, which is postulated to signify preferential amplification of repetitive sequences, DOP-PCR products exhibiting this band can still be used for CGH analysis. Lane 3 is the negative control. Lanes 4, 5, 8, 9, and 10 show amplification of DNA extracted from fresh tissue. Lane 7 shows amplification of microdissected DNA. Lane 6 is the molecular weight marker

polymerase adds nucleotides to the strand, incorporating the fluorescently labeled dUTP. The following protocol is a modification of the nick translation method outlined by Kallioneimi et al. [1, 2]. This protocol uses FITC and Texas Red fluorophores, although many other fluorophores can be used for labeling providing the microscope used for detection has the appropriate excitation and emission filters and that these do not overlap greatly. Some protocols use an indirect labeling protocol (incorporating biotin), which may increase the signal. However, direct labeling limits background signal and usually provides signals of adequate intensity for analysis. As such, this is the protocol that is given here.

1. Prepare sufficient bulk mix for your samples:

Reagent	1× bulk mix (μl)	13.5× bulk mix (μl)
10× dNTP mix	5	67.5
10× BioNick enzyme mix	1.5	20.25
DNA polymerase I/ DNase I enzyme mix	1.5	20.25
DNA polymerase	1.0	13.5
dUTP (FITC- or Texas Red-labeled)	1.0	13.5
Final Volume	10	135.0

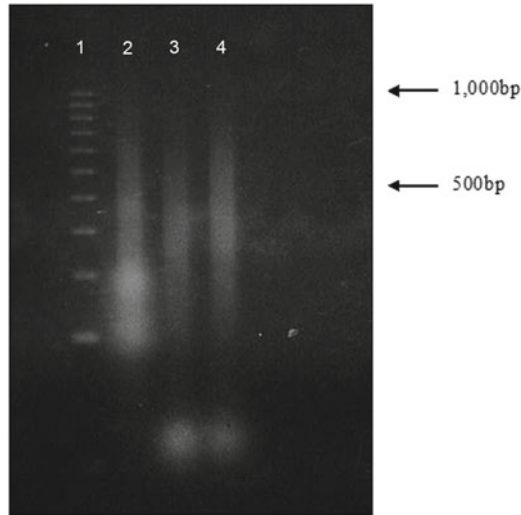

Fig. 2 Typical nick translation products. Lane 1 is the molecular weight marker. Lane 2 is labeled with Texas Red and lanes 3 and 4 are labeled with FITC. Both dyes fluoresce under UV light and as such can be seen in the gel picture

2. For DOP-PCRed samples obtained from fresh tissue add 10 μl bulk mix and 17.5 μl DNase/RNase-free water to 22.5 μl sample. For DOP-PCRed samples obtained from microdissected tissue add 10 μl bulk mix to 40 μl sample (*see* **Note 7** for additional comments on nick translation).
3. Incubate at 15 °C for 60 min and halt the reaction by heating the samples to 75 °C for 15 min (*see* **Note 7**). Nick translation products must be stored in the dark at −20 °C to prevent degradation of both the product and the fluorophore.
4. Electrophorese 5 μl of each sample on a 2 % agarose gel at 90 V for 45–60 min to ensure nick translated product is of appropriate length (*see* **Note 7**). *See* Subheading 3.5 for agarose gel preparation. Figure 2 shows an example of typical nick translated products.

3.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis can be used to determine the quality of DNA, DOP-PCR amplified products, and nick translated products used for analysis. Electrophoresis is carried out using 2 % agarose gels. The following protocol is sufficient for one 7 cm × 10 cm tray, if a larger gel is needed adjust the volumes accordingly.

1. Add 50 ml 1× TAE to 1.0 g of agarose in a conical flask and heat in microwave until agarose is well dissolved (about 2 min, watch it to ensure it does not boil over, you may need to take the flask out every 30 s or so and swirl to dissolve the agarose).
2. When cooled, add 2.5 μl 10 mg/ml ethidium bromide (0.5 μg/ml final), mix gently.

- As ethidium bromide is mutagenic, it is best to perform this step in a chemical fume hood if possible.

3. Pour agarose solution into gel tray and insert comb, allow gel to set before loading samples. The gel will take approximately 30 min to set fully but this time can be reduced if the gel is placed in a 4 °C refrigerator to set.
4. While gel is setting, add 1 μl of 6× loading dye to 5 μl of your sample.
5. Remove comb from gel when set: load 5 μl 100 bp molecular weight marker (containing loading dye) in the first lane then load all samples and your negative control.
6. Place gel into electrophoresis tank and cover with 1× TAE and run the gel at 80–90 volts for about 45–60 min or until you can clearly see the size range of your DNA smears using the UV light box. To test for DNA quality 20–30 min is sufficient.
   - If you check the gel and the size range is not sufficient, the gel can go back into the electrophoresis tank and be run for an additional time. Be sure not to run the gel too long or the ethidium bromide will run off the gel and the DNA in the lower range of the molecular weight marker will not be stained. If this occurs, you can try staining the gel in a TAE solution containing 0.5 μg/ml ethidium bromide for 5–10 min. Be sure to dispose of ethidium bromide liquid solutions in the correct manner (such as charcoal filtration).
7. Capture an image of your gel for future records.

3.6 In Situ Hybridization

Labeled test and reference DNA are precipitated with COT-1 DNA to generate a probe for the hybridization. The probe and metaphase chromosomes are both denatured prior to hybridization. Following hybridization, slides are washed to remove excess probe and DAPI is added in the presence of an antifade solution. All denaturation and washing of the slides is done in coplin jars and no more than four slides should be processed at a time. Metaphase slides can be made in the laboratory or purchased from Abbott Molecular. Each slide has 2 target areas and thus two experiments can be performed per slide. An initial experiment should be performed by hybridizing differentially labeled male and female DNA (normal karyotype) to generate a ratio threshold. Also, positive control hybridizations should be performed incorporating a cell line with known cytogenetic aberrations and a reference DNA. This will indicate how sensitive your technique is and can also be used as a quality-control hybridization across experiments.

1. Prewarm denaturation solution in a coplin jar to 73 ± 1 °C.
2. Precipitate the labeled test and reference DNA with COT-1 DNA by combining 22.5 μl of the test DNA and 40 μl of the

reference DNA (labeled by nick translation) with 30 µl of COT-1 DNA, 9.5 µl 3.0 M sodium acetate (pH 5.2) and 237.5 µl cold 100 % ethanol.

3. Vortex the mix briefly, freeze in liquid nitrogen, and store at −80 °C for 2–3 h.
4. Centrifuge at 11,000 rpm at 4 °C for 30 min, remove ethanol, and air-dry the pellet in the dark.
5. Resuspend the pellet/probe in 10 µl hybridization buffer. The probes will need to be heat denatured at 73 °C for 5 min just prior to hybridization to the target metaphase slide (while metaphase slides are drying—**step 8** later in this protocol).
6. Label target metaphase slides (using pencil, not pen) and circle the target areas (underneath the slide) using a diamond tipped scribe.
7. Soften target metaphase slides in 2× SCC at 37 °C for 5 min then dehydrate them using an ethanol series of 70 %, 85 % and 100 % for 2 min each (RT).
8. Place slides in the denaturation solution at 73 ± 1 °C for 5 min (±1 min) (*see* **Note 8**) then dehydrate the slides using an ethanol series of 70 %, 85 % and 100 % for 2 min each (RT). Slides are air-dried vertically on paper towel for 10 min.
9. Place dried slides on a slide warmer set to 37 °C, then drop the entire denatured probe (10 µl) onto a target area (two probes per slide). Make sure the probe has been denatured for 5 min at 73 °C immediately prior to dropping on the target area (**step 5**), which can be done towards the end of the slides drying. Cover each target area with a 22 mm × 22 mm square coverslip, (two per slide), ensuring that no air bubbles are formed (*see* **Note 9**). Seal all edges using rubber cement.
10. Place slides in a sealed humidified chamber (containing 2× SSC soaked paper towel) and into a hybridization oven set to 37 °C for 3 days. If the chamber is not sealed well, additional 2× SSC will need to be added each day.
11. Following hybridization, remove coverslips and rubber cement from slides with a pair of tweezers (the rubber cement will be soft and will come off easily).
12. Wash slides three times in wash solution (50 % formamide, use 3 separate coplin jars) at 45 °C for 10 min each (to denature poorly bound probe).
13. Wash slides in 2× SCC for 10 min at 45 °C then for 10 min at RT.
14. Wash slides in PN buffer for 10 min at RT (to remove poorly bound probe).

15. Rinse slides in DNase/RNase-free water for 5 min and dry slides vertically in the dark for 5–10 min or until dry.
16. Counterstain metaphase chromosomes by adding 20 µl DAPI/Vectashield mix and placing 22 mm × 22 mm coverslips on each of the target areas. Store slides at 4 °C in the dark until ready for digital analysis (*see* **Note 10**).

3.7 Fluorescence Microscopy and Image Analysis

Images of the DAPI, FITC, and Texas Red stains can be captured using a fluorescence microscope and a CCD camera. An initial experiment, performed by hybridizing differentially labeled male and female DNA (normal karyotype), should generate a ratio threshold between 0.8 and 1.2, as seen on all chromosomes except for the X chromosome. This means that normal variation will fall between 0.8 and 1.2 (intensity is normalized to 1.0). Deviations from this ratio threshold indicate deletions (those below 0.8) or amplifications (those above 1.2). A male versus female hybridization should clearly show an amplification of the X chromosome (if female DNA is considered as test (Texas Red) and male DNA as reference (FITC)). Analysis of positive control cell lines should reveal cytogenetic aberrations typical of that cell line. The ATCC usually contains some information, or references to information on cytogenetic abnormalities associated with each cell line. However, it is important to remember that cytogenetic anomalies will vary (typically increasing in number) with increasing passage number of the cell line, as long as you get consistent abnormalities across different experiments, this is OK.

Each metaphase spread contains two homologues of each chromosome, therefore the ratio profile generated for each metaphase spread is an average of these. An average (mean) ratio profile is generated from 6 to 12 homologous chromosomes derived from 5 to 10 metaphase spreads across each hybridization. The average fluorescence intensity ±1 standard deviation is displayed for each region of all chromosomes. Copy number changes are assumed to be real if both the mean and one standard deviation are either above or below the set ratio threshold, with the other standard deviation above or below a ratio of one, respectively. Results need to be interpreted with caution in repetitive regions (e.g. centromeres) and also in regions affected by fluorophore artifacts (*see* final comments in **Note 7**).

1. All digital analysis should be performed in a dark room to prevent degradation of the fluorophores. Turn on the mercury burner lamp and allow it to stabilize (about 5 min) then place the slide on the microscope.
2. Using the 10× objective lens first, locate metaphase spreads using the DAPI excitation filter (*see* **Note 11**). Once located, increase the magnification to 40× and then 60× (using oil

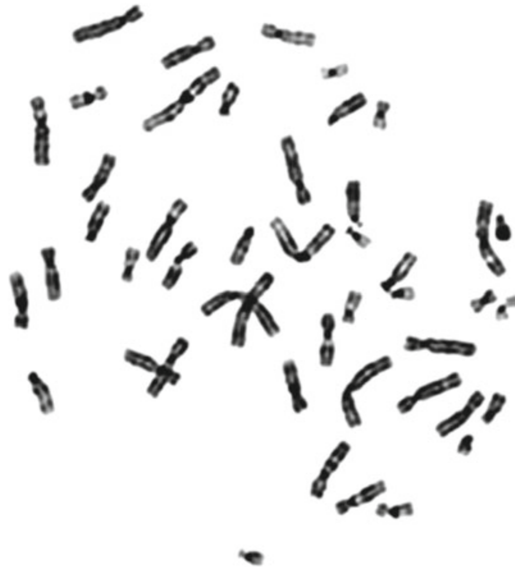

Fig. 3 Example of a good quality metaphase spread. Chromosomes should be long and banding should be distinct

immersion). Each time the magnification changes, the metaphase spread of interest needs to be re-centered and focused. Before capturing the images, ensure that the metaphase spread is of good quality (*see* **Note 11**). Figure 3 gives an example of a good quality metaphase spread.

3. Ensure the microscope is in camera mode. Using your chosen software package, a live preview can be used to finely focus and center the metaphase spread image (*see* **Note 12**).
4. Using the computer software, capture an image using the DAPI excitation filter (approximately 0.5 s exposure time), this will be pseudo-colored blue. Capture additional images with the FITC (about 6 s exposure, pseudo-colored green) and Texas Red (about 2 s exposure, pseudo-colored red) excitation filters (*see* **Note 13**). Although the images are analyzed in gray-scale, a red/green/blue (RGB) composite image can be displayed at this point.
5. Image processing (by the software package) allows: background flattening and subtraction, standardization of the red and green signal intensities to 1.0 (to limit differences in hybridization intensities across metaphase spreads) and grayscale conversion.
6. The DAPI stain produces a weak G-banding pattern that can be enhanced (by the user) for karyotyping. After enhancement, chromosomes (and often nonchromosomal material) are automatically selected. De-selection of nonchromosomal material

can be done manually. Although bad quality or overlapping chromosomes can be de-selected, it is often best to leave them as they can aid in karyotyping of other chromosomes. Also, touching chromosomes or single chromosomes that have been automatically selected as two chromosomes (due to light banding) can be outlined manually.

7. An automated karyotype is generated based on pattern recognition, however this usually requires further manual manipulation. Correction of miscalled chromosomes and adjustment of chromosome orientation is done manually and any chromosomes that cannot be reliably interpreted are excluded from analysis. For help in karyotypic analysis, it is best to use established ideograms and guidelines, outlined in the ISCN 2013 [15] (*see* also [16, 17]). It may also be appropriate or necessary to get advice from a cytogeneticist on karyotypic analysis.
8. This process is repeated for 5–10 metaphase spreads, to ensure that at least 6–12 copies of each chromosome are used for interpretation.
9. A ratio profile is generated from each karyotype (by overlaying normalized red and green images) (*see* **Note 14**). A ratio of 1 indicates equal copy number whereas ratios deviating from this indicate gains and losses of chromosomal material. Due to the genetic heterogeneity of tumor tissue cutoff values of <0.8 for losses and >1.2 for gains are recommended.
10. An average ratio profile ± 1 standard deviation is generated from 5 to 10 metaphase spreads to indicate regions of chromosomal gain or loss. Figure 4 demonstrates a typical average ratio profile generated.

4 Notes

1. Cells can be stored at −20 °C in 10 ml of fixative. If cells are stored, the fixative must be replaced before preparing the slides (i.e. centrifugation, removal of supernatant, resuspension in 5 ml fixative). Although 5 ml of fixative is recommended, the amount actually required may need to be adjusted if there are too few or too many (crowded) metaphase spreads on the slide.
2. Metaphase chromosome spreads within the target areas need to be well separated. Viewing a target area with a DAPI stain will indicate if metaphase spreads are appropriate for CGH analysis. You will see many interphase cells (which appear as small circles) as well as metaphase spreads (appear as larger circles at 10× and individual chromosomes at 40× and 60×). Chromosomes should be long enough for karyotyping and chromosomes that are medium to dark in color (phase contrast

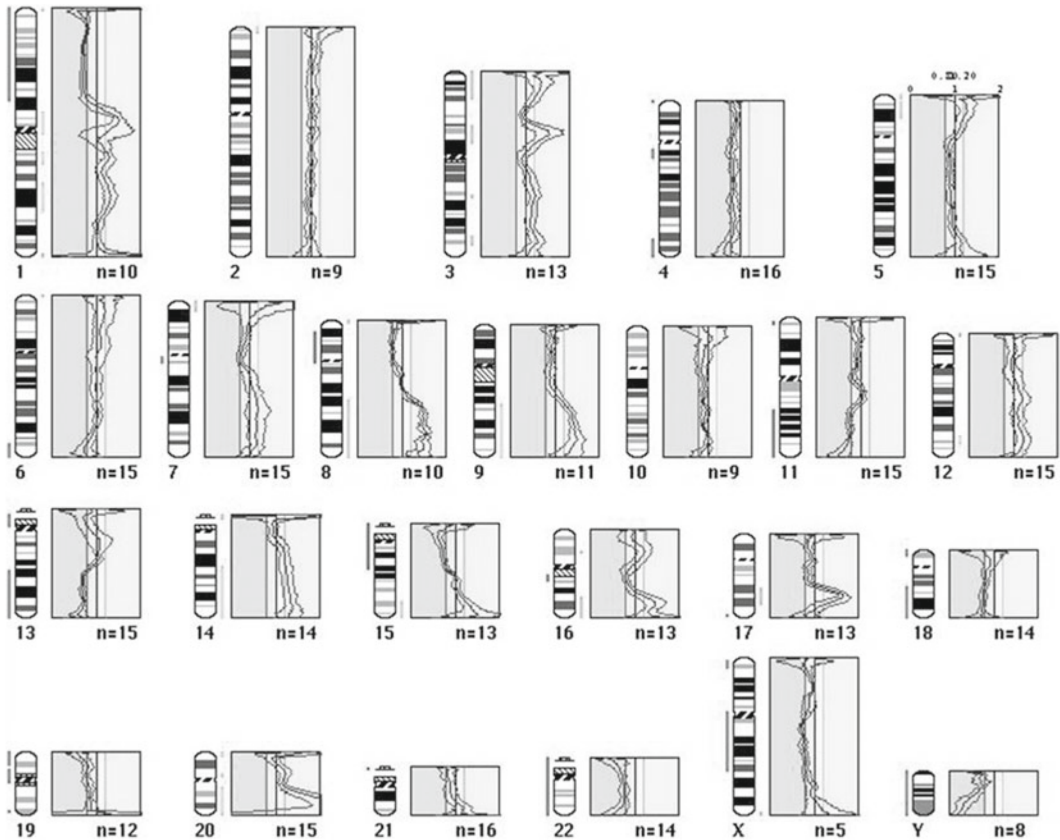

Fig. 4 Example of an average ratio profile. If the reference sample is labeled with Texas Red and the test sample is labeled with FITC, amplifications will be indicated by a *green line* to the *right* of each ideogram and deletions will appear as a *red line* to the *left* of each ideogram. For example, in this *grayscale* Figure, chromosomal location 8q is amplified and 1p is deleted (color figure online)

microscopy, not fluorescence) may give better hybridization. As four different conditions are tried (for the four flasks), one of these is likely to produce quality metaphase spreads, however, the preparation may need to be repeated with different Colcemid incubation times (step 8). If the cells are too crowded within the target areas, then more fixative can be added to dilute the cells. If there are too few cells on the slide, then centrifuge the samples and resuspend in a smaller volume of fixative to concentrate the cells. If metaphase spreads are not spread out enough (i.e. too many chromosomes are overlapping) then try dropping the cells from more of a height (e.g. drop from 3 to 5 cm above the slide) or try adding a second drop of fixative on top of the drop of cells to spread them out further. Remember that you want at least 5–10 good metaphase spreads, this means that you may require up to 50 spreads or more within a target area. If suitable metaphase

spreads cannot be established then use commercially available slides from Abbott Molecular (although these are not guaranteed, quality control usually means good metaphase spreads).

3. Banding patterns seen by the DAPI stain should be distinct so that the chromosomes can be easily karyotyped. Typically, chromosomes should have sharp boundaries and weak DAPI banding.
4. To make microdissection easier, it is necessary to put a small amount of PKTB on the scalpel blade to moisten the tissue. If the tissue is not moistened, it is likely to flake off and is difficult to recover. It is also best to remove the tissue surrounding the tumor first and then remove the isolated tumor sample.
5. Do not add more than 1 μl of microdissected DNA as it may inhibit the reaction (possibly due to products from the proteinase K reaction). If using fresh DNA for a positive control or reference sample, use 50 ng in a 1 μl reaction. During nick translation we typically use 40 μl of microdissected DOP-PCRed DNA but only 25 μl of fresh genomic DOP-PCRed DNA. Thus, if using fresh DNA for reference DNA (recommended), a single DOP-PCRed sample is sufficient for two nick translation reactions.
6. There should be no DNA smear present in the negative control. DOP-PCRed DNA obtained from fresh tissue is typically 200–2,000 bp in size, from microdissected tissue it is about 100–1,000 bp in size (at least some of the DNA should be greater than 300 bp in size). If the DNA fragments obtained from microdissected samples are less than 400 bp or are low in intensity, multiple (usually two to four) DOP-PCRed samples can be pooled together and the DNA precipitated. Precipitation is performed by adding one-tenth the pooled sample volume of 3.0 M NaAc (pH 5.2) and 2.5× the volume of ice cold 100 % ethanol. Samples are stored at −80 °C overnight and centrifuged the following day for 30 min at 11,000 rpm at 4 °C. The supernatant is removed and the pellet washed with 70 % ethanol and centrifuged for 15 min at 11,000 rpm at 4 °C. The supernatant is removed; the pellet air-dried for about 10 min and then resuspended in 50 μl DNase/RNase-free water. Once again, 5 μl of the product is electrophoresed to check for quality.
7. The combination of enzymes used (BioNick enzyme mix, DNA polymerase I/DNase I enzyme mix and DNA polymerase) will need to be optimized for each new batch of enzymes. The volumes given here are an example and can be used as a good starting point. The size of the nick translated product for DNA extracted from fresh tissue should be 300–2,000 bp and from microdissected tissue 100–1,000 bp (with at least some

of the product greater than 300 bp). This protocol uses a 75 °C incubation to halt the reaction, which will allow additional nick translation to be performed if the resulting product is too large in size. An alternative approach is to halt the reaction by adding EDTA, although if this approach is used additional nick translation cannot be performed. If the product is too small in size, the ratio of DNase I and polymerase I can be adjusted (a decrease in the DNase I concentration should result in a larger product). Although nick translated products can be stored at −20 °C, better results often ensue when hybridization proceeds immediately after. Typically, test DNA is labeled with FITC-dUTP and reference DNA is labeled with Texas-Red in the initial experiment, however reverse experiments also have to be performed as fluorophores often give misleading results at specific chromosomal regions. FITC is known to give inaccurate results at 1p32-pter, 16p, 17p, 19, 22, 7q21, 9q34, 16q, and 17q, whereas 4q13-21, 11q21-23, 13q21-qter, Xq21-q22, and 19p need to be interpreted with caution when using Texas Red for labeling.

8. If metaphase chromosomes are denatured for too short a time, the probe will not bind easily and signal intensity will be low. Also, if metaphase chromosomes are over-denatured they will appear distorted. If chromosomes do appear distorted either decrease the denaturation time (usually by 30 s—1 min) or decrease the temperature of the solution to 72 °C.
9. It is important to avoid air bubbles when placing coverslips on the slides as the metaphase spreads will not be exposed to the probe. As a maximum number of metaphase spreads is required, the entire target area should be exposed to the probe and therefore air bubbles should be avoided.
10. During the first analysis if you notice that the DAPI counterstain is too strong or too weak you can adjust the concentration of DAPI in the DAPI/Vectorshield mix. Although Vectorshield protects the fluorophores (FITC, Texas Red, and DAPI), they are light sensitive and it is best to proceed to digital analysis quickly. It is recommended to leave the slides at 4 °C in the dark overnight (or at least for 1 h) and then analyze the metaphase spreads or if this is not possible, analysis should occur within a few days.
11. Although the conditions under which the slides are made allow for an increased number of cells in metaphase, the number of interphase nuclei will heavily outweigh the number of metaphase spreads. Under the 10× objective metaphase spreads look larger and less distinct than interphase nuclei. Although

the 40× magnification can give an idea of whether a metaphase spread is of good quality, the chromosomes will have to be viewed at 60× to ensure this. The metaphase spread must be intact (46 chromosomes present), chromosomes must be minimally overlapping, chromosome arms should be of appropriate length (not too long or short) and distinct and the metaphase spread should not be too close to other interphase nuclei or artifacts. Artifacts, such as dust, are often stained with the fluorophore and emit a very strong signal under the different excitation filters. It is therefore recommended that each metaphase spread is viewed briefly under each of the excitation filters to make sure that there is no artificial fluorescence that would interfere with analysis of the metaphase spread. Viewing the metaphase spread with the different excitation filters at this point will degrade the fluorophores slightly and should therefore not last more than a few seconds. It is very rare that a metaphase spread will have well defined chromosomes that do not overlap at all, for this reason multiple metaphase spreads are analyzed to ensure good coverage of all chromosomes.

12. As various computer software packages are available for image capture and analysis, detailed instructions on the use of any one software package are not provided. Instructions will vary according to the software used and should be provided with the software itself. This section focuses on the types of images to be captured and the analysis that is required. When choosing a software package, ensure that it meets the requirements for image capture and CGH analysis.
13. These exposure times are suggestions only and will typically be different for each metaphase spread on a slide and will vary across experiments. Most software packages should allow each image (i.e. blue, green, red) to be captured independently so that different exposure times can be optimized for each of the filters without interfering with the previously captured image. To prevent degradation of the fluorophores, after each exposure the filter should be removed/blocked so that the slide is not exposed to light while viewing the captured image.
14. The software package may allow a color image to be overlaid on the karyotype before the generation of a ratio profile. This is an additional measure to check for correct karyotyping as gains and losses should be displayed on both chromosomes in the same position. If there is a discrepancy between homologous chromosomes, then karyotyping is likely incorrect or one of the chromosomes may be in close proximity of an artificial fluorescent spot.

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Chapter 7

Histone Deacetylase Activity Assay

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Abstract

Histone deacetylases (HDACs) are enzymes that catalyze the removal of acetyl groups from the ε-amino groups of conserved lysine residues in the amino terminal tail of histones. Accumulating evidence suggests that many, if not all, HDACs can also deacetylate nonhistone proteins. Through deacetylating histones and nonhistone proteins, HDACs regulate a variety of cellular processes including gene transcription, cell differentiation, DNA damage responses, and apoptosis. Aberrant HDACs are implicated in many human diseases and, therefore, it is important to have a consistent and reliable assay for analyzing HDAC activities. The focus of this chapter is to provide up-to-date, easy-to-follow, approaches and techniques, for the assay of HDAC enzymatic activities.

Key words HDAC assay, HDAC2, Sirtuin, SIRT1, Protein deacetylation, Core histone, Boc-Lys(Ac)-AMC, Radioactive assay, Fluorometric assay

1 Introduction

Reversible lysine acetylation is an important posttranslational regulatory mechanism in cells. The lysine acetylation status of proteins is regulated dynamically through the opposing action of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HDAC enzymes catalyze the removal of acetyl groups from lysine residues in histone and nonhistone proteins. Deacetylation may lead to a change in the conformation and/or activity of the substrates. Through deacetylation, dynamic modification of lysine residues near the N-termini of histones plays an important role in the regulation of chromatin structure and gene expression. In general, hypoacetylation of histones is linked to transcriptional repression. Besides histones, HDACs also regulate biological processes, including DNA repair, cell cycle control, cell differentiation, and apoptosis, through deacetylating nonhistone proteins [1–3].

Eighteen mammalian HDACs have been identified so far. Based on protein size, sequence similarity, and organization of the protein domains, they are divided into three classes [1, 2].

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Class I HDACs, which include HDAC1, HDAC2, HDAC3, and HDAC8, are highly related to the yeast RPD3 protein. Class II HDACs contain HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10, and are homologues of the yeast HDA1 protein. The Class II enzymes have been subdivided further into two subclasses, IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6 and 10). HDAC11 uniquely shares sequence homology within the catalytic domains of both Class I and II HDACs. Class III HDACs, also called sirtuins (SIR2-like proteins), include SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7. Sirtuins are structurally and evolutionarily unrelated to and mechanistically distinct from the Class I and II HDACs. They couple deacetylation of substrates to cleavage of NAD^+ to form nicotinamide and O-acetyl-ADP-ribose. Therefore, the Class III HDAC/sirtuin activity assay requires the addition of NAD^+ as a cofactor.

With increased interest in HDAC enzymes over the last decade, it is very important to have readily available, reliable, and efficient approaches to analyze their activities. Currently, several different kinds of HDAC activity assays have been established, including radioactive and nonradioactive methods [4–7]. The classic radiometric HDAC activity assay directly detects the release of free radioactive isotope-labeled acetate from the deacetylation reaction using a scintillation counter. In contrast, the colorimetric, fluorogenic, and bioluminogenic methods are based on a two-step enzymatic reaction. The HDAC substrate (an acetylated lysine side chain or peptide) is conjugated with either a colored compound (i.e., para-nitroanilide), a fluorescent group (i.e., 7-amino-4-methylcoumarin [AMC]), or an aminoluciferin. After deacetylation by an HDAC, the substrate becomes sensitive to trypsin. Once the deacetylated substrate is cleaved by trypsin, a free chromophore, fluorophore, or aminoluciferin is released. In the colorimetic assay, the colored product absorbs a certain wavelength that can be recorded by a spectrometer. In the fluorescent assay, the production of fluorophore can be measured using a fluorescence reader. In the bioluminescent assay, the free aminoluciferin is processed by luciferase to generate a photophore that can be recorded by a luminometer. The signal of photophore and fluorophore is directly proportional to the deacetylation activity of the sample.

For analysis of sirtuin activity, another way is to employ nicotinamidase to measure the product of nicotinamide from the sirtuin-mediated deacetylation reaction (*see* the SIRTainty™ Class III HDAC Assay kit of Millipore).

In this chapter, we present practical step-by-step protocols for scientists outside of the HDAC field who are interested in incorporating HDAC studies into their research. We describe both the classic radioactive method and the widely used fluorometric method, using representative members of classical and sirtuin families: HDAC2 and SIRT1.

2 Materials

For the radioactive HDAC activity assay, researchers need to prepare isotope-labeled substrates by themselves. For the nonradioactive assay, dozens of colorimetric and fluorometric HDAC/Sirtuin activity assay kits are available from multiple companies (e.g., Millipore, Active Motif, Enzo Life Sciences, and Sigma). Substrates, reagents, and even enzymes can be purchased with a kit or separately. Researchers can choose the products and the commercial source according to the choice of substrate and HDACs. However, all of the reagents and materials, except substrates, can be easily prepared in a laboratory following the protocols described here.

2.1 Reagents for Purification of Enzymatically Active HDACs

The biological activity of HDACs is tightly modulated by the binding of cofactors and posttranslational modifications, particularly phosphorylation [8]. Although all Class I and II HDACs can be expressed easily as recombinant proteins in bacteria, we have found that, with the exception of HDAC8, HDAC proteins produced in *E. coli* display very little enzymatic activities. Unlike bacterially expressed HDACs, HDAC proteins isolated from mammalian or insect cells retain their native conformation, modification, and interaction with cofactors. Therefore, enzymatically active HDACs that are useful for most activity assays can be obtained from immunoaffinity-purified proteins expressed in mammalian cells or insect cells [9]. Researchers can decide the source of HDACs according to their own need and limitations. We present protocols for the expression and purification of enzymatically active HDAC2 from mammalian and insect cells, and enzymatically active SIRT1 from bacteria. Identical approaches can be applied to other HDAC members. Whole HDAC activity from cells could also be examined using nuclear extracts according to the protocol described here.

2.1.1 To Purify HDAC2 from HEK293T Cells

1. Mammalian expression plasmid pME18S-Flag-HDAC2 that expresses a Flag-tagged HDAC2.
2. HEK293T cells for the transfection recipient.
3. Lipofectamine 2000 (Invitrogen, cat # 11668019).
4. Phosphate-buffered saline (PBS): 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.4).
5. Cell lysis buffer: 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5 % NP-40, 10 % glycerol.
6. EDTA-free protease inhibitor cocktail (Roche, cat # 11617900).
7. Anti-Flag M2 agarose (Sigma, cat # A2220).
8. 1× HDAC assay buffer: 10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10 % glycerol.
9. Flag peptide to elute Flag-HDAC2 from agarose (Sigma, cat # F3290).

2.1.2 To Purify HDAC2 from Sf9 Insect Cells

1. Baculovirus expression plasmid pFastBac-HTa-HDAC2 that expresses a 6× His-tagged HDAC2. Recombinant baculovirus encoding HDAC2 generated according to the manufacturer's protocol.
2. *Sf*9 cells.
3. Cell lysis buffer: 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10 % glycerol, and 0.5 % Triton X-100.
4. Ni-NTA (nitrilo-triacetic acid) resin (Qiagen).
5. Buffer A: 20 mM Tris–HCl (pH 8.5), 1 M KCl, 20 mM imidazole, 10 % glycerol, 5 mM β-mercaptoethanol.
6. Buffer B: 20 mM Tris–HCl (pH 8.5), 1 M KCl, 10 % glycerol, 5 mM β-mercaptoethanol.
7. Buffer C: 20 mM Tris–HCl (pH 8.5), 100 mM KCl, 200 mM imidazole, 10 % glycerol, 5 mM β-mercaptoethanol.

2.1.3 To Purify SIRT1 from E. coli

1. Bacterial expression plasmid pGEX-5X1-SIRT1 that generates a GST-tagged SIRT1 recombinant protein.
2. Competent BL21/DE3 bacteria.
3. 0.1 M IPTG.
4. STE buffer: 10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA.
5. 10 % *N*-lauryl sarcosine.
6. 1 M DTT.
7. Sonicator (Sonic Dismembrator 60, Fisher Scientific).
8. 10 % Triton X-100.
9. Glutathione-agarose beads (Sigma, cat # G4510).
10. Elution buffer: 10 mM glutathione in 50 mM Tris–HCl (pH 8.0), 1 mM DTT (freshly made).

2.1.4 HeLa Nuclear Extract

HeLa nuclear extracts (2.5 mg/ml, 20 mM HEPES [pH7.5], 350 mM NaCl, 20 % glycerol, 1 mM MgCl2, 0.1 mM EGTA, 5 mM DTT, 1 % NP-40, proteasome inhibitor) will be used as a positive control of enzymatic active HDACs. The extract can be purchased from Millipore (cat # 12-309) or other companies or it can be prepared as previously described [10].

2.2 Substrates

Acetylated core histones or synthetic acetylated histone H4 peptides were originally used as substrates for HDACs [4, 11]. However, besides histone tails, an increasing number of nonhistone proteins are found to be the natural substrates for HDACs. Different substrates, p53 peptides for example, have been developed to test the deacetylase activity of HDACs [7, 12, 13]. Non-peptide-acetylated

lysine moieties, i.e., Boc-Lys(Ac) (Nα-[t-butoxycarbonyl]-Nω-acetyl lysine amide), are widely used in commercial HDAC activity assay kits because they work for most HDACs. HDACs differ in substrate affinity towards different substrates. Based on Enzo Life Sciences literatures, Class I, IIb, and III HDACs are generally most sensitive to acetylated p53 (379-382) peptides, next to acetylated p53 (317-320) peptides, followed by acetylated lysine 16 of histone H4, and the least to Lys-Ac. Class IIa HDACs are the exception. Class IIa HDACs (HDAC4, 5, 7, and 9) have undetectable activity with peptide substrates in vitro, but are active with acetylated lysine moieties, i.e., trifluoroacetyl-lysine [14]. Therefore, when analyzing the activities of Class IIa HDACs, Boc-Lys(Tfa)-AMC ([S]-tert-butyl1-[4-methyl-2-oxo-^{2}H-chromen-7-ylamino]-1-oxo-6-[2,2,2-trifluoroacetamido]hexan-2–ylcarbamate) can be chosen as the substrate. Sirtuins are more sensitive to acetylated p53 peptides than to acetyl-lysine side chains (Lys-Ac) and H4-AcK16 peptides. Individually, HDAC8 has a higher activity with the dually acetylated p53 peptides (379-382: RHK[Ac]K[Ac]) and the H4-AcK16 peptides than with singly acetylated p53 peptides (379-382: RHKK[Ac] and 317-320: QPKK[Ac]). HDAC10 and HDAC11 have higher preference for p53 (379-382) peptides than p53 (317-320) peptides. These substrates can be radioactively, colorimetrically, or fluorescently labeled dependent on the choice of assays.

2.2.1 Radioactive Substrates

Radioactive Acetylated Core Histones

1. HeLa cells.
2. 10 mg/ml cyclohexamide (CHX).
3. 2 M sodium butyrate (NaB).
4. 1.0 mCi/ml [^{3}H] acetic acid (Perkin Elmer, cat # 3499232).
5. IB buffer: 10 mM Tris–HCl (pH 7.4), 2 mM $MgCl_2$, 3 mM $CaCl_2$, 10 mM sodium butyrate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Prepare immediately prior to use.
6. NIB buffer: 1 % Nonidet P-40 in IB buffer. Prepare immediately prior to use.
7. Storage buffer: 10 mM HEPES (pH 7.5), 1 mM EDTA, 10 mM KCl, 0.2 mM PMSF, and 10 % glycerol.
8. 5 M NaCl.
9. SnakeSkin® Pleated Dialysis Tubing (Pierce, cat #68053).

2.2.2 Nonradioactive Substrates

Although radioactive HDAC assays are very sensitive, they have many shortcomings including labor consumption, batch-to-batch variation of labeling, exposure to radioactivity, and the need for radioactive waste management [15, 16]. As alternatives to radiolabeled histones or peptides, nonradioactive labeled substrates have been developed. HDAC activity can be readily detected by measuring the optical density or fluorescence using a spectrometer.

In this protocol, the fluorophore-conjugated Boc-Lys[Ac]-AMC (N-[4-Methyl-7-coumarinyl]-Nα-[t-butoxycarbonyl]-Nω-acetyl lysine amide) is used as the substrate. We have used the product from Enzo Life Biosciences (cat # ALX-260-137-M001) in this protocol. However, it is also available from other companies including Biovision (cat # K330-100) and BACHEM (cat # I-1875.0250).

2.2.3 Materials Needed for Enzymatic Reaction and Detection

Radioactive Assay

1. 5× HDAC buffer: 50 mM Tris–HCl (pH 8.0), 750 mM NaCl, 50 % glycerol.
2. Stop solution: 1 M HCl and 0.4 M acetic acid.
3. Ethyl acetate.
4. Plastic scintillation vials (Pony vial, 6 ml, Perkin Elmer, cat # 6000592).
5. Gas scintillation counter (Matrix 96 beta counter, Canberra Packard).

Nonradioactive Assay

1. 10× HDAC assay buffer: 250 mM Tris–HCl (pH 8.0), 1.37 M NaCl, 27 mM KCl, 10 mM $MgCl_2$, 10 mg/ml BSA.
2. Developer buffer: 10 mg/ml trypsin (Sigma, cat # T4799) in 50 mM Tris–HCl (pH8.0), 10 mM NaCl, 2 μM Trichostatin A, or 5 mM nicotinamide (*see* **Note 1**).
3. 50 mM β-nicotinamide adenine dinucleotide hydrate (NAD^+) (Sigma, cat # N1636, only added for Class III sirtuin assays).
4. Nunc™ 96 well polystyrene plates, black (Sigma, cat # P8741).
5. A plate reader capable of the excitation of 350–380 nm and the detection of the emission of 450–480 nm (Envision 2103 multilabel reader, Perkin Elmer).

3 Methods

3.1 Express and Purify Enzymatic Active HDACs

3.1.1 Purification of HDAC2 from HEK293T Cells

1. Transfect one 10-cm plate of HEK293T cells with 10 μg of pME18S-Flag-HDAC2 plasmid using Lipofectamine 2000 according to the manufacturer's manual. In a separate transfection, use pME18S vector as a negative control.
2. At 36 h post-transfection, wash cells twice with 1× PBS. Harvest cells in 1 ml fresh 1× PBS in a 1.5-ml tube. After centrifugation at 1,500×*g* for 5 min, lyse cell pellet in five packed-cell-pellet volumes of cell lysis buffer in the presence of an EDTA-free protease inhibitor cocktail for 30 min at 4 °C. (It is preferable not to include EDTA in the preparation of cell lysate.) Remove cell debris by centrifugation at 10,000×*g* for 15 min at 4 °C, and transfer the supernatant to a prechilled 1.5-ml tube (*see* **Note 2**).

3. Subject the supernatant to immunoprecipitation reactions with anti-Flag M2 agarose. Add 30 μl anti-Flag M2 agarose (prewashed with cell lysis buffer) per ml of cell lysate, and agitate for 2 h at 4 °C.
4. Two hours later, pellet immune complexes by centrifugation at 10,000 × *g* for 2 min at 4 °C. Carefully remove supernatant, and wash immune complexes four times using cell lysis buffer. Subsequently, wash the immune complexes three times in 1× HDAC assay buffer. Beads containing the immunoprecipitated HDAC2 are now ready to be used in the enzymatic assay.
5. (Optional) Flag-HDAC2 can be eluted with 50 μl of 1 mg/ml of Flag peptide in 25 mM Tris–HCl (pH 7.1), 120 mM NaCl, and 10 % glycerol. Dialyze protein against 25 mM Tris–HCl (pH 7.1), 120 mM NaCl, and 10 % glycerol, and distribute into 1.5-ml centrifuge tubes. Store materials at −80 °C. The HDAC sample should be refrozen immediately at −20 or −70 °C after each use to reduce loss of activity.

3.1.2 Purification of HDAC2 from Sf9 Insect Cells

1. To produce a high-titer stock baculovirus encoding HDAC2, infect *Sf*9 cells with recombinant virus at a multiplicity of infection (MOI) of 0.01–0.1. Once 15–20 % cell survival is achieved, harvest the virus-containing cell supernatant by centrifugation at 2,000 × *g* for 3 min at room temperature.
2. Infect *Sf*9 cells with viral particles (virus/cell ratio of 10:1), and harvest cells at 48 h post-infection by centrifugation at 500 × *g* for 2 min at room temperature.
3. Lyse cells in ice-cold cell lysis buffer containing 1× EDTA-free protease inhibitor. Centrifuge at 10,000 × *g* for 10 min.
4. Incubate the supernatant with Ni-NTA resin on a rotator for 1 h at 4 °C.
5. Wash resin successively with ten volumes of buffer A and two volumes of buffer B.
6. Elute protein with buffer C.
7. Determine protein concentration by the Bradford method using the Bio-Rad dye reagent with BSA as a standard. Analyze purified HDAC2 protein by SDS-PAGE and Coomassie blue staining.

3.1.3 Purification of SIRT1 from E. coli

1. Transform BL21/DE3 bacteria with pGEX-5X1-SIRT1. Grow a 6-ml culture in the presence of antibiotics overnight.
2. Dilute 100-fold of the overnight bacteria culture into 250 ml LB broth with antibiotics, and incubate at 37 °C until the OD600 reaches 0.6 (about 2 h 15 min).
3. Add IPTG to the culture to a final concentration of 0.5 mM, and incubate bacteria for 4–8 h.

4. Pellet cells by centrifugation at 6,500 × g for 10 min at 4 °C.
5. Resuspend pellet in 5 ml of ice-cold STE buffer. Addition of 100 μg/ml lysozyme and 1× protease inhibitor to the STE buffer is optional.
6. Add DTT to a final concentration of 5 mM.
7. Add *N*-lauryl sarcosine to a final concentration of 1.5 % from a 10 % stock.
8. Mix cells thoroughly and sonicate at 4 °C until the lysate becomes clear. Typically, sonicating the bacteria five times for 15 s each at the output setting of 4 is sufficient.
9. Add Triton X-100 to a final concentration of 3 % from a 10 % stock.
10. Centrifuge insoluble fractions at 10,000 × g at 4 °C for 10 min.
11. Aliquot supernatants into microfuge tubes (100–200 μl each), snap freeze in liquid nitrogen (or dry ice with ethanol), and store at −80 °C (optional).
12. Pre-swell 30 μl glutathione-agarose bead slurry, and mix with the supernatant. Rotate to mix at 4 °C for 2 h or overnight.
13. Wash beads with PBS five times to remove unbound and non-specifically bound proteins.
14. Elute fusion protein by adding 100 μl elution buffer. Repeat at least twice.
15. Analyze individual fractions by SDS–PAGE and Coomassie blue staining; pool fractions containing the highest amounts of purified recombinant protein, aliquot, and store at −80 °C (*see* Fig. 1).

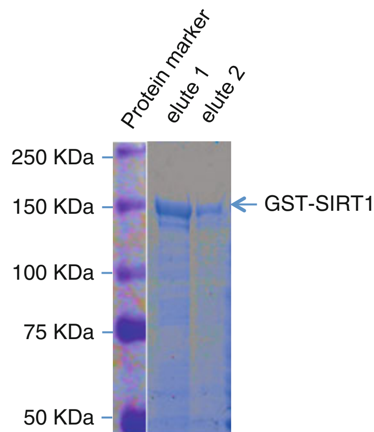

Fig. 1 A representative experiment to show purification of an enzymatically active GST-SIRT1. Coomassie *blue staining* showing the purified GST-SIRT1

3.2 Prepare [^{3}H]-Labeled Acetylated Core Histones

1. Prepare one confluent 15-cm plate of HeLa cells.
2. Wash cells once with 1× PBS.
3. Incubate cells with 20 ml PBS containing 200 μl of 10 mg/ml CHX for 10 min at 37 °C.
4. Aspirate PBS and incubate cells with 20 ml PBS containing 200 μl of 10 mg/ml CHX, 100 μl NaB, and 400 μl of 1.0 mCi/ml [^{3}H] acetic acid for 1 h at 37 °C.
5. Collect cells and chill on ice for 10 min.
6. Centrifuge at 1,500×*g* for 5 min at 4 °C to pellet cells. The following steps are done at 4 °C or on ice.
7. Wash cells with 5 ml PBS containing 25 μl of 2 M NaB three times.
8. Wash cells twice with 1 ml NIB buffer (*see* **Note 3**).
9. Wash cells once with 1 ml NIB buffer containing 100 mM NaCl.
10. Wash cells once with 1 ml IB buffer containing 100 mM NaCl.
11. Wash cells twice with 500 μl of IB buffer containing 400 mM NaCl.
12. From **steps 7–11**, pellet cells by centrifugation at 500×*g* for 5 min at 4 °C.
13. Extract the nuclei pellet with 0.25 ml of 0.2 N H_2SO_4 for 90 min on ice.
14. Centrifuge at 30,000×*g* for 90 min at 4 °C.
15. Dialyze supernatant against 500 ml of storage buffer overnight at 4 °C. Aliquot purified core histones and store at −80 °C (*see* **Notes 3–5**).

3.3 HDAC Activity Assay

3.3.1 Radioactive HDAC Activity Assay

Here we describe a detailed protocol for small-scale labeling and isolation of core histones from HeLa cells with [^{3}H] acetic acid, which is performed routinely in our laboratory and is adapted from Carmen et al. [4]. In this protocol, we have used radiolabeled acetylated core histones as the substrates. However, histones can be replaced with radiolabeled acetylated H4 or p53 peptide substrates in this assay.

1. For each reaction, the following reagents are mixed in a 1.5-ml tube in a final volume of 200 μl, containing 40 μl of 5× HDAC buffer, [^{3}H]-labeled core histones (about 10,000 cpm), 0.5 μg of HDAC2 or 1 μg of GST-SIRT1 (add 1 mM NAD^{+} cofactor if the analyzed enzyme is sirtuin), and ddH_2O (*see* **Note 6**). For the negative control, HDAC reaction can be heated to 95 °C for 5 min to destroy any enzymatic activity or skip the addition of the enzyme into the reaction.
2. Incubate reactions for 2 h at 30 °C.
3. Stop reaction by the addition of 50 μl of stop solution.

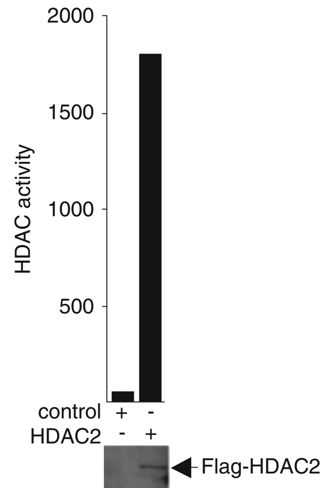

Fig. 2 A representative experiment to determine HDAC activity using radiolabeled core histones. Cells were transfected with either Flag-HDAC2 or empty vector for 48 h, lysed, and immunoprecipitated with anti-Flag M2 agarose beads. Immunoprecipitated samples then were subjected to HDAC assays as described using approximately 8,000 cpm core histone as substrate. The expression of Flag-HDAC2 was monitored by Western blotting using anti-Flag antibodies

4. Extract released [^{3}H]-acetate by adding 0.4 ml ethyl acetate with mixing. Centrifuge at 10,000 × *g* for 3 min at room temperature to separate phases.
5. Transfer 200 μl of the upper (organic) phase to a scintillation vial with 3 ml scintillation liquid and measure CPM in a scintillation counter (*see* **Note 7**).
6. HDAC activity = (counts from test sample [cpm] − counts from control [cpm]) / volume of sample (μl) (*see* Fig. 2).

3.3.2 Fluorometric HDAC Activity Assay

This fluorometric protocol is described using the GST-SIRT1 enzyme and the Boc-Lys(Ac)-AMC substrate. However, Boc-Lys(Ac)-AMC can be replaced with p53-AMC (Enzo Life Sciences, BML-KI177-0005) in this assay. This protocol can also be modified to evaluate the activity of Class I/II HDACs with the omission of NAD$^+$ (*see* **Notes 8** and **9**).

1. Dissolve the substrate of Boc-Lys(Ac)-AMC in DMSO to give a 50 mM solution.
2. Add 0.4 μl of substrates and 6 μl of 50 mM NAD$^+$ (NAD$^+$ could be omitted if the HDAC of interest belongs to Class I/II) in 1× HDAC buffer within a 96-well black plate.
3. Add 1 μg of GST-SIRT1 and 1× HDAC buffer to give a total volume of 100 μl per well with final concentration of 200 μM Boc-Lys(Ac)-AMC and 3 mM NAD$^+$ (*see* **Notes 10** and **11**), and mix well. For the background control, add 1× HDAC

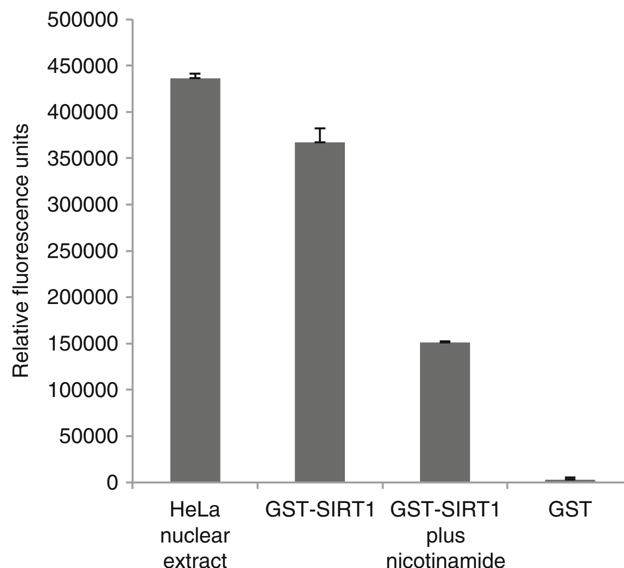

Fig. 3 A representative fluorometric experiment using non-radiolabeled substrates to measure GST-SIRT1 activity

buffer into the substrates. For the negative control, add GST instead of GST-SIRT1 into the reaction. In addition, GST-SIRT1 is added into a well together with 5 mM nicotinamide to set another negative control (*see* Fig. 3). For the positive control, add 5 μg of HeLa extract per well.

4. Incubate the plate for 1 h at 32 °C.
5. Add 100 μl of developer buffer and mix well (*see* **Note 1**). Incubate the plate for 20 min at 37 °C.
6. Read plate within 60 min. Measure the release of fluorescence in a fluorimeter plate reader with the excitation wavelength of 350–380 nm and the emission wavelength of 440–480 nm.
7. The fluorescent signals are recorded against background wells that contain substrates but without the enzyme. All fluorescence readings are expressed in relative fluorescence units (RFU) (*see* **Note 12, 13,** and Fig. 3).

Overall, there are different methods to examine the activity of HDACs, especially for sirtuins. Researchers can choose the assay according to their needs. Radioactive methods are sensitive but labor consuming. Nonradioactive methods are particularly more suitable for large-scale drug screenings. Alternatively, if a researcher wants to detect the deacetylation activity of an HDAC against a specific target, i.e., the deacetylation effect of SIRT2 on tubulin, Western blotting would be a viable choice. The acetylated targets can be detected by Western blotting with target-specific acetylation antibody or pan-acetylated lysine antibodies. Pan-acetylated lysine

antibodies are available from multiple companies, including Millipore and Cell Signaling, and make up one of the most convenient reagents to study acetylation/deacetylation. It is wise to test several different pan-acetylated lysine antibodies against the protein of interest since each antibody targets different acetylated lysines with different efficiencies. Since the basal acetylation level of many proteins may be too low to be detectable by standard procedures, a common strategy to induce acetylation is by treating cells with 10 mM nicotinamide, 100 ng/ml Trichostatin A, or both, before cell harvest.

4 Notes

1. For the sirtuin assay, 5 mM nicotinamide will be added into the developer buffer to inhibit the enzymatic reaction. For the Class I/II HDAC assay, 2 μM Trichostatin A should be added instead.
2. To maximize the release of HDACs from nuclei, cells can be sonicated three times for 15 s each at the output setting of 4 (Sonic Dismembrator 60, Fisher Scientific).
3. Since PMSF is readily degraded in water-based solutions, it is important that IB buffer, NIB buffer, and storage buffer are freshly made. Alternatively, complete EDTA-free protease inhibitor cocktail tablets (Roche, cat # 11836170001) can be used in place of PMSF.
5. To ensure that the isolated core histones are labeled correctly, an aliquot of core histones can be measured for specific activity using a scintillation counter. Using this protocol, the counts for 10 μl of core histones are approximately 10,000 cpm.
6. For many HDACs, core histones or histone H4 peptides work well as a substrate. However, some HDACs may require mononucleosome instead of free histones as a substrate. The preparation of mononucleosome substrates has been described elsewhere [3, 10, 14].
7. The upper phase should be very carefully transferred to the scintillation vial using a pipet tip to avoid background count carryover from interphase contamination.
8. GST-SIRT1 used in the assay can be replaced with an immunoprecipitated SIRT1 protein from transfected mammalian cells as described for HDAC2.
9. To maintain the enzymatic activity, aliquot the purified HDACs or HeLa nuclear extract, snap freeze in liquid nitrogen, and store at −80 °C. Thaw reagents just before use. Aliquot unused material into prechilled microcentrifuge tubes and snap freeze in liquid nitrogen prior to storage at −80 °C.

10. It is recommended to run every sample and control at least in duplicate.
11. Although the described fluorometric assay is performed within a regular 96-well plate, it could also be modified to be performed on a ½-volume 96-well plate by decreasing half of the reaction volume and half of the developer buffer.
12. For the fluorometric assay, black plate is often used. However, white plates may be chosen to a get higher signal. If the signal is too strong, clear plates could be used instead, and the reading would decrease about fivefold.
13. Developer buffer and NAD^+ may exhibit some autofluorescence. Background reading of the substrates without enzymes is usually high. Therefore, it is necessary to subtract the reading of the HDAC samples to that of no-enzyme control. Different readers and 96-well plates have different sensitivities in the detection of fluorophores. Researchers can optimize the instrument setting and the choice of plates by using non-acetylated Boc-Lys-AMC standards (Enzo Life Sciences). In brief, incubate different dilutions of a non-acetylated substrate standard (i.e., 0–50 μM) with an equal volume of developer buffer and read the fluorescence as described in the protocol. Adjust the parameters of the reader according to the range of the standards.

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Chapter 8

In Vitro Histone Demethylase Assays

Kenji Kokura, Lidong Sun, and Jia Fang

Abstract

Histone methylation plays pivotal roles in modulating chromatin structure and dynamics and in turn regulates genomic processes that require access to the DNA template. The methylation status at different sites is dynamically regulated by histone methyltransferases and demethylases. During the past decade, two classes of proteins have been characterized to actively remove methyl groups from lysine residues through different mechanisms. The LSD1/KDM1 family of amine oxidases require flavin adenine dinucleotide (FAD) for reaction, while the larger Jumonji C (JmjC) family of hydroxylases utilize Fe(II) and α-ketoglutarate as cofactors to demethylate histones. Since their discoveries, histone demethylases have been implicated in the precise control of gene expression program during development, cell identity, and fate decision. Several demethylases have also been linked to various human diseases such as neurological disorders and cancer. This chapter describes several in vitro assay conditions and detection methods for two classes of histone demethylases. We also discuss the protocols to prepare various substrates for different histone demethylase assays.

Key words Epigenetic regulation, Histone methylation, Histone demethylation, Histone demethylase, Oxidative hydroxylation, Chromatin, Nucleosomes, Histone

1 Introduction

Various covalent modifications on histones play essential roles in chromatin organization and function [1, 2]. Among them, histone methylation occurs on lysine and arginine residues and regulates fundamental processes such as transcription, heterochromatin formation, X inactivation, genomic imprinting, cell cycle, and DNA damage response [3]. Lysine residues can be mono-, di-, or tri-methylated by SET domain-containing histone methyltransferases (HMTases) and DOT1/DOT1L proteins, while arginine residues can be mono- or di-methylated by PRMT proteins. The methylation marks at different sites or different methylation states at the same residue serve as recognition sites to recruit different methyl-binding proteins to various genomic regions. Therefore, histone methylation functions as a complex regulatory platform for downstream signaling and biological consequences [4]. Although histone methylation was historically assumed as an irreversible

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epigenetic mark, tremendous recent studies have demonstrated that it can be erased through different enzymatic reactions [5–7].

Seeking for histone demethylases lasted over 40 years since Paik and colleagues reported in 1964 that purified N6-methyl-lysine oxidase can demethylate mono- and di-methylated lysines [8]. In 2004, Allis and colleagues uncovered that human arginine deiminase PADI4/PAD4 can turn over methyl groups on histones by converting mono-methylated arginine to citrulline [9, 10]. In the same year, Shi and colleagues demonstrated that a nuclear amine oxidase LSD1 is the first bona fide histone lysine demethylase, which removes methyl groups from H3K4 through a flavin adenine dinucleotide (FAD)-dependent amine oxidation [11]. As formed imine intermediate in this reaction requires a lone pair of electrons in the nitrogen atom of methyl-lysine, LSD1 only demethylates mono- and di-methylated lysines. Based on the reaction mechanism used by AlkB to demethylate damaged DNA [12, 13], Zhang and colleagues later developed a novel in vitro histone demethylase assay and identified a large family of JmjC domain-containing demethylases [14, 15]. This family of proteins use Fe(II) and α-ketoglutarate as cofactors to catalyze a hydroxylation-based oxidative reaction and thus are able to demethylate all methylation states. Since then, many proteins in this family have been demonstrated to demethylate histones spontaneously in a site- and state-specific manner and regulate various important functions [5–7].

Here, we describe substrate preparation and several in vitro histone demethylase assays based on different detection methods. Native core histone octamers or nucleosomes can be purified from large-scale cultured HeLa-S3 cells. By incubating with different HMTases, ^{3}H-labeled methyl groups can be added to specific lysines on these histones, which can next be used as substrates for different demethylation reactions. In the radical-based oxidative hydroxylation catalyzed by JmjC domain-containing demethylases (type I lysine demethylation), methyl-lysine is converted to a carbinolamine intermediate. The hydroxyl-methyl groups are next released as formaldehyde while α-ketoglutarate is converted to succinate and carbon dioxide. Ascorbic acid is also included in the reaction to regenerate Fe(II) from Fe(III) [14, 16]. In the LSD1-catalyzed type II demethylation, methyl-lysine is oxidized by FAD to form an imine intermediate, which is further hydrolyzed to yield unmodified lysine and formaldehyde [11]. To monitor the released [^{3}H]-formaldehyde in both reactions, proteins can be removed through trichloroacetic acid (TCA) precipitation. Formaldehyde in the supernatant is then converted to 3,5-diacetyl-1,4-dihydrolutidine (DDL) using the modified NASH method [17]. DDL stays in the organic phase during the following extraction step using 1-pentanol and thus can be separated from other radioactive contaminations and accurately measured by liquid scintillation counting. As histones are methylated in vivo, purified

native histones can also be used directly as substrates in demethylase assays. In this case, the change of methylation levels can be monitored by western blot using site- and state-specific methyl-histone antibodies. In addition, we briefly describe the recently developed Shokat's reactions which allow a specific and complete installation of methyl-lysine analogs (MLAs) into purified recombinant histones [18, 19]. Similarly, these histones can serve as substrates in demethylase assays followed by immunoblotting analyses. Complementing these in vitro assays, we also discussed a transfection-based immunofluorescence assay which can be used to test whether a protein has the histone demethylase activity in cells.

2 Materials

2.1 Purification of Nucleosomes and Histone Octamers

1. Cultured HeLa-S3 cells (20 l), harvested at mid-log phase (5×10^5 cells/ml).
2. 40 ml Dounce homogenizer with type B pestle.
3. Histone purification buffer I: 10 mM MES, pH 6.5, 0.5 mM sodium metabisulfite, 0.5 mM benzamidine-HCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 15 mM NaCl, 60 mM KCl, 0.25 M sucrose, 0.5 % Triton X-100, 0.1 mM PMSF, 0.5 mM DTT.
4. Histone purification buffer II: 10 mM PIPES, pH 6.5, 0.5 mM sodium metabisulfite, 0.5 mM benzamidine–HCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF, 0.5 mM DTT.
5. Nuclease, Micrococcal (MNase, Worthington Biochemical): Dissolve at 10 U/μl in 50 mM Tris-Cl, pH 7.9, 5 mM $CaCl_2$, aliquots are stored at −80 °C.
6. Histone purification buffer III: 1 mM EDTA, pH 8.0, 0.2 mM PMSF.
7. SG gradient makers (Hoefer SG100, GE Healthcare).
8. Sucrose gradient buffer: 5 % or 30 % (w/v) sucrose in 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 0.75 M NaCl, 0.3 mM PMSF, stored at 4 °C.
9. 5× SDS loading buffer: 0.25 M Tris–HCl, pH 6.8, 0.5 M DTT, 10 % SDS, 0.25 % bromophenol blue, 40 % glycerol.
10. Histone storage buffer: 10 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1 mM EDTA, 0.2 mM PMSF, 10 % glycerol.
11. Hydroxyapatite Bio-Gel® HT Gel (BioRad).
12. Histone purification buffer IV: 40 mM Na_2HPO_4, pH 6.8, 1 mM DTT, 0.2 mM PMSF.

2.2 In Vitro Installation of Methyl-Lysine Analogs into Histones

1. Purified and lyophilized recombinant Xenopus histone H3 (C110A mutant) or H4 full-length proteins, harboring the lysine-to-cysteine mutation at the desired methylation site (*see* **Note 1**).

2. Alkylation buffer: 1 M HEPES pH 7.8, 4 M guanidinium chloride, 10 mM D/L-methionine.
3. 1 M (2-chloroethyl)-methylammonium chloride (Karl Ind.), dissolved immediately before use.
4. 1 M (2-chloroethyl)-dimethylammonium chloride (Aldrich), dissolved immediately before use.
5. (2-Bromoethyl)-trimethylammonium bromide (Aldrich).

2.3 In Vitro Histone Methyltransferase Assays

1. 5× HMTase buffer: 100 mM Tris–HCl, pH 8.0, 20 mM EDTA, 2.5 mM DTT.
2. S-Adenosyl-L-[methyl-^{3}H]methionine (Perkin Elmer, 5–15 Ci/mmol).
3. Histone methyltransferases: Active HMTases can be endogenously purified from cultured cells, recombinant proteins/complexes purified from transfected 293T cells or infected Sf9 cells, or recombinant proteins purified from bacteria with different tags (*see* **Note 2**).
4. EN^{3}HANCE or ENLIGHTNING gel autoradiography enhancer (Perkin Elmer).
5. BioRad Model 583 Gel Dryer.

2.4 Histone Demethylase Assays

1. Buffer C: 40 mM HEPES–KOH, pH 7.9, 50 mM KCl, 0.2 mM PMSF, 1 mM DTT, 10 % glycerol.
2. 5× Histone demethylase buffer type I: 250 mM HEPES–KOH, pH 8.0, 7–700 μM $Fe(NH_4)_2(SO_4)_2$, 5 mM alpha-ketoglutarate, 10 mM ascorbate acid (*see* **Note 3**).
3. 5× Histone demethylase buffer type II: 0.5 M glycine, pH 8.0, 250 mM KCl (*see* **Note 4**).
4. NASH buffer: 3.89 M ammonium acetate, 0.1 M acetic acid, 0.2 % 2,4-pentandione, stored at 4 °C.
5. 1-Pentanol (Sigma).
6. 4 % paraformaldehyde (w/v) in PBS, dissolved immediately before use.
7. Blocking buffer: 1 % BSA in PBS.
8. Secondary antibodies: Donkey anti-mouse, rabbit or chicken, conjugated with Rhodamine, FITC, or HRP (Jackson ImmunoResearch Lab, Inc).
9. 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma), dissolved in water at 1 mg/ml as stock solution (1,000×).
10. Fluorescent Mounting Medium (Dako Cytomation).

3 Methods

3.1 Histone Octamer and Nucleosome Purification from HeLa-S3 Cells

Suspension-cultured HeLa-S3 cells are good source to obtain native histones and nucleosomes for various applications. Oligo- and mononucleosomes can be purified from MNase-digested nuclei by sucrose gradient ultracentrifugation and core histone octamers can be further purified by hydroxyapatite chromatography. The following purification protocol is derived from previous published methods [20–22]. Purified histones and nucleosomes can next be radioactively labeled or directly applied as substrates for different histone demethylation assays (*see* **Note 5**).

3.1.1 Nuclei Preparation and Digestion

1. Resuspend HeLa-S3 cells (~1×10^{10} total) in 100 ml histone purification buffer I and lysate cells with a Dounce homogenizer (B type pestle, 10 strokes). Pellet nuclei by centrifugation at $3724 \times g$ for 10 min at 4 °C (Beckman SX4750). Wash one time with the same buffer and resuspend nuclei in 12 ml histone purification buffer II (*see* **Note 6**).
2. Take out 0.5 ml nuclei suspension and warm up to 37 °C. Add $CaCl_2$ to 5 mM and 50 U MNase for digestion at 37 °C. Transfer 50 μl suspension every 5 min into a tube containing 1 μl 0.5 M EDTA and add 75 μl H_2O, 30 μl 10 % SDS, 25 μl 4 M NaCl, and 200 μl phenol/chloroform in order to extract DNA. Analyze 10 μl sample on a 1 % agarose gel to determine the optimal digestion time which should yield majority of DNA around 2 Kb.
3. Warm up the bulk nuclei for 10 min at 37 °C and carry out digestion under the same settings for the optimized time. Stop the digestion by adding 0.5 M EDTA to final concentration of 4 mM.
4. Centrifuge at $2988 \times g$ for 5 min at 4 °C (Sorvall SS-34) and save supernatant as S1. Take out 10 μl sample into 1 ml 1 M NaOH and measure the A_{260} units.
5. Resuspend the pellet with equal volume of histone purification buffer III. Incubate on ice for 15–30 min with extensive vortexing at every 5–10 min to extract chromatin.
6. Spin at $11952 \times g$ for 15 min at 4 °C (Sorvall SS-34). Save supernatant as S2 and measure the A_{260} units. Repeat this step if the A_{260} units are low in S1/S2. Combine DNA-enriched samples.
7. Add 5 M NaCl dropwise to the final concentration of 0.75 M with vortexing (*see* **Note 7**).

3.1.2 Purification of Nucleosomes and Histone Octamers

1. Prepare 35 ml linear sucrose gradients (5–30 %) in 40 ml polyallomer tubes (Beckman) using an SG gradient maker with 5 % and 30 % sucrose gradient buffer (*see* **Note 7**).

2. Gently load 4–5 ml chromatin samples (up to 300 A_{260} units) to each gradient and centrifuge at 121570 × *g* for 16 h at 4 °C (Beckman SW-28). Carefully fractionate gradients by manually siphoning 1 ml fractions from top to bottom of each gradient.
3. Take out 50 μl samples from each fraction and add 100 μl of 25 % TCA to precipitate proteins on ice for 30 min. After centrifugation, wash with 0.5 ml of cold acetone and air-dry the pellet. Examine the purity of histone proteins by 18 % SDS-PAGE.
4. Take out another 50 μl samples to examine the size of DNA (**step 2**, Subheading 3.1.1).
5. Combine fractions containing histones with good purity and DNA with 1–2.5 Kb size as oligo nucleosomes. Dialyze against histone storage buffer (*see* **Notes 8** and **9**).
6. Combine fractions containing histones with good purity and DNA with >2.5 kb size. Dialyze against 10 mM Tris-Cl (pH 8.0) containing 1 mM PMSF and concentrate by ultrafiltration (Amicon Ultra-15, Millipore).
7. Add $CaCl_2$ to 5 mM and MNase for a complete digestion. Purify mononucleosomes using the same sucrose gradient protocol (*see* **Notes 8** and **10**).
8. Combine all other histone-containing fractions and dialyze against 10 mM Tris-Cl (pH 8.0) with 1 mM PMSF. Load sample onto a hydroxyapatite column equilibrated with histone purification buffer IV with 300 mM NaCl. After washing with 500 mM NaCl, elute proteins with the same buffer containing 2.5 M NaCl into 0.5–1 ml factions. Check protein purity by SDS-PAGE and combine fractions as histone octamers. Dialyze against histone storage buffer (*see* **Note 8**).

3.2 In Vitro Histone Methylation

HMTase assay is adapted from a published protocol [23] and carried out as previously described [21, 24]. Recombinant HMTases can be purified through standard protein purification protocols to methylate their preferred substrates using S-adenosyl-L-[*methyl*-^{3}H]-methionine as methyl donor. [*methyl*-^{3}H]-histones can next be used as substrates for demethylase assays. Because of variations among different purifications, optimizing the HMTase/substrate ratio is strongly recommended.

1. Incubate 3–5 μg histone octamers or nucleosomes, 1 μl S-adenosyl-L-[*methyl*-^{3}H]-methionine, 1/5 volume of 5× HMT buffer, and different amount of purified HMTase at 30 °C for 1 h.
2. Stop the reaction by adding 1/5 volume of 5× SDS loading buffer and analyze the reaction by 18 % SDS-PAGE. Stain the gel with Coomassie blue R-250 for 30 min and destain it well.

3. Treat the gel with autoradiography enhancer for 30–45 min with gentle shaking and wash with deionized water for 5–10 min. Dry the gel on filter paper for 2.5 h at 70 °C using a gel dryer.
4. Expose the dried gel to an X-ray film with intensifying screens overnight at −80 °C. Develop the film and determine the optimized HMTase/histone ratio based on the activity.
5. Scale up the reaction 10–50-fold under the optimized conditions.
6. Dialyze the reaction against 4 l histone storage buffer without EDTA at 4 °C overnight to remove free S-adenosyl-L-[*methyl*-^{3}H]-methionine (in tubing with 3.5 kDa MW cutoff).
7. Analyze 1 μl dialyzed sample by liquid scintillation counting and store at 4 °C (*see* **Note 11**).

3.3 Installation of Methyl-Lysine Analogs into Full-Length Histones

HMTase assay is a simple and effective approach to introduce methyl groups on histones at the particular site; however, it is limited by the availability of HMTases to methylate the specific lysine to different states. Shokat and colleagues recently developed a chemical approach to install MLAs into recombinant histone proteins [18, 19]. The installation site is specified by mutating the lysine to cysteine, which can next be alkylated by different alkylating reagents to produce different *N*-methyl aminoethylcysteines. Given that their biochemical properties are similar to the natural methyl-lysines [18, 19, 25, 26], MLA-installed histones have been successfully used as substrates in various histone demethylase assays [25, 27, 28]. Moreover, MLA-installed histones can be assembled into core octamers or further reconstituted into nucleosomes [29–31] to determine the substrate specificity of histone demethylases.

1. Dissolve 5 mg lyophilized histone protein containing specific lysine-to-cysteine mutation using 900 μl (for me1), 900 μl (for me2), or 980 μl (for me3) alkylation buffer. Add 20 μl 1 M DTT to reduce histone at 37 °C for 1 h.
2. To generate mono-methylated MLA, add 100 μl 1 M (2-chloroethyl)-methylammonium chloride to proceed reaction for 4 h at room temperature. Add another 10 μl 1 M DTT and allow the reaction for at least another 10 h at room temperature.
3. To install di-methylated MLA, add 50 μl 1 M (2-chloroethyl)-dimethylammonium chloride and allow the reaction for 2 h at room temperature. Add 10 μl 1 M DTT and incubate the reaction for 30 min at room temperature. Add 50 μl 1 M (2-chloroethyl)-dimethylammonium chloride and proceed the reaction for another 2 h at room temperature.
4. To produce tri-methylated MLA, add 100 mg (2-bromoethyl)-trimethylammonium bromide and carry out the reaction at 50 °C

for 2.5 h with occasional mixing. Add 10 μl 1 M DTT and allow the reaction for another 2.5 h at 50 °C.

5. Quench the reaction with 50 μl 2-mercaptoethanol (14.2 M) and purify histone from the reaction mixture using PD-10 column pre-equilibrated with 3 mM 2-mercaptoethanol (*see* **Note 12**).

3.4 Histone Demethylase Assays

The methyl groups on histone lysine residues can be dynamically removed by LSD1/2 and JmjC domain-containing demethylases through two different oxidation reactions. The following protocols describe two reaction conditions. The type I reaction system is developed for various JmjC domain histone demethylases which use Fe(II), alpha-ketoglutarate, and ascorbic acid as cofactors, whereas the type II reaction condition is optimized for LSD1/2 histone demethylases. Given that both reactions release formaldehyde, the most sensitive detection method is to monitor the release of radiolabeled formaldehyde from [*methyl*-^{3}H]-histones [14, 16, 32–39]. However, generating [*methyl*-^{3}H]-histones highly depends on the availability of specific HMTases. Moreover, purified histone octamers/nucleosomes or MLA-installed recombinant histones can be directly used as substrates in the reaction [25, 28]. In this case, demethylase activities at every methylation site can be determined by western blotting using commercially available site- and state-specific methyl-histone antibodies [5, 14, 16, 28, 33–35, 37, 39–58]. Although it is easy to perform, this detection method requires relatively large amount of demethylase to detect robust activities. When the peptide substrates are used in the reactions, mass spectrometry can also be used to quantitatively measure the loss of methyl groups [5, 11, 14, 27, 35–42, 44, 46–49, 51, 54–56, 59]. Additionally, transfection-based immunofluorescence approach has also been widely used to evaluate the demethylase activity in cells [5, 11, 14, 34–43, 45–57, 60]. Because of these advantages and limitations, different assays have been combined in most demethylase studies. Importantly, Triever and colleagues recently applied a formaldehyde dehydrogenase (FDH)-coupled assay [61] in histone demethylation by including FDH and NAD^{+} in the reaction [27, 62]. In this modified assay, formaldehyde produced by histone demethylases is further oxidized to formate by FDH, with reduction of the nonfluorescent NAD^{+} to the fluorescent NADH [27, 59, 62]. Given that NADH production can be monitored continuously at 340 nm, this assay has been further applied in the quantitative high-throughput screenings for different histone demethylase inhibitors [63, 64].

3.4.1 In Vitro Radioactive Formaldehyde Release Assays

1. Dialyze recombinant histone demethylases or the demethylase-containing protein fractions against 4 l buffer C at 4 °C (*see* **Note 13**).
2. Incubate [*methyl*-^{3}H]-histone octamers or nucleosome substrates (about 10,000 cpm total, *see* Subheading 3.2), demethylase

samples, and 1/5 volume of 5× histone demethylase buffer I or II. Proceed the reaction at 37 °C for 1–2 h (*see* **Notes 14–16**).

3. Add water into reaction to bring up the total volume to 100 μl. Add 100 μl 25 % TCA to precipitate proteins for 20 min on ice.
4. Spin at top speed for 10 min at 4 °C and carefully transfer 180 μl supernatant into a microtube containing 200 μl NASH buffer. Proceed the conversion reaction at 37 °C for 50 min.
5. Add 0.4 ml 1-pentanol and vortex well. Centrifuge at top speed for 5 min at room temperature.
6. Carefully transfer 350 μl organic phase (top) into a scintillation counting vial containing 1 ml scintillation cocktail. Mix thoroughly by inverting vials more than five times and measure counts using the scintillation counter.

3.4.2 Western Blot-Based Histone Demethylase Assays

1. Dialyze histone demethylase samples and histone substrates, such as purified histone octamers, nucleosomes, or MLA-installed recombinant histones against 4 l buffer C at 4 °C.
2. Incubate 1–5 μg purified histone substrates (*see* Subheadings 3.1 and 3.3), histone demethylase samples, and 1/5 volume of 5× histone demethylase buffer I or II. Proceed the reaction at 37 °C for 1–2 h (*see* **Notes 13–17**).
3. Stop the reaction by adding 1/5 volume of 5× SDS loading buffer and resolve proteins in the reactions by 18 % SDS-PAGE.
4. Transfer proteins onto a polyvinylidene difluoride (PVDF) membrane and proceed western blot analyses using site- and state-specific methyl-histone antibodies. Histone H3 or H4 antibodies should be used in parallel in immunoblotting as the loading controls.

3.4.3 Immuno-fluorescence-Based Histone Demethylase Assays

1. Plate 293T or NIH-3T3 cells on 0.2 % gelatin-coated glass coverslips in 12-well plates 1 day before the transfections (*see* **Note 18**).
2. Transfect cells with expression plasmids (Flag, HA, myc, or GFP tagged) harboring different histone demethylases/candidates, or the control plasmid using appropriate transfection reagent. Allow protein expression for 24–48 h.
3. Wash coverslips with PBS and fix cells for 20 min in 4 % paraformaldehyde (*see* **Note 19**).
4. Permeabilize cells with cold PBS buffer containing 0.5 % Triton X-100 for 5 min on ice.
5. Wash cells with blocking buffer for 5 min three times and incubate coverslips in the blocking buffer at room temperature for 30 min.

6. Incubate coverslips with primary antibodies diluted in blocking buffer for 1 h in a humidified chamber with gentle rocking motion.
7. Wash coverslips with blocking buffer for 5 min three times and incubate with fluorescent-labeled secondary antibodies diluted in blocking buffer for 1 h with gentle rocking motion.
8. Wash cells with PBS for 5 min two times and stain with DAPI.
9. Wash cells with PBS for 5 min two times and water one time. Mount coverslips on slides. Dry slides in the dark place overnight and exam under fluorescent microscope.

4 Notes

1. Detailed protocols for expression and purification of recombinant Xenopus histones have been described previously by Luger and colleagues [29].
2. For histone lysine methylation, the following recombinant HMTases can be used to generate different [*methyl*-^{3}H]-histone substrates for HMTase assays: GST-SET7/9 (H3K4me1, core histone octamers), GST-SET7/9-Y245A (H3K4me2&3, core histone octamers), GST-G9a (621-1000aa) (H3K9me1 and H3K9me2, core histone octamers) and GST-SUV39H1(82-412)-H320R (H3K9me, nucleosomes), GST-Dim5 (*N. crassa*, H3K9me3, core histone octamers), Ezh2 complex (H3K27me, nucleosomes), CBP-Set2-Flag (*S. pombe*, H3K36me2, nucleosomes), GST-DOT1L (1-416) (H3K79me, nucleosomes), GST-SET8 (H4K20me1, core histone octamers, and nucleosomes), GST-Suv4-20 h1, and GST-Suv4-20 h2 (H4K20me2 and 3, nucleosomes).
3. Type I histone demethylase buffer should be made freshly from 1 M HEPES-KOH (pH 8.0), 0.5 M $Fe(NH_4)_2(SO_4)_2$, 0.5 M α-ketoglutarate, and 0.5 M ascorbate acid stock solutions. Aliquot of stock solutions of cofactors should be stored at −20 °C.
4. Alternatively, another reaction buffer was initially reported for LSD1 family of histone demethylases: 50 mM Tris-HCl pH 8.5, 50 mM KCl, 5 mM MgCl, 0.5 % BSA, and 5 % glycerol [11].
5. Core histone octamers and nucleosomes can be purified from chicken red blood cells following same protocols.
6. Generation of nuclei should be verified under microscope.
7. This step is to remove histone H1 from nucleosomes. Omit this step and remove 0.75 M NaCl in the sucrose gradient buffer if histone H1 is needed. Alternatively, 0.75 M NaCl can be included directly in histone purification buffer III during extraction steps to simplify the procedure if histone H1 is not required.

8. Dialyze to histone storage buffer in the absence of 1 mM EDTA if histones will be directly applied to histone demethylase assays under type I reaction condition.
9. Purified nucleosomes can be stored for up to 6 months at 4 °C and for longer at −80 °C.
10. A couple of top gradient fractions contain mononucleosomes; however, these fractions are not recommended for HMTase assays directly because of impurity.
11. ^{3}H-labeled methyl-histone octamers or nucleosomes should be stored at 4 °C for up to 1 month. Avoid freeze-thaw steps. Longer storage could result in higher background in the histone demethylase assays for unknown reasons.
12. MLA-installed histone can also be purified from the reaction mixture by dialysis.
13. The enzymatic activity of JmjC domain-containing histone demethylases is inhibitored divalent transition metal ions such as Co(II), Cu(II), and Ni(II). Purification of His-tagged JmjC demethylases using immobilized metal ion affinity chromatography (IMAC) is not recommended [27, 65–67].
14. Avoid EDTA in type I histone demethylase reactions. EDTA does not affect the type II histone demethylase reactions.
15. 70 μM $Fe(NH_4)_2(SO_4)_2$ in 5× type I histone demethylase buffer is suitable for most of the cases; however, titration of $Fe(NH_4)_2(SO_4)_2$ concentration is recommended to get optimized assay condition for different histone demethylases.
16. 10 mM $MgCl_2$ is included in a similar type I reaction buffer for the core histone octamer substrates, but not for the nucleosome substrates [40–42].
17. Commercial or customer-synthesized site-specific methyl-histone peptides can also be applied as substrates using dot blot-based detection method. Mass spectrometry can also be employed to detect reduction of histone peptide masses [14, 15, 33–36, 38–41, 47, 54, 56].
18. Other cell lines have also been used for the histone demethylase assays including HeLa, U2OS, COS-7, and iMEF.
19. Alternatively, cells can be pre-extracted with Triton X-100 prior to fixation [68].

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Chapter 9

Integrated DNA Methylation and Chromatin Structural Analysis at Single-Molecule Resolution

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Abstract

Chromatin limits the accessibility of DNA to *trans*-acting factors in transcription, replication, and repair. Although transcriptional variation between cells in a population may contribute to survival and disease, most assays of chromatin structure recover only population averages. We have developed DNA methyltransferases (MTases) as probing agents of DNA accessibility in chromatin, either expressed in vivo in budding yeast or as recombinant enzymatic probes of nuclei isolated from mammalian cells. In this chapter, we focus on the use of recombinant MTase (M) M.CviPI to probe chromatin accessibility in nuclei isolated from mammalian cell lines and animal tissue. This technique, named methylation accessibility protocol for individual templates (MAPit), reports protein–DNA interactions at single-molecule resolution. The single-molecule readout allows identification of chromatin subpopulations and rare epigenetic variants within a cell population. Furthermore, the use of M.CviPI in mammalian systems gives a comprehensive view of both chromatin structure and endogenous DNA methylation in a single assay.

Key words Chromatin, DNA methylation, DNA methyltransferases, Footprinting, Nucleosomes, Single-molecule methods, Transcription

1 Introduction

Site-specific protein–DNA interactions and chromatin structure have emerged as central aspects of chromosome biology in all eukaryotic organisms. Hence, precise mapping of protein–DNA interactions is necessary to unravel the complexities of genome function in living cells. Classical footprinting techniques infer sites of protein binding to DNA from spans of protection against enzymatic (i.e., DNase I or micrococcal nuclease) or chemical probing reagents (e.g., dimethylsulfate). Accessibility to these probes is usually detected by mapping cuts in the DNA strand. While extremely informative, this approach breaks apart the individual elements or modules within each molecule or functional unit.

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Moreover, molecular heterogeneity within a population of cells is obscured by averaging the contribution of every molecule at each specific site of cleavage; that is, differences between individual gene copies cannot be visualized (reviewed in ref. 1). To overcome these disadvantages, we have developed MAPit for mapping protein–DNA interactions with high spatial resolution on single molecules while preserving the integrity of individual DNA molecules [2, 3]. In this chapter, we describe a detailed MAPit protocol to probe chromatin structure in intact nuclei isolated from cultured cell lines and tissue, using purified M.CviPI that methylates the C in accessible GpC dinucleotides (hereafter, GC) [4]. MAPit is based on the observation that association of histones and site-specific DNA-binding factors with DNA hinders the accessibility of exogenously added DNA MTases [5–9], including M.CviPI [2, 3]. After probing, protein–DNA interactions are located by determining the pattern of accessible and inaccessible GC sites by bisulfite sequencing. In this procedure (Fig. 1), denatured DNA is treated with bisulfite ion under conditions in which C residues that are unmethylated (i.e., due to factor-binding impeding MTase access to DNA) are converted by hydrolytic deamination to uracil, while accessible or methylated C residues (m^5C) resist chemical conversion [10]. During PCR of the region of interest, m^5C is amplified as C, whereas U base pairs with A in the first PCR cycle and is subsequently replaced by T [11–13]. Sequencing individual DNA products after amplification of bisulfite-converted sequences permits assignment of the methylation status of every enzyme target site along a single continuous DNA strand.

The premise of mapping protein–DNA interactions with DNA MTases is identical to probing with conventional nucleases, except that footprints arise from protection against DNA methylation rather than scission. This presents a distinct advantage over classic footprinting techniques because the integrity of each DNA molecule is preserved. In addition, the methylation status of each site is determined independently from other sites on individual DNA strands, enabling m^5C at multiple sites on the same molecule to be scored. This eliminates concerns regarding single-hit kinetic levels of methylation and provides absolute rather than relative methylation frequencies. Analysis of sets of cloned molecules yields a quantitative view of chromatin structure and also discerns variation between molecules from different cells [2, 14–19]. Importantly, probing mammalian samples with the GC-methylating enzyme M.CviPI allows simultaneous mapping of chromatin accessibility and detection of endogenous methylation of CpG (hereafter, CG) [2, 20, 21]. Thus MAPit is able to detect subpopulations of molecules with distinct patterns of protein binding or chromatin architecture, and correlate them directly with the occurrence of endogenous DNA methylation. The single-molecule readout allows identification of chromatin subpopulations and rare epigenetic variants within a cell population [2, 17, 18, 22–25].

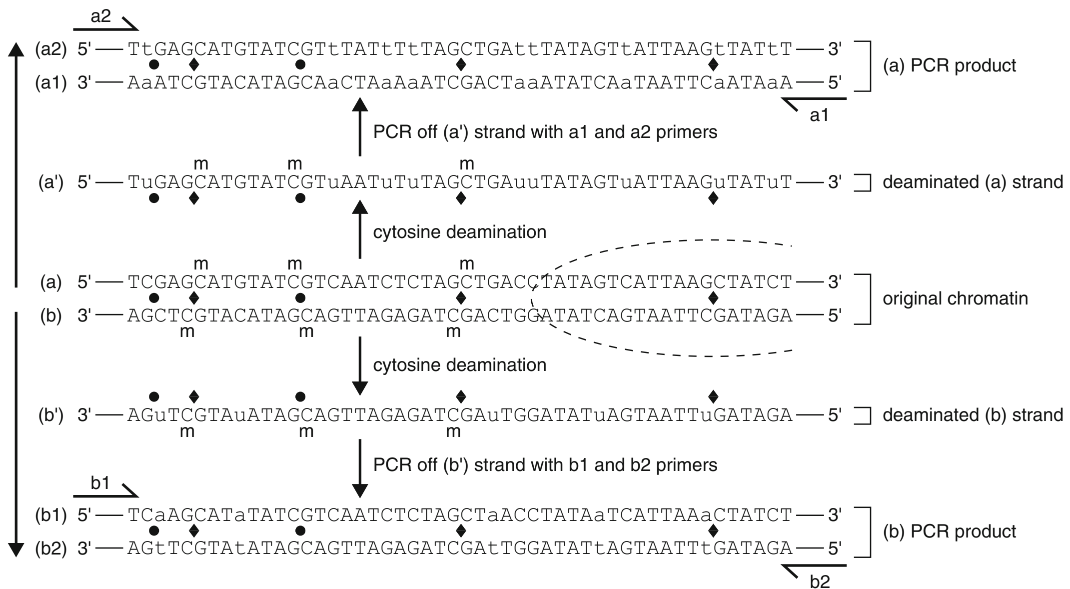

Fig. 1 Integrated detection of chromatin accessibility and DNA methylation by MAPit. M.CviPI sites (GC) are marked by *filled diamonds*, whereas CG sites are marked by *filled circles*. The original segment of M.CviPI-probed chromatin is shown in the *middle*, with a DNA-bound protein depicted as a *dashed partial ellipse*. DNA occupancy by this protein protects a GC target site against methylation by M.CviPI. The protected and hence unmodified C in this GC site, an unmethylated CG site at *left*, as well as other C residues in non-GC and non-CG sites are subsequently deaminated to uracil (u) by treatment with bisulfite. Conversely, cytosines in GC sites exogenously methylated (m) by M.CviPI and an endogenously methylated CG site in the original chromatin sample resist chemical deamination. Note that the a′ and b′ DNA strands are no longer complementary following deamination, and thus can and must be amplified separately with dedicated primer pairs (a1/a2 or b1/b2; *upper* and *lower*, respectively). PCR products are then either cloned and subjected to dideoxy terminator sequencing, as in conventional bisulfite genomic sequencing, or subsequently amplified with primers for a specific next-generation platform (not shown). Alternatively, genomic DNA isolated from probed chromatin can be subjected directly to next-generation bisulfite sequencing. Sites on the upper DNA strand that sequence as GC and CG indicate sites methylated in the original probed chromatin sample. In contrast, sites that sequence as Gt and tG indicate unmethylated sites. In this manner, the methylation status of all GC and CG sites along a single DNA strand can be assigned, providing single-molecule, simultaneous profiling of chromatin accessibility and DNA methylation. See text for treatment of overlapping GCG sites

MAPit has been used to demonstrate heterogeneity in chromatin remodeling at the induced *PHO5* promoter of budding yeast [3, 7, 9]. Similarly, single-molecule MTase footprinting of isolated mammalian nuclei has been used to show sequential co-occupancy by site-specific activators at an induced promoter [15], and to characterize chromatin structures associated with different transcriptional states [16, 17]. We have also used MAPit to characterize chromatin structural diversity in populations of KSHV episomal genomes [19] and to discover epigenetic heterogeneity within populations of glioblastoma in contrast with normal cells [26]. Finally, we envision that, due to the high heterogeneity characteristic of human tumor cell populations, the new adaptation of

MAPit in tissue samples will help decipher chromatin heterogeneity and epigenetic diversity associated with disease states. Previous chapters can be consulted for a full discussion of MAP analysis of populations and for in vivo chromatin probing in yeast cells [2, 3, 7] as well as a review on the adaptation of MAPit to high-throughput sequencing platforms for detection of rare epigenetic variants [25].

2 Materials

2.1 Equipment

(a) Micropipettes.
(b) 37 °C, 5 % CO_2 incubator.
(c) 37 °C incubator.
(d) Leica LMID inverted microscope.
(e) Dounce homogenizer.
(f) TC-10 automated cell counter (BioRad) or hemocytometer.
(g) Incubator-shakers.
(h) Water baths.
(i) Hot-stir plate.
(j) Heat block.
(k) Benchtop vortexer.
(l) Refrigerated centrifuge.
(m) Microcentrifuge.
(n) DNA engine Peltier thermocycler with heated lids (BioRad or similar).
(o) pH meter.
(p) Mini-gel boxes.
(q) UV transilluminator.
(r) StepOnePlus™ real-time PCR system (Life Technologies or similar).
(s) ABI 3130 DNA sequencer or other sequencing platform.
(t) Comparable equipment may be substituted.

2.2 Sterile Consumables

(a) 100 mm cell culture dishes.
(b) 1,250 μL micropipette tips.
(c) 200 μL micropipette tips.
(d) 10 μL micropipette tips.
(e) 50 mL conical centrifuge tubes.
(f) 15 mL conical centrifuge tubes.
(g) 1.7 mL microcentrifuge tubes.
(h) 0.65 mL microcentrifuge tubes.
(i) 0.2 mL thin-wall PCR tubes.

(j) 10 mL pipettes.
(k) 5 mL pipettes.
(l) 96-MicroWell plates, conical bottom.
(m) AirPore™ tape sheets (Qiagen 19571).
(n) Aluminum tape.
(o) Parafilm®.
(p) Wood applicator sticks.
(q) 0.45 μm syringe filters.
(r) MicroAmp® fast optical 96-well reaction plate (Life Technologies 4346906).
(s) MicroAmp® optical adhesive film (Life Technologies 4311971).
(t) Comparable consumables may be substituted.

Unless otherwise specified, solutions are prepared using water that has been purified to a resistance of 18.2 MΩ-cm (dH_2O).

Caution: A number of hazardous compounds are used in these protocols, including but not limited to chloroform, phenol, sodium metabisulfite, sodium hydroxide, and hydroquinone. Personnel should be trained in the use of the materials listed herein and should take appropriate precautions as set forth by the manufacturer and institutional policy.

2.3 Cell Culture/ Animal Tissue

1. Cell lines and growing conditions will vary depending on the experimental goals. This protocol can be carried out with adherent or suspension cells.
2. Appropriate cell culture media and additives, if required.
3. Phosphate-buffered saline (PBS) 1×.
4. Trypsin–EDTA 1×.
5. Trypan blue, 0.4 % (w/v).
6. 100–200 mg freshly collected, flash-frozen, or cryopreserved animal tissue.

2.4 MTase Mapping of Protein–DNA Interactions

2.4.1 Nuclei Isolation

1. Ice-cold 1× PBS.
2. Cell resuspension buffer: 20 mM HEPES, pH 7.5, 70 mM NaCl, 0.25 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 0.5 % (v/v) glycerol, 10 mM DTT (always add fresh immediately before use, store at −20 °C), 0.25 mM PMSF (always add fresh immediately before use, store at −20 °C).
3. Cell lysis buffer: Cell resuspension buffer + 0.19 % (v/v) Nonidet P-40 (*see* **Note 1**).
4. Methylation buffer: Cell resuspension buffer + 290 μM *S*-adenosyl-*L*-methionine (SAM) (always add fresh immediately before use, store at −20 °C) (*see* **Note 2**).

2.4.2 M.CviPI Probing of Chromatin Structure in Isolated Nuclei

1. GpC MTase M.CviPI (New England Biolabs M0227).
2. DNA MTase storage buffer (M.CviPI): 15 mM Tris–HCl, 0.2 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 200 μg/mL acetylated bovine serum albumin (BSA), 50 % (v/v) glycerol, pH 7.4 at 25 °C.
3. DNA MTase dilution buffer: Dilute M.CviPI storage buffer 1:7 with nuclei methylation buffer.
4. Methylation stop buffer: 100 mM NaCl, 10 mM EDTA, pH 8.0, 1 % (w/v) SDS.
5. Proteinase K 20 mg/mL.

2.4.3 DNA Isolation from M.CviPI-Probed Nuclei

1. 1× TE, pH 8.0: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0.
2. Phenol/chloroform/isoamyl alcohol (25:24:1 liquid mixture, pH 6.7 ± 0.2; Fisher BP1752I100): Equilibrate by adding the entire contents of the accompanying small bottle of equilibration buffer to increase pH from 6.7 to 7.9. Mix the two phases thoroughly and allow them to separate. The aqueous buffer will lie on top of the equilibrated solution. Store at 4 °C.
3. 5 M ammonium acetate.
4. 200-proof absolute EtOH.
5. 70 % (v/v) EtOH: Aliquot 13 mL 1× TE, pH 8.0 to a 50 mL centrifuge tube, qs to 50 mL with 200-proof absolute EtOH, and mix thoroughly. The final EtOH concentration is 70 % (v/v).
6. 0.1× TE, pH 8.0.

2.4.4 Quantitative Methylation-Sensitive Restriction Enzyme (qMSRE) Assay Verification of GC Methylation by M.CviPI

1. Methylation-sensitive restriction enzyme, such as R.HaeIII (New England Biolabs R0108) with accompanying 10× NEB4 buffer.
2. 50 % (v/v) glycerol.
3. SYBR® green PCR master mix (Life Technologies 4344463).
4. qMSRE primers: Herein we report an assay that scores a site in the promoter region of the human *GAPDH* gene. Forward primer 5′-TACTAGCGGTTTTACGGGCG-3′ and reverse primer 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′.

2.4.5 Bisulfite Deamination of Isolated DNA

1. 1× TE, pH 8.0.
2. 20 mL glass scintillation vial.
3. Unopened vial containing 5 g aliquot of sodium metabisulfite (Sigma 255556) (*see* **Note 3**).
4. 3 mg/mL molecular biology-grade glycogen (optional, to be used as a carrier if less than 200 ng of DNA is to be bisulfite treated).

5. Degassed distilled water (dg.dH_2O): Boil >200 mL dH_2O for 20 min in a glass beaker. After 20 min, carefully pour the boiling water into a 125 mL bottle until it is completely full (above the lip using surface tension) and screw the cap on tightly. Cool overnight on the benchtop.
6. 3 N NaOH: Make fresh shortly before use. Weigh out approximately 0.4 g NaOH pellets into a 15 mL centrifuge tube. Add the appropriate volume of dg.dH_2O (8.333 × number of grams of NaOH = mL of dg.dH_2O). Dissolve the pellets by gently rocking the tube rather than by vortexing in order to minimize aeration.
7. 100 mM hydroquinone (HQ): Make fresh shortly before use. Weigh out approximately 0.04 g HQ (Sigma) into a 15 mL centrifuge tube. Add the appropriate volume of dg.dH_2O (90.827 × number of grams of HQ = mL of dg.dH_2O). Dissolve the HQ by gently rocking the tube rather than by vortexing in order to minimize aeration.
8. 0.5 M EDTA, pH 8.0.
9. Sample denaturation buffer (SDB): Make fresh shortly before use. Mix buffer components in the following ratios (5 μL total volume per sample to be deaminated, plus a little excess): 3.25 μL dg.dH_2O, 1.5 μL 3 N NaOH, 0.25 μL 0.5 M EDTA, pH 8.0. Use the 3 N NaOH made in **step 6** (*see* **Note 4**).
10. Saturated sodium metabisulfite solution (SMBS): Make fresh shortly before use:
    (a) Put a small stir bar in a clean 20 mL scintillation vial and place the vial in the center of a stir plate next to the pH meter.
    (b) Take all reagents to be used (100 mM HQ, an unopened 5 g vial of sodium metabisulfite, dg.dH_2O, and 3 N NaOH) to the bench.
    (c) Take P100 and P1000 micropipettes, tips, and a 5 mL pipette and pipette-aid to the bench.
    (d) Calibrate and prepare the pH meter.
    (e) Prepare appropriate volumes of reagents prior to beginning preparation of SMBS as follows.
    (f) Draw 7 mL dg.dH_2O into the pipette and set aside.
    (g) Draw 1 mL 3 N NaOH using the P1000 and set aside.
    (h) Draw 100 μL 100 mM HQ using the P100 and add to the scintillation vial.
    (i) Open the 5 g vial of sodium metabisulfite and quickly dump the entire contents into the scintillation vial.

(j) Immediately and in rapid succession add the 7 mL dg.dH_2O, begin stirring, and add the 1 mL 3 N NaOH.

(k) Begin taking the pH of the solution. Adjust the pH to 5.0 using 3 N NaOH (it usually requires an additional 200–300 μL). Record the final pH.

(l) Since this is designed to be a saturated solution at room temperature, there will probably be some undissolved sodium metabisulfite remaining.

11. EZ bisulfite DNA clean-up kit™ (Zymo Research D5026): This kit contains all the reagents necessary for desalting and desulfonation of bisulfite-treated DNA.

2.4.6 PCR Amplification of Deaminated Template DNA

1. HotStar Taq Plus DNA polymerase (Qiagen) with accompanying 10× coral buffer and 25 mM $MgCl_2$ (*see* **Note 5**).
2. 2.5 mM deoxynucleoside triphosphates (dNTPs): The solution is prepared by mixing 360 μL dH_2O with 10 μL of each 100 mM stock of dNTPs (New England Biolabs) to yield a 2.5 mM solution with respect to individual nucleotides. Store at −20 °C.
3. 20 μM primers for PCR of deaminated DNA (b1/b2 or a1/a2 pair) (*see* **Note 6**).
4. Electrophoretic gels: 1 % (w/v) agarose (BioRad 162-0138), 1× Tris–acetate–EDTA (TAE), 0.5 μg/mL ethidium bromide (EtBr).
5. Electrophoretic buffer: 1× TAE, 0.5 μg/mL EtBr.
6. 10× loading buffer for agarose gel electrophoresis.
7. QIAEX II gel extraction kit (Qiagen 20021).

2.4.7 Cloning PCR Products Amplified from Deaminated Template DNA

1. Chemically or electro-competent strain of *E. coli* cells. We have used competent TOP10 cells (Invitrogen) with success.
2. Appropriate cloning vector: We typically use the pGEM®-T Easy system (Promega) for TA cloning (*see* **Note 7**) that confers ampicillin resistance and its accompanying 5× rapid ligation buffer and T4 DNA ligase. Blue/white screening is used to select white bacterial colonies that are enriched for recombinant plasmids (*see* **Note 8**).
3. Lysogeny broth (LB) + amp plates.
4. 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) 20 mg/mL.

2.4.8 Sequencing Cloned DNA Molecules

1. LB + amp liquid media.
2. 50 % (v/v) glycerol (*see* **Note 9**).

3 Methods

3.1 Cell Culture/ Animal Tissue

It is desirable to test more than one MTase concentration to select the ideal probing conditions and determine the extent of saturation of methylation by the exogenously provided M.CviPI chromatin probe. We usually use two MTase probe concentrations and an untreated sample. The untreated sample serves as a control for background false-positive GC methylation and shows the pattern of endogenous methylation (CG) before probing, which in some cases can help extract useful information from GCG sites. One may also omit GCG sites from the analysis. Here we describe how to process enough cells for two M.CviPI doses and the untreated control.

1. Grow cells under appropriate conditions for the experimental goals using standard cell culture techniques. Approximately 1.1×10^6 cells are needed for each dose of M.CviPI to be used.
2. Tissue type will vary depending on researcher needs. We have tested this protocol in rat brain, colon, and liver tissue (Fig. 2), as well as in fresh, flash-frozen, and cryopreserved tissue.

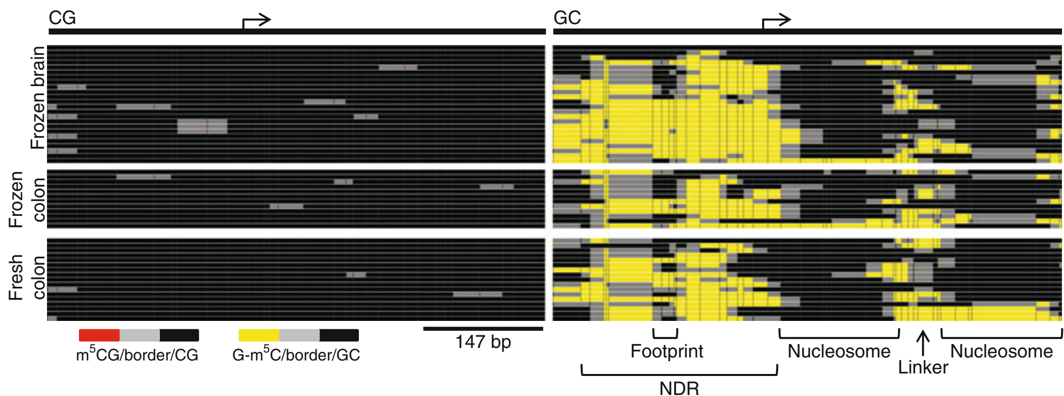

Fig. 2 MAPit analysis of the rat *MLH1* locus probed in tissue. Schematic of the locus is shown at *top*, with *bent arrow* representing the TSS. MAPit experimental procedure for tissue was performed as described in the text. In each panel, each row of pixels corresponds to one cloned and sequenced molecule analyzed from frozen brain (24 molecules shown), frozen colon (12 molecules), and fresh colon (17 molecules) rat tissues as indicated. The profiles of endogenous DNA methylation (CG; shown in *red* in the *left panels*) and chromatin accessibility (GC sites accessible to and hence methylated by M.CviPI; colored *yellow* in the *right panels*) are shown separately. However, the profiles for each molecule correspond directly to each other; that is, each molecule has the same *y*-axis coordinate on the *left* and *right*. As expected for an actively transcribed gene, the *MLH1* locus shows almost no or background levels of endogenous methylation (*left*). In addition, there is an open chromatin structure characterized by a nucleosome-depleted region (NDR) immediately upstream of the TSS, with a region of protection against GC methylation on some molecules, i.e., a footprint of an unidentified factor (*right*). Downstream of the TSS, two nucleosome-sized areas of protection are observed, separated by accessible linker DNA of variable size. Keys are shown at the *bottom*. A bar of nucleosome core particle size (147 bp) is shown to indicate scale

We have also used the protocol on normal and tumor cells obtained from fresh human mammary tissues. Cell yield varies substantially depending on the tissue type and the sampling technique. We recommend, when possible, to start with 100–200 mg of tissue.

3.2 MTase Mapping of Protein–DNA Interactions

3.2.1 Nuclei Isolation

Nuclei Isolation from Cultured Cells

1. For adherent cells, wash cells twice with 1× PBS. Detach cells from culture dishes by adding the appropriate volume of 1× trypsin. Incubate cells at 37 °C or room temperature until cells start to detach from the culture dish. Harvest cells with the appropriate volume of cell culture medium and centrifuge for 5 min at $1{,}000 \times g$ at 4 °C. For suspension cells, transfer the desired amount of growth media containing cells to a conical tube and collect by centrifugation for 5 min at $1{,}000 \times g$ at 4 °C. For all subsequent steps, keep samples on ice at all times.
2. Wash cells twice with ice-cold 1× PBS. After the final wash, resuspend cells in 1 mL ice-cold 1× PBS.
3. Mix 1:1 cell suspension to 0.4 % (w/v) trypan blue solution and count the number of live cells using a hemocytometer or an automated counting device. Aliquot 1.1×10^6 cells per enzyme dose of M.CviPI (total of 3.3×10^6 cells) into a prechilled 1.7 mL microcentrifuge tube. Pellet cells by centrifugation for 5 min at $1{,}000 \times g$ at 4 °C.
4. Gently aspirate the supernatant and resuspend the cells with 500 μL of ice-cold 1× cell resuspension buffer. Pellet cells by centrifugation for 5 min at $1{,}000 \times g$ at 4 °C.
5. Gently aspirate the supernatant and resuspend the cells in 115.5 μL of ice-cold 1× cell lysis buffer. Gently tap the tube to resuspend cells. Incubate on ice for 10 min or until complete cell lysis has been achieved (*see* **Note 10**).
6. When complete cell lysis has been achieved stop the reaction by adding 181.5 μL of ice-cold 1× nuclei methylation buffer. Tap the tube to mix.

Nuclei Isolation from Animal Tissue

A single-cell suspension must first be made for probing chromatin structure from tissue samples. Depending on researcher needs and the origin of the tissue, enzymatic and/or mechanical disaggregation may be needed to obtain a single-cell suspension from primary tissue (*see* **Note 11**). Nuclei are then prepared and used for the chromatin probing with purified M.CviPI. When processing tissue all steps must be performed on ice.

1. On a sterile petri dish placed on ice or frozen block, use two sterile scalpels or razor blades to finely mince tissue into 1–3 mm^3 pieces (*see* **Note 12**).
2. For mechanical disaggregation, collect the minced tissue in a prechilled dounce homogenizer with a loose-fitting pestle and add 0.5–1 mL of ice-cold 1× PBS.

3. Homogenize the tissue by gently moving the pestle up and down several times. Check the cell suspension every few strokes to ascertain if a single-cell suspension has been obtained (*see* **Note 13**).
4. Transfer the cell suspension to a prechilled 1.7 mL microcentrifuge tube. Pellet the cells by centrifugation for 5 min at 1,000 × *g* at 4 °C.
5. Wash the cells 2–4 times with 1× PBS. After the last wash, resuspend the cell pellet in 1 mL ice-cold 1× PBS.
6. Count the cells as in Subheading 3.2.1.1, **step 3**.
7. At this point, the probing protocol can be followed as is in Subheading 3.2.1.1, **steps 4–6** (*see* **Note 14**), and then proceed to Subheading 3.2.2.

3.2.2 M.CviPI Probing of Chromatin Structure in Isolated Nuclei

1. Aliquot 10^6 nuclei (90 μL at this point) per M.CviPI dose to be used.
2. Pre-warm the nuclei for 2 min at 37 °C in a water bath.
3. Add 10 μL of the appropriate M.CviPI dilution to the nuclei. Stagger the addition of M.CviPI by 30 s so that the time of incubation in the presence of the probing enzyme is held constant. Probe nuclei for 15 min at 37 °C (*see* **Note 15**).
4. Stop methylation reaction by adding 100 μL of pre-warmed 2× methylation stop buffer per reaction, follow the staggered addition time used in **step 3**, and vortex each sample immediately.
5. Add 20 mg/mL proteinase K to a final concentration of 100 μg/mL. Incubate overnight at 50 °C (*see* **Note 16**).

3.2.3 DNA Isolation from M.CviPI-Probed Nuclei

1. Add 100 μL or one equal volume of phenol:chlorophorm:isoamyl solution to the proteinase K-treated samples. Vortex vigorously for 30 s or until a homogeneous suspension is observed.
2. Separate the aqueous and organic phase by centrifugation for 10 min at 14,000 × *g* at 4 °C.
3. Transfer the aqueous phase to a new labeled 1.7 mL microcentrifuge tube.
4. Precipitate DNA by adding 5 M ammonium acetate to a final concentration of 2.5 M and two volumes of 200-proof absolute ethanol. Mix by gentle inversion. Incubate samples for 10–120 min at −20 °C.
5. Pellet the precipitated DNA for 10 min at 14,000 × *g* at 4 °C.
6. Add 0.4 mL of 70 % (v/v) EtOH and vortex briefly to wash pellet.
7. Re-pellet the DNA for 10 min at 14,000 × *g* at 4 °C. Carefully aspirate the supernatant and air-dry the pellet for 10–15 min.
8. Resuspend the genomic DNA with 60 μL of 0.1× TE, pH 8.0.

3.2.4 qMSRE Assay Verification of GC Methylation by M.CviPI

At this point, successful probing by M.CviPI can be verified by several methods. Here, we describe a qMSRE assay that we use on a regular basis. Methylation-sensitive R.HaeIII can digest unmethylated GGCC sites but not GG-m^{5}CC sites (*see* **Note 17**). If the restriction site to be interrogated is in an accessible region of chromatin, M.CviPI will methylate the GC dinucleotide resulting in increased protection to R.HaeIII digestion. The percent of GG-m^{5}CC at the site is proportional to the percent protection from R.HaeIII, as determined by quantitative real-time PCR (qPCR) by comparing the digested sample to the mock digestion.

1. Digest 500 ng of genomic DNA (non-deaminated) in a 19 μL reaction containing 1× NEB4 (supplied as 10× by the manufacturer).
2. Aliquot 9.5 μL into two 1.7 mL tubes. To one, add 0.5 μL (5 U) of R.HaeIII. To the other, add 0.5 μL of 50 % (v/v) glycerol to perform a mock digestion. Incubate both tubes for 2 h at 37 °C (*see* **Note 18**).
3. Use 3 μL of the R.HaeIII-digested DNA and mock reaction (75 ng) as templates for a 20 μL qPCR reaction consisting of 10 μL of SYBR® green PCR master mix (Life Technologies), and 500 nM each of forward and reverse primers. Set up three technical replicates for the R.HaeIII-digested DNA and mock reaction.
4. Thermocycle the samples in a real-time PCR machine and determine the percent protection to R.HaeIII digestion.

3.2.5 Bisulfite Deamination of Isolated DNA

1. Prepare the degassed water the prior evening.
2. Prepare the 3 N NaOH and 100 mM hydroquinone solutions.
3. Prepare the sample denaturation buffer (SDB) and aliquot 5 μL to a 0.65 mL microcentrifuge tube for each sample.
4. Add up to 2 μg of DNA solution (in 10 μL 0.1× TE) to the SDB and mix by pipetting. The samples should be left at room temperature while preparing subsequent reagents.
5. Prepare the sodium metabisulfite solution (SMBS) and prewarm it to 50 °C. It is not necessary to confirm the temperature prior to use.
6. While the SMBS is warming, denature the samples in a thermocycler for 5 min at 98 °C.
   (a) At about the 4-min point, bring the SMBS to the thermocycler, uncap it, and stir briefly.
   (b) At 5 min, open the thermocycler while maintaining block temperature at 98 °C and, working rapidly, open the first tube in the block, add 100 μL SMBS, cap and remove the tube, vortex it immediately, and place it in a rack or float on the bench top.

(c) Proceed likewise for all the samples, using a new tip for each sample.

(d) After all samples have been prepared, incubate them and the remaining SMBS at 50 °C for 6 h in the dark (*see* **Note 19**).

(e) Near the end of the incubation, read and record the final pH of the unused SMBS (*see* **Note 20**).

7. For DNA desalting and desulfonation, we have successfully used the EZ bisulfite DNA clean-up kit™ (Zymo Research). Near the end of the 6-h bisulfite conversion, prepare the necessary components for the kit.
8. Elute the DNA in 25 μL of elution buffer (supplied). Store the deaminated DNA at −20 °C.

3.2.6 PCR Amplification of Deaminated Template DNA

1. Use 2–4 μL deaminated DNA as template in the following 50 μL PCR reaction: dH_2O to volume, 1× Qiagen HotStar Coral PCR buffer, 2.25 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 μM b1 (or a1) primer, 0.8 μM b2 (or a2) primer, and 1.25 U Qiagen HotStar Plus Taq DNA polymerase (*see* **Note 21**). Thermocycle as follows: 1 cycle of 5 min at 94 °C, 30 cycles of 45 s at 94 °C, 45 s at 5 °C below the calculated T_m of the primers, 30 cycles of 1 min/kb at 72 °C, and 1 cycle of 5 min at 72 °C (*see* **Notes 22** and **23**).
2. Check for successful amplification of PCR product by electrophoresis of 1 μL of the unpurified reaction on a 1 % (w/v) agarose–TAE–EtBr mini-gel for 30 min at 100 V.
3. Visualize by UV transillumination.
4. Gel purify the PCR product using the QiaEX II gel purification system. Elute the PCR product in 15 μL 0.1× TE. Store at −20 °C.
5. In preparation for cloning of the individual templates, quantify the relative product concentration by electrophoresing 2 μL of each PCR product to be cloned on a 1 % (w/v) agarose–TAE–EtBr mini-gel for 30 min at 100 V followed by UV imaging. Obtain a linear digital image of the gel (i.e., not saturated due to overexposure) under UV transillumination. Using image analysis software, calculate the relative intensities of the product bands. Alternatively determine DNA concentration by spectrometry with a NanoDrop instrument. Samples can be stored at −20 °C (*see* **Note 24**).

3.2.7 Cloning PCR Products Amplified from Deaminated Template DNA

1. Ligate 1 μL (50 ng) of pGEM®-T Easy vector (Promega) and 40–60 ng of insert. Ligate the insert and vector at room temperature for 1–3 h in a reaction containing 1× rapid T4 ligation buffer and 200 U T4 DNA ligase, according to the manufacturer's recommendations (*see* **Note 25**).

2. Transform a chemically or electro-competent *E. coli* strain. Select transformants on LB plates with the corresponding selection for the chosen plasmid (*see* **Note 26**). Incubate for 12–16 h at 37 °C.
3. Transformation efficiency can be determined by blue/white screening, colony PCR, or plasmid isolation followed by screening with restriction enzymes (*see* **Note 27**).
4. Sequence positive transformants.

3.2.8 Sequencing Cloned DNA Molecules

At this point, the cloned inserts can be sequenced by several standard sequencing technologies. In this chapter, we used rolling circle amplification (*see* **Note 28**) and conventional automated BigDye sequencing (Applied Biosystems). For this protocol, the plasmid from the frozen bacterial cultures is sequenced at the core facility in the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida. The steps to prepare these bacterial cultures are outlined below.

1. In a 96-well plate, inoculate a single-transformant colony positive for cloned insert per well containing 100 μL of LB + amp. Cover the plate with AirPore™ tape sheets (Qiagen). Incubate without shaking at 37 °C for 12–16 h (*see* **Note 29**).
2. Add 16 μL 50 % (v/v) glycerol to obtain 8 % (v/v) final concentration of glycerol. Cover and seal the plate with aluminum tape (we purchase standard aluminum insulation tape at a home supply center). Close the plate with the corresponding lid and seal it with Parafilm™.
3. Incubate at −80 °C for at least 30 min (*see* **Note 30**).

4 Notes

1. Detergent concentration may need optimization for different cell types. If a higher detergent concentration is used, the final Nonidet P-40 concentration needs to be diluted to <0.07 % (v/v) in the methylation reactions.
2. Methylation buffer contains 290 μM SAM, which will be diluted to a final concentration of 160 μM in the methylation reactions.
3. We purchase 0.5 kg and repackage ~5 g amounts of sodium metabisulfite in glass 5 g vials in an H_2O- and oxygen-free safety hood. The vials are stored tightly capped in the dark in a sealed bottle with Drierite® desiccant to protect against oxidation. Artifacts due to poor deamination efficiency have been widely reported [27], but we have found that measures taken to protect the reagents from oxidation during storage and use [10]

eliminate such artifacts or greatly reduce them to non-detectable levels. As a simpler alternative, commercially available bisulfite deamination kits such as the EZ DNA methylation-direct™ kit (Zymo Research D5020) or EpiTect® bisulfite kit (Qiagen 59104) achieve comparable results.

4. When less than 200 ng of DNA is bisulfite treated 0.7 μL of 3 mg/mL glycogen should be added to the SDB as a carrier for the DNA.

5. Due to lower sequence complexity and the single-stranded nature of bisulfite-treated DNA, it is prone to adopt secondary structures. Hot-start PCR must be performed to achieve adequate yields of specific PCR product.

6. Primers are designated as a1/a2 or b1/b2 pairs according to the convention of Frommer et al. [11]. Primers must be designed to account for deamination of unmethylated residues. For best results, primers should discriminate between deaminated and undeaminated template, and should contain as many C (a1/b1) or G (a2/b2) residues as possible while avoiding potential methylation sites for both endogenous and exogenous MTases. If C residues within endogenous and probing MTase target sites cannot be avoided, the appropriate degeneracy (C/T or G/A) should be inserted at this position. We usually design primers to have a T_m of 55–60 °C at 0.8 μM primer in 0.1 M NaCl. It becomes difficult to obtain high yield for amplicons longer than 1 kb because the DNA is partially degraded by the bisulfite conversion procedure. Warnecke et al. [27] should be consulted for help in avoiding and resolving artifacts associated with bisulfite genomic sequencing.

7. As an alternative to the pGEM®-T TA cloning system, we have also used the TOPO® TA cloning kit (Invitrogen) with similar results. If desired, directional cloning can be performed. For this, incorporate different restriction sites at the 5′ end of each primer for PCR of deaminated DNA for cloning of the individual templates in the vector of choice.

8. Blue/white screening is used to enrich for insert-positive recombinant plasmids; however, a significant number of false negatives occur when cloning PCR amplicons generated from bisulfite-treated DNA. For this reason, we recommend verification of cloned inserts by a different method, such as colony PCR or plasmid isolation, before inoculating transformants for sequencing.

9. As described herein, individual molecules from PCR amplicons derived from bisulfite-treated DNA can be cloned and sequenced, as originally described for bisulfite genomic sequencing [11–13]. Depending on researcher needs, several sequencing strategies can be adopted and individual DNA reads can be obtained by

preparing libraries for high-throughput sequencing for any platform of choice (e.g., Roche 454 or Illumina) [25].

10. After 8 to 10 min of incubation, check sample for complete cell lysis. Mix 2 μL of cell lysis reaction with 2 μL of 0.4 % (w/v) trypan blue and observe the nuclei under the light microscope. When lysis is complete, nuclei should stain blue, and appear round and granular, with no attached debris. If intact cells are observed, continue lysis reaction and recheck in 2 min.
11. Enzymatic disaggregation can be conducted according to established protocols that generate single-cell suspensions. Examples include, but are not limited to, trypsin, collagenase, and hyaluronidase. Single cells obtained by enzymatic disaggregation should be washed with PBS prior to preparation of nuclei and chromatin probing. If disaggregation was performed on fresh tissue, single-cell suspensions may be cryopreserved for probing at a later date. Additional purifications may also be performed to isolate particular cell types within a tissue. For example, immunostaining using an antibody to a particular cell surface marker followed by fluorescence-activated cell sorting may be used to purify a cellular population of interest.
12. Frozen tissue must be thawed on ice before processing. Samples must be kept on ice at all times and all steps should be done quickly. In our experience, smaller tissue pieces obtained from the initial mincing are easier to disaggregate into single cells.
13. The number of strokes needed to disaggregate the tissue into single cells will vary according to the type of tissue. We recommend checking under the light microscope for a unicellular suspension after every few pestle strokes. Tissue disaggregation is rarely 100 % efficient, necessitating a compromise to minimize processing time and maximize the amount of obtained single cells. Some tissue clumps would remain in the cell suspension. After homogenizing the tissue, leave the homogenizer on ice for 2 min; tissue clumps will sink to the bottom of the homogenizer, allowing transfer of most of the cell suspension while avoiding tissue clumps.
14. In our experience, when the starting material is tissue, cell lysis will take longer than when starting with cultured cell lines. An approximate good starting time frame for lysis is 15–20 min.
15. Pilot experiments should be performed to optimize both enzyme concentration and probing time. We suggest performing an initial pilot experiment probing with 30 and 60 U of M.CviPI per one million cells for 15 min at 37 °C.
16. Complete degradation of DNA-bound proteins is required to obtain high-quality DNA for bisulfite treatment. Incomplete proteinase K treatment can interfere with bisulfite conversion [27]. We recommend digesting with proteinase K for at least 16 h.

17. Of the methods detailed herein, bisulfite genomic sequencing is the most sensitive for determining DNA methylation status. However, the more rapid and cost-effective screening approach presented in Subheading 3.2.4 is generally sufficient for assaying MTase activity. Any commercially available methylation-sensitive restriction enzyme can be used for the qMSRE assay, as long as its digestion is blocked (or enhanced) by the MTase used as a chromatin probe.
18. When digests are to be performed on multiple samples, make a cocktail of the common constituents to ensure homogeneity among reactions.
19. Incubation for 4–6 h is sufficient to achieve efficiencies for deamination of unmodified cytosine residues of approximately 99 %.
20. Significant deviation from the initial pH of 5 of the SMBS may indicate oxidation of the reactive sulfite to inert sulfate ion, which could lead to poor deamination efficiency.
21. Approximately 5–10 ng/μL of PCR product template is generally sufficient, but a higher concentration can be helpful when feasible.
22. When primers have different melting temperatures (T_m), use the lower of the two for determining the thermocycling parameters. When amplifying multiple samples, make a cocktail of all reagents, except template, and then aliquot to separate reactions to ensure homogeneity among reactions with respect to common constituents.
23. To minimize stochastic differences between PCR reactions, products of at least three different PCR reactions are pooled together at the PCR purification step before cloning the individual templates.
24. In our experience, better results are obtained when PCR products are used immediately for TA cloning. Freeze–thaw cycles affect the A overhangs required for TA ligation, resulting in lower ligation efficiency.
25. Several protocols exist for insert-vector ligation in which incubation is carried out overnight at 4 or 16 °C. We have found that the conditions described in this chapter yield good results in a relatively short time.
26. When using a vector that allows blue/white screening, 20 μL 50 mg/mL X-gal and 40 μL 100 mM IPTG (if required by the bacterial strain used; for example, TOP10 cells do not require IPTG induction) should be spread on plates before plating the transformations.
27. Usually one transformation reaction per insert yields a sufficient number of cloned PCR products for the analysis. The number of single molecules to be analyzed depends on the experimental goals.

28. Rolling circle amplification [28] is performed with TempliPhi™ reagent (GE Healthcare), which contains Phi29 DNA polymerase that exponentially replicates the circular plasmid from cells frozen in the glycerol stocks. Subsequently, the amplified single-stranded DNA is sequenced by conventional BigDye methodology (Applied Biosystems).
29. Grow the cultures until the media appears cloudy, which is usually accomplished with overnight incubation.
30. Frozen cultures can be stored at −80 °C for several months before sequencing. They can also be stored at −80 °C after sequencing and reused for further sequencing at a later date.

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Chapter 10

Determination of DNA Methylation Levels Using Illumina HumanMethylation450 BeadChips

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Abstract

DNA methylation is a modifiable epigenetic phenomenon that has a strong influence over transcriptional regulation and as such has been consistently implicated in development and disease. Several platforms are targeted toward the identification of DNA methylation changes that might be pertinent to the disease process and include regional analysis (e.g., pyrosequencing) as well as genome-wide analysis (e.g., next-generation sequencing and microarray). The Illumina HumanMethylation450 BeadChip is one of the most comprehensive microarray platforms available, and due to the high costs associated with next-generation sequencing, it is becoming a widely used tool for the analysis of genome-wide DNA methylation levels. Providing quantitative DNA methylation levels at 482,421 CpG sites within CpG islands, shores, and shelves, as well as intergenic regions, the HumanMethylation450 BeadChip can allow accurate assessment of differential methylation across large studies. This chapter outlines the laboratory methodologies associated with performing the Illumina Infinium Methylation Assay, including bisulfite conversion, whole-genome amplification, BeadChip hybridization, XStain procedures, and imaging systems. Furthermore, this chapter provides an outline of data analysis tools, including the GenomeStudio pipeline, quality control measures, and additional statistical considerations. This comprehensive overview can aid not only in performing the Illumina Infinium Methylation Assay but also in the interpretation of data derived from this platform.

Key words DNA methylation, Bisulfite conversion, Infinium assay, CpG site, GenomeStudio

1 Introduction

DNA methylation occurs at the C(5) position of cytosine molecules in cytosine-guanine dinucleotides (CpG) and is known to be intimately involved in genetic regulation. Often, such CpG sites are found within clusters or "islands," which are defined (for the purposes of the Illumina HumanMethylation450 BeadChips) as 500 bp or greater regions of DNA that have a G + C content equal to or greater than 50 % and an observed/expected CpG ratio of 0.6 [1, 2], although stricter definitions are also often employed [3]. CpG islands, particularly within the promoter regions of genes, efficiently repress gene transcription when methylated, but these islands only represent approximately 1 % of the genome [2, 4].

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In addition to transcriptional repression caused by methylation at CpG islands, intragenic methylation has been shown to both repress [5] and increase [6, 7] gene expression and methylation at the 3′ end of genes (although not well understood) may be involved in the suppression of antisense transcripts, regulation of polyadenylation, or transcription termination [7]. In order to detect pertinent DNA methylation changes that might influence biological processes and contribute to disease, several platforms and methodologies have been developed. This chapter focuses on a microarray platform developed by Illumina for genome-wide detection of DNA methylation levels at ~480,000 CpG sites.

The Illumina Infinium HumanMethylation450 BeadChips were designed to detect DNA methylation via quantitative "genotyping" of C/T polymorphisms; this is based on the principle that following bisulfite conversion, unmethylated cytosines are converted to uracils (detected as thymines) whilst methylated cytosines are protected and are detected as cytosines. The BeadChip includes assay probes for 482,421 CpG sites, 3,091 non-CpG sites, and 65 random SNPs, and content includes coverage of 99 % (n = 21,231) of RefSeq genes, with multiple probes per gene (average of 17.2 probes per gene region) [2]. In total, 200,339 of the CpG sites are located in the promoter regions of genes, 150,212 are located in the body of genes, 15,383 are located in the 3′UTR of genes, and 119,830 are intergenic [8]. The content focuses on providing high coverage of ***CpG islands*** (26,658 islands, 96 % coverage, average of 5.6 probes per island) as well as the 2 kb flanking regions known as ***CpG island shores*** (26,249 upstream shores, 95 % coverage, average of 2.9 probes per shore; 25,761 downstream shores, 93 % coverage, average of 2.8 probes per shore) and the additional 2 kb flanking regions (2–4 kb from CpG island) referred to as ***CpG island shelves*** (23,965 upstream shelves, 86 % coverage, average of 2.1 probes per shelf; 24,018 downstream shelves, 87 % coverage, average of 2.0 probes per shelf) [2]. In addition, based on Consortium selection, several other genomic regions are covered on the array, including differentially methylated regions, informatically predicted enhancers, and DNase hypersensitive sites, amongst others [2].

The Illumina HumanMethylation450 BeadChips utilize both Infinium I (135,476 probes, 28 %) and Infinium II (346,945 probes, 72 %) chemistry for the assay probes. The Infinium I assay utilizes two bead types for a single CpG locus, corresponding to a "methylated" state (C) and an "unmethylated" state (T), and incorporation of a fluorescently labeled nucleotide at each bead type during the single-base extension indicates the percentage of that base (or methylation) within the sample. The Infinium II assay utilizes a single-bead type at each CpG locus which will incorporate either the C or T base; these are then labeled with different

fluorescent bases during the single-base extension stage and the detection of each fluorophore indicates the percentage of that base (or methylation) present in the sample [2]. The percentage of methylation, which is based on the detection of fluorescence intensity, is calculated as a “beta” value, which is the ratio of methylated to combined intensity values and is represented by the equation ***beta = methylated intensity/unmethylated intensity + methylated intensity + 100*** (a small correction term to regularize probes of low signal intensity) [2, 9]. Beta values are constrained to lie between 0 (representing an unmethylated locus) and 1 (representing a methylated locus). Each beta value is associated with a detection *p*-value and upon assessment, Sandoval and colleagues found that a *p*-value above 0.01 indicated unreliable beta values in 0.16 % of analyzed sites and suggest that this should be used as a threshold value [8]. Data derived from the HumanMethylation450 BeadChips is highly reproducible ($R^2 = 0.992$) and shows good concordance with both whole-genome bisulfite sequencing (beta correlations of 0.95–0.96) and pyrosequencing (Spearman $r = 0.86$–0.88) [2, 10].

Although a highly powerful and efficient means for the detection of genome-wide DNA methylation, the Illumina Human Methylation450 BeadChips do have some limitations. Several studies, including one by Illumina, have identified differences in the performance of the two assays in terms of the beta value distributions [2, 11]. Specifically, beta values generated from Infinium II probes (accounting for 72 % of the content) were less accurate and reproducible than those obtained from Infinium I probes; application of a correction technique may however improve the quality of Infinium II data [9, 11–13]. A recent study identified potential ambiguity in probe binding for a significant proportion (~29 %) of the probes on the Illumina HumanMethylation450 BeadChips [14]. The authors found that ~140,000 of the BeadChip probes ambiguously mapped to multiple locations in the human genome using Bowtie 2 [15] and allowing for up to two mismatches [14]. Although these probes will not necessarily bind ambiguously under experimental conditions, caution should be taken in the interpretation of data arising from such probes and measures taken to ensure findings are legitimate (for example replication with another technology such as pyrosequencing).

This chapter gives an overview of the Illumina Human Methylation450 BeadChip technology, including bisulfite conversion, the Illumina Infinium methylation assay, and data visualization and analysis. The Illumina Infinium assay has been carefully optimized by Illumina and the protocol given closely matches that provided by the manufacturer, with only minor modifications made and additional notes included.

2 Materials

All reagents used throughout these protocols should be of molecular biology grade where applicable. Illumina supplies most reagents in prepackaged kits. In cases where a company is preceded by "e.g.," the reagent may be purchased from other vendors; companies that are not preceded by "e.g." should be used for purchasing of reagents to ensure high-quality results. Sterile techniques should always be used in preparation of reagents to prevent contamination during the protocol. Ensure that water used is DNase/RNase free. Ensure that gloves are worn for the duration of the experiment (use powder-free gloves whilst handling the BeadChips and glass back plates).

2.1 Sodium Bisulfite Conversion

1. 500 ng DNA for conversion, diluted in 45 μl water, 10 mM Tris-Cl or 1× TE buffer (accurately quantitated via PicoGreen or similar; should be of good quality as assessed by agarose gel electrophoresis).
2. *Optional*: Methylated and unmethylated DNA controls (e.g., Zymo research, Qiagen, New England Biolabs, Life Technologies, Active Motif, Diagenode).
3. EZ DNA Methylation™ Kit, or EZ-96 DNA Methylation™ Kit Deep Well Format (Zymo Research), contains:
   - CT conversion reagent.
   - M-dilution buffer.
   - M-binding buffer.
   - M-wash buffer.
   - M-desulfonation buffer.
   - M-elution buffer.
   - Zymo-Spin™ IC columns (EZ DNA Methylation™ Kit), or Zymo-Spin™ I-96 binding plates (EZ-96 DNA Methylation™ Kit).
   - Conversion plates with pierceable cover film (EZ-96 DNA Methylation™ Kit only).
   - Collection tubes (EZ DNA Methylation™ Kit), or collection plates (EZ-96 DNA Methylation™ Kit).
   - Elution plates (EZ-96 DNA Methylation™ Kit only).
4. DNase/RNase-free water (e.g., Fisher Scientific, Life Technologies).
5. 100 % Ethanol (e.g., Fisher Scientific, Sigma-Aldrich).
6. PCR strip tubes or plate (EZ DNA Methylation™ Kit only) (e.g., Fisher Scientific, USA Scientific).

7. Thermal Cycler (e.g., Life Technologies, Bio-Rad).
8. Centrifuge for plates or microfuge for tubes (e.g., Thermo Scientific, Eppendorf).
9. Pipettes and pipette tips (e.g., Eppendorf, Fisher Scientific, USA Scientific).

2.2 Illumina Infinium Methylation Assay

1. HumanMethylation450 DNA Analysis BeadChip Kit (available in 24, 48, or 96 sample kits) (Illumina), contains:
   - HumanMethylation450 BeadChips (1 chip is sufficient for 12 samples).
   - MA1 (multi-sample amplification 1 mix).
   - RPM (random primer mix).
   - MSM (multi-sample amplification master mix).
   - FMS (fragmentation solution).
   - PM1 (precipitation solution).
   - RA1 (resuspension, hybridization, and wash solution) (NOTE: contains aliphatic amide, which is a probable reproductive toxin; hazard classification: category 2).
   - PB1 (reagent used to prepare BeadChips for hybridization).
   - PB2 (humidifying buffer used during hybridization).
   - XC1 (XStain BeadChip solution 1).
   - XC2 (XStain BeadChip solution 2).
   - TEM (two-color extension master mix).
   - XC3 (XStain BeadChip solution 3).
   - STM (superior two-color master mix).
   - ATM (anti-stain two-color master mix).
   - XC4 (XStain BeadChip solution 4).
2. 0.8 ml storage plate, conical well bottom, and 96-well cap mats (pierceable, non-autoclavable) (Abgene).
3. Reservoirs/pipetting trough (optional but useful for high numbers of samples where multichannel pipettes should be used) (e.g., Fisher Scientific, VWR).
4. 0.1 N sodium hydroxide: Should be made (or diluted) fresh on the day of the experiment (e.g., Sigma-Aldrich, Fisher Scientific).
   - *Alternative*: If only processing a small number of samples, you may wish to make a larger stock of 0.1 N sodium hydroxide and store at −20 °C to reduce variation across experiments.

5. Vortexer suitable for plates: Must be accurately calibrated or an optical tachometer/stroboscope used to identify true vortex speed (if an Illumina service agreement is in place they will likely do this for you) (e.g., VWR).
6. Centrifuge fitted with rotors for plates and 15 ml conical tubes (e.g., Thermo Scientific, Eppendorf).
7. Hybridization oven, capable of rocking (e.g., Illumina, Fisher Scientific, VWR).
8. 100 % isopropanol (e.g., Sigma-Aldrich, Fisher Scientific).
9. Heat sealing foil sheets, Thermo-Seal (Abgene).
10. Heat sealer (Thermo Scientific).
11. Heat block with insert for 0.8 ml plates (Illumina).
12. Hybridization chambers, one per four BeadChips (Illumina).
13. Hybridization chamber gaskets, one per four BeadChips (Illumina).
14. Hybridization chamber inserts, one per BeadChip (Illumina).
15. 100 % Ethanol (e.g., Fisher Scientific, Sigma-Aldrich).
16. Kimwipe tissue (e.g., Fisher Scientific, VWR).
17. Compressed air can (e.g., VWR).
18. Forceps (e.g., Fisher Scientific, VWR).
19. Multi-sample BeadChip alignment fixture.
20. Te-Flow flow through chambers: one black frame, one spacer, one glass back plate, two clamps required per BeadChip (Illumina).
21. Scissors (e.g., Illumina).
22. Tube rack.
23. Wash dishes (×2) and rack (e.g., wash rack and glass tray from Illumina, glass staining dish with removable rack from Fisher Scientific).
24. Alconox powder detergent (e.g., VWR, Fisher Scientific).
25. 95 % formamide/1 mM EDTA: Should be made fresh on the day of experiment or stored at −20 °C (15 ml is sufficient for eight BeadChips).
    - Add 14.25 ml formamide (e.g., Sigma-Aldrich, Fisher Scientific), 150 μl of 100 mM EDTA (e.g., Fisher Scientific), and 600 μl molecular grade water (e.g., Fisher Scientific, Life Technologies).
    - *Alternative*: You can make a larger batch of this reagent and store at −20 °C in aliquots (we recommend this; light sensitive, should be stored in the dark).

26. Freedom Evo® robotic liquid handling system (*optional*) (Tecan).
    - Infinium Automation Control software required (Illumina).
    - *Alternative*: Infinium Water Circulator and Teflow Rack Kit (Illumina).
27. 1× Tris-EDTA (TE) buffer (if using the Tecan Freedom Evo® robot).
    - Add 1,800 ml water to 200 ml of 10× TE (e.g., Fisher Scientific); you may need several batches of this to ensure that the buffer bottle is sufficiently filled.
28. Temperature probe (Illumina).
29. Reservoirs and frames to be used on Tecan robot (quarter, 40 ml; half, 75 ml; full, 150 ml) (Beckman Coulter).
30. Wash dishes (×2) and staining rack (to accommodate upright BeadChips) (Illumina).
31. Dismantling tool (Illumina, provided with hardware starter kit) or narrow laboratory spatula (e.g., Fisher Scientific, VWR).
32. Desiccator: One desiccator is required per 12 BeadChips (e.g., Fisher Scientific, VWR).
33. Isopropyl (70 %) alcohol wipes **or** use a lint-free tissue moistened with 70 % ethanol or isopropanol (e.g., VWR, Fisher Scientific).
34. Desiccator cabinet (*optional*: for storage of BeadChips, up to 3 days) (e.g., Fisher Scientific, VWR).
35. iScan instrument (or HiScan/HiScanSQ) with air table and Decode File Client software (Illumina).
    - *Optional*: AutoLoader2.x (for automated processing of chips).
    - *Note*: A BeadArray Reader could be used in place of an iScan or HiScan; however these instruments are no longer sold by Illumina, and as of 30th December 2013 (30th December 2016 for Gold-level service contracts), servicing of instruments will no longer be supported by Illumina.
    - *Note*: Within a project, you should not switch between different platforms (e.g., BeadArray Reader and iScan) as they may have significantly different detection systems. We also recommend not to switch between different instruments (of the same platform) as they may be calibrated slightly differently.
36. *Optional*: Detailed Illumina documentation.
    - Illumina Infinium HD Methylation Assay, Manual Protocol.
    - Illumina Infinium HD Methylation Assay, Automated Protocol.

- Infinium HD Assay Methylation protocol guide (includes part numbers for consumable items).
- Decode File Client User Guide.
- Infinium Assay Lab Setup and Procedures Guide.
- iScan System User Guide or HiScan®SQ System User Guide.
- GenomeStudio Methylation Module User Guide.

2.3 Data Analysis

1. GenomeStudio Software (Illumina).
2. Secondary software package (*optional*), for example:
   - R-based packages (e.g., lumi [16], methylumi [17], minfi [18]).
   - Alignment software (e.g., Bowtie 2 [15]).
   - Association analysis software (e.g., SOLAR [19], CpGassoc [20]).

3 Methods

3.1 Sodium Bisulfite Conversion (Days 1–2)

The Zymo EZ and EZ-96 DNA Methylation™ Kits are based on the principle that sodium bisulfite, under acidic conditions, converts unmethylated cytosine residues into uracil, whilst methylated cytosines are protected from this reaction. The Zymo kits have a high conversion efficiency (>99 % of unmethylated cytosines are converted to uracil) as well as high protection of methylated cytosines (>99 %). Furthermore, DNA recovery is greater than 80 %. Bisulfite conversion will only take place on single-stranded DNA and as such, the samples must first be denatured using the dilution buffer provided in the kit. Additionally, during the conversion process, heat will be used intermittently to maintain single-strand conformation. Following denaturation, a conversion reagent is added and a reaction takes place overnight whereby unmethylated cytosines are sulfonated and spontaneously deaminate under acidic conditions, resulting in a sulfonated uracil. After this overnight reaction, samples are bound to spin columns (or spin plates) and desulfonated under alkaline conditions, so that cytosines in the original sequence are left as uracil bases. As these uracil bases are no longer complementary to their previously bound DNA sequence, the template remains single stranded and either the top or bottom strand of the DNA can be analyzed. Samples are washed and eluted in an elution buffer and the entire sample is used for methylation profiling using the Illumina Infinium protocol. Bisulfite-converted DNA can be stored at −20 °C for up to 1 month. The following is based on the protocol provided by

Zymo Research, with modifications for the Illumina Infinium protocol.

1. Prepare CT Conversion Reagent (light sensitive; *see* **Note 1**):
   - EZ DNA Methylation™ Kit: Add 750 μl water and 210 μl M-Dilution buffer to a tube of CT Conversion Reagent; mix at room temperature with frequent vortexing/shaking for 10 min.
   - EZ-96 DNA Methylation™ Kit: Add 7.5 ml water and 2.1 ml M-Dilution buffer to a bottle of CT Conversion Reagent; mix at room temperature with frequent vortexing/shaking for 10 min.
2. Prepare M-Wash buffer.
   - EZ DNA Methylation™ Kit: Add 24 ml of 100 % ethanol to 6 ml M-Wash buffer concentrate (or add 96 ml ethanol to 24 ml M-Wash buffer concentrate).
   - EZ-96 DNA Methylation™ Kit: Add 144 ml of 100 % ethanol to 36 ml M-Wash buffer concentrate.
3. Add 5 μl M-Dilution buffer to each high-quality DNA sample (500 ng in 45 μl water/Tris/TE; *see* **Note 2**) and mix by gently pipetting up and down or flicking the tube. If using the full Illumina Infinium HD Methylation Assay automated protocol (as opposed to the protocol listed below), you will use 1,000 ng DNA.
4. Incubate at 37 °C for 15 min.
5. Add 100 μl of the CT Conversion Reagent (prepared in **step 1**) to each sample and mix.
6. Incubate the samples in a thermal cycler overnight for 16 cycles of 95 °C (30 s) and 50 °C (60 min), and hold at 4 °C.
7. Add 400 μl of M-Binding buffer to a Zymo-Spin™ IC column and place in collection tube (EZ DNA Methylation™ Kit), or to each well of a Zymo-Spin™ I-96 binding plate and place in a collection plate (EZ-96 DNA Methylation™ Kit).
8. Load the samples (from **step 6**) onto the column/plate and mix with the M-Binding buffer by inversion (tubes) or pipetting up and down (plates). Ensure that you do not puncture the filter with a pipette tip whilst loading the samples.
9. Centrifuge spin columns at full speed (≥10,000 × *g*) for 30 s (EZ DNA Methylation™ Kit), or centrifuge plates at ≥3,000 × *g* (maximum of 5,000 × *g*) for 5 min (EZ-96 DNA Methylation™ Kit); discard the flow through.
10. Add 100 μl M-Wash buffer to each column and centrifuge at full speed (≥10,000 × *g*) for 30 s (EZ DNA Methylation™ Kit), or add 500 μl M-Wash buffer to each well of a plate and centrifuge at ≥3,000 × *g* (maximum of 5,000 × *g*) for 5 min (EZ-96 DNA Methylation™ Kit); discard the flow through.

11. Add 200 μl M-Desulfonation buffer to each column/plate well and incubate at room temperature for 15 min.
12. Centrifuge spin columns at full speed (≥10,000×*g*) for 30 s (EZ DNA Methylation™ Kit), or centrifuge plates at ≥3,000×*g* (maximum of 5,000×*g*) for 5 min (EZ-96 DNA Methylation™ Kit).
13. Add 200 μl M-Wash buffer to each column (EZ DNA Methylation™ Kit) or 500 μl M-Wash buffer to each well (EZ-96 DNA Methylation™ Kit) and centrifuge according to **step 12**. Discard flow through.
14. Repeat **step 13**, but centrifuge the plates for a total of 10 min (EZ-96 DNA Methylation™ Kit). Alternatively, we prefer to spin the plate for 1 min initially, discard the supernatant, and continue to spin for an additional 9 min.
15. To elute the DNA, place the column into a collection tube, add 10 μl M-Elution buffer directly to the column, and centrifuge at full speed (≥10,000×*g*) for 30 s (EZ DNA Methylation™ Kit), or place the plate onto a collection plate, add 15 μl M-Elution buffer directly to each well, and centrifuge at ≥3,000×*g* (maximum of 5,000×*g*) for 3 min (EZ-96 DNA Methylation™ Kit). If using the Illumina Infinium HD Methylation Assay automated protocol (as opposed to the protocol listed below), you will need to elute the samples in 22 μl. Although bisulfite DNA can be stored at −20 °C for up to 1 month, we recommend proceeding directly to the Illumina Infinium Protocol (*see* **Note 3**). The protocol listed will only utilize 4 μl of the bisulfite-converted DNA; the remainder may be stored for any necessary repeats or for verification of results via a different methodology (*see* **Note 3** for storage information).

3.2 Illumina Infinium HD Methylation Assay (Days 2–4)

The Illumina Infinium HD Methylation Assay protocol can be performed in either manual or automated mode; the automated mode requires a Tecan instrument. Most reagents required for the Illumina Infinium HD Methylation Assay are provided in the HumanMethylation450 DNA Analysis BeadChip Kit. Experienced User Cards for each of these protocols, as well as a more detailed protocol guide (covering both the manual and automated mode), can be found on the Illumina Web site (www.illumina.com; requires free registration/login):

Illumina Infinium HD Methylation Assay, Manual Protocol.

Illumina Infinium HD Methylation Assay, Automated Protocol.

Infinium HD Assay Methylation protocol guide (includes part numbers for consumable items).

The protocol outlined here is a combination of the manual and automated modes, which has been optimized for protocol efficiency and accuracy. Following bisulfite conversion, DNA samples

are denatured (using sodium hydroxide) and neutralized, prior to whole-genome amplification, which is performed isothermally to increase the DNA quantity by several thousandfold whilst limiting amplification bias. Amplified DNA is fragmented, precipitated, and resuspended in a hybridization buffer. Samples (12 per BeadChip) are hybridized to BeadChips, where they anneal to locus-specific 50mer probes. BeadChips are washed to remove any poorly bound or nonspecifically bound DNA and samples undergo staining and single-base extension, which incorporates fluorescent labels for detection of methylation status.

There are several points in the protocol at which it is safe to stop the protocol and store the samples, which I will state in the given protocol; however in our experience we find that conducting the protocol from start to finish, without stopping, provides good-quality data and can easily be accomplished within the 3-day time frame (4 days if including bisulfite conversion). It is important to keep track of the reagents (in case of assay failure) and BeadChips (to analyze data); Illumina provides lab tracking sheets on their Web site (Infinium HD Methylation Lab Tracking Form). As an alternative, we have designed our own Excel spreadsheet that allows entry (either by a barcode scanner or manual) of reagent lot numbers, BeadChip barcodes, and incubation times. This reduces space, can easily be adjusted for the number of BeadChips run, and allows direct and easy transfer of sample numbers from a database to reduce potential errors in rewriting information. Illumina provides barcodes for tracking plates at each stage of the protocol; however in our experience we have found it sufficient to label each plate (particularly if running more than one plate) with a permanent marker (ensure that this is not removed by alcohol during the experiment); the protocol below assumes adequate labeling so that plates can be differentiated if necessary and identified if stored at any point in time.

3.2.1 Denaturation and Amplification of Samples (Day 2)

This process takes approximately 30 min to 1 h, followed by an overnight incubation. *See* **Note 4** for general considerations throughout the protocol.

1. Preheat the hybridization oven to 37 °C; thaw RPM and MSM tubes; MA1 is stored at room temperature (one tube of each for 24–48 samples, two tubes for 96 samples; record barcodes on your tracking sheet). If the bisulfite DNA has been frozen, thaw it. Prepare fresh 0.1 N NaOH (we make 2 ml per 192 samples, to reduce errors in pipetting small amounts).
2. Dispense 20 μl MA1 in each well of a 0.8 ml storage plate/s and add 4 μl of bisulfite-converted DNA into each well (one sample per well; ensure that you keep track of the location of samples; ensure that DNA is placed in the bottom of well so that it cannot contaminate pipette tips in subsequent steps). Alternatively, you can plate the DNA first and then add MA1.

3. Denaturation: Add 4 μl 0.1 N NaOH to each sample (be sure not to touch the sample with your pipette tip, but place tips against the top edge of the wells to ensure steady dispensing of reagents: follow these recommendations for all subsequent dispensing steps), place a cap mat/s on the plate/s, and seal well (pushing on the individual caps until they click into place is easy and will seal the plate tightly); vortex at 1,600 rpm for 1 min; centrifuge at 280 × *g* for 1 min; and incubate at room temperature for 10 min (*see* **Note 5**).
4. Amplification: Remove cap map, add 68 μl RPM to each sample, and then add 75 μl MSM to each sample; reseal plate/s with cap mat (ensure that orientation is correct) and invert the plate at least ten times to mix (do not shake or vortex plate); centrifuge at 280 × *g* for 1 min; and incubate for 20–24 h at 37 °C in the hybridization oven to amplify the DNA. We recommend incubation for 22 h (*see* **Note 6**).

3.2.2 Fragmentation, Precipitation, and Hybridization (Day 3)

This process takes approximately 6 h (including several short incubation times), followed by an overnight incubation. *See* **Note 4** for general considerations throughout the protocol.

1. Thaw FMS (one tube for 24–48 samples, two tubes for 96 samples; record barcode); set heat block to 37 °C (*optional*: the hybridization oven can be used at this point instead).
2. Remove the samples from the hybridization oven (keeping oven set to 37 °C if using this for incubations), centrifuge plate/s at 50 × *g* for 1 min, and remove the cap mat (but keep for use later).
3. Fragmentation: Add 50 μl FMS reagent to each sample and reseal with the cap mat.
4. Vortex the plate/s at 1,600 rpm for 1 min, centrifuge at 50 × *g* for 1 min, and incubate on the 37 °C heat block **or** in the hybridization oven set to 37 °C for 1 h. If using the hybridization oven this can be extended to 1 h, 10 min to ensure complete fragmentation, but we have found that 1 h in the hybridization oven provides high-quality data. *This is a safe stopping point in the protocol; the plate can be stored at −20 °C.*
5. Whilst the plate is incubating, thaw the PM1 for use in **step 6** (one tube each for 24–48 samples, two tubes for 96 samples; record barcodes) and RA1 for use in **step 11** (one bottle provided, which is used for this step and for the XStain procedure on day 4, record barcode); if the sample plate/s has/have been frozen, thaw to room temperature and set heat block or hybridization oven to 37 °C; if proceeding directly from fragmentation step, remove plate/s from heat block or oven (but keep set to 37 °C); centrifuge plate/s at 280 × *g* for 1 min and remove cap mat (keep for next step).

6. Precipitation: Add 100 μl PM1 reagent to each sample, reseal plate/s with cap mat, vortex at 1,600 rpm for 1 min, incubate at 37 °C for 5 min (heat block or hybridization oven), centrifuge at 50×*g* for 1 min, remove and discard cap mat, add 300 μl 100 % isopropanol to each well, seal well with a *new* cap mat, invert at least ten times to mix, and incubate at 4 °C for 30 min.
7. Whilst the plate/s is/are incubating at 4 °C, set the centrifuge to 4 °C; after the incubation, push down on the cap mat to ensure that it is fully sealed (sometimes the mat lifts up a little whilst incubating) and centrifuge at 4 °C, 3,000×*g*, for 20 min (be sure that the centrifuge is properly balanced if you are processing only one plate or a partial plate); during centrifugation, prepare absorbent pads for next step; you will need to layer 5–6 pieces of paper towel together and fold in half for the first absorbent pad (not necessary if you use a container in the next step); you will need to layer 2–3 paper towels together and fold in half for the second and third absorbent pads.
8. Immediately following centrifugation remove the cap mat/s from the plate/s and discard; decant the supernatant by quickly inverting the plate over an absorbent pad (or you can do this over a container to collect the waste, which we prefer), firmly tap the plate/s over the second absorbent pad several times, and then firmly tap the plate/s over the third absorbent pad for about 30 s (*see* **Note 7**).
9. Allow the samples to dry on a rack, with the plate inverted for 1 h (carry the plate to the rack whilst still inverted); alternatively we allow the plate to dry inverted for 30 min and then tap the plate gently on a new absorbent pad and flip the plate upright to dry for an additional 30 min (at this point most supernatant is gone and only small droplets are usually present around the rim of the plate, which do not fall back into the pellet); we find that this method allows for better evaporation of any remaining supernatant. In our experience, if this drying time is extended a little (e.g., an additional 15 min), we have not seen any detriment to the quality of results, providing the pellets are still well resuspended in the next step. *This is a safe stopping point in the protocol; the plate can be stored at −20 °C (with cap mat in place).*
10. Preheat the hybridization oven to 48 °C; turn on heat sealer; if plate/s was/were stored at −20 °C following the last step, thaw to room temperature.
11. Resuspension: Ensure that the RA1 is thawed and all crystals are dissolved (takes ~3 h at room temperature). Add 46 μl RA1 reagent (this reagent is also utilized during the XStain procedure, so only pour out as much RA1 as is necessary for this step),

apply a heat foil seal (Thermo-Seal) to the plate, and seal it using the heat sealer (hold down heat sealer for ~5 s; afterwards lift at edges gently to ensure that the foil has properly sealed); place the plate/s in the hybridization oven at 48 °C for 1 h; vortex the plate/s at 1,800 rpm for 1 min and centrifuge at 280×*g* for 30 s. If plates were stored at −20 °C, or if they are a little overdried, the pellet may not be fully resuspended at this point (although we have never seen this occur); if pellets are not fully resuspended, incubate for an additional 30 min at 48 °C and repeat the vortex and centrifugation steps (repeat 30-min incubation again if necessary). *This is a safe stopping point in the protocol; the plate can be stored at −20 °C overnight or at −80 °C for more than 24 h.*

12. Whilst plate/s is/are incubating, preheat heating block to 95 °C; if plate/s was/were stored at −20 °C or −80 °C, allow them to thaw to room temperature; set the hybridization oven to 48 °C.
13. Remove plate/s from the hybridization oven (but keep it set to 48 °C), place them on the heat block at 95 °C for 20 min, then place on the bench at room temperature for 30 min, and centrifuge the plate/s at 280×*g* for 1 min; if you have more than one plate and only one heat block the second plate can be heated whilst samples from the first plate are at room temperature; the second plate can sit at room temperature whilst samples from the first plate are being loaded onto the BeadChips (**step 15**).
14. Whilst samples are incubating at room temperature, set up hybridization chambers (one chamber per four BeadChips; *see* Fig. 1): place the hybridization chamber gasket on the base of the hybridization chamber (the wider edge of the gasket should be matched to the side of the base that shows barcode ridges; if the gasket is not in the correct orientation, it will not sit flush on the base and the chamber will not be sealed correctly); dispense 400 μl PB2 reagent (record barcode) into each of the eight reservoirs in the base of the hybridization chamber; if samples are not ready to load at this point, place the lid on the base and seal the hybridization chamber by snapping the clamps shut (*see* **Note 8**; if samples are almost ready to load you can just place the lid gently on top without snapping it shut); BeadChips should be loaded into the chamber within an hour.
15. Whilst the plate/s is/are cooling on the bench, take out the packets containing the BeadChips from 4 °C (but do not remove the BeadChips from the packet). After the plate/s has/have been cooled and centrifuged, remove BeadChips from their packet (keep BeadChips in their packets until ready for sample loading). BeadChips include an IntelliHyb seal,

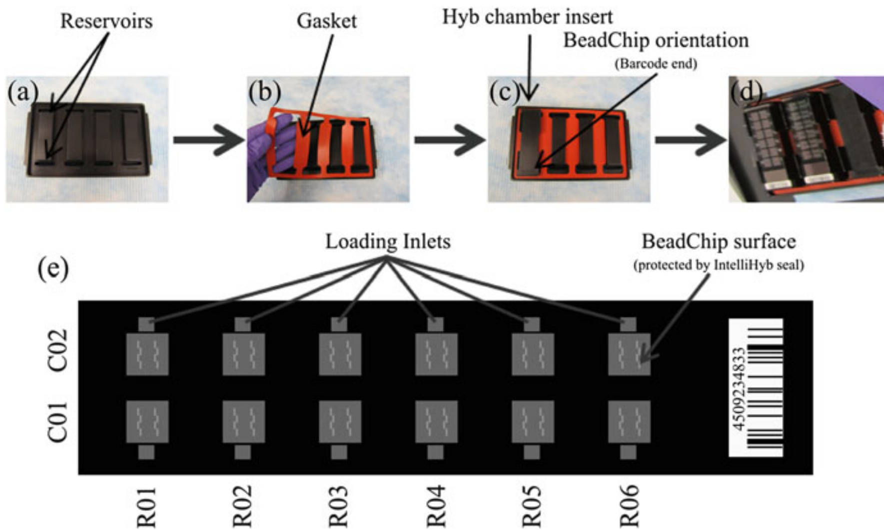

Fig. 1 Setup of hybridization chamber. (**a**) Hybridization chamber base; (**b**) gasket; (**c**) hybridization chamber insert; (**d**) loaded BeadChips; (**e**) BeadChip layout, including BeadChip positions (required for tracking and SampleSheet setup)

which isolates regions of the BeadChip so that 12 samples can be loaded onto a single BeadChip. The six positions on the left-hand side of the BeadChip correspond to column 1 (rows 1–6, from top to bottom (barcode)) and the six positions on the right-hand side of the BeadChip correspond to column 2. Each position is labeled as R01C01, for example, to represent row 1, column 1 (*see* Fig. 1e). Place the BeadChips into a hybridization chamber insert such that the BeadChip barcode matches the barcode symbol on the insert; place the insert into the hybridization chamber (after removing the lid) such that the barcode matches the barcode ridges on the base of the chamber (*alternatively*: BeadChips and inserts can be placed on the hybridization chamber base after samples have been loaded). Ensure that BeadChips are handled by the sides (i.e., edge) and ends and that you do not touch the regions where samples will be loaded; avoid touching the beadstripe area and sample inlets. ***Important:*** Either at this point or after **step 16**, it is imperative that you record the BeadChip barcodes and which position corresponds to each sample; this is the only way that you can track your samples; typically we have an Excel sheet setup so that we copy and paste sample numbers from a plate setup to a column; we type the corresponding chip position (i.e., R01C01, R01C02, R02C01…R06C01, R06C02 where R designates the row number 01–06 and C designates the column number 01 and 02) next to each of the samples and then scan the BeadChip barcode (using a barcode scanner, although this can be typed manually) that corresponds to each

sample (12 samples per BeadChip); we have designed these Excel sheets so that the same process is followed for all projects to reduce potential sources for error.

16. Load samples: Dispense 15 μl of each sample into the appropriate BeadChip section; this should correspond to what has been (or will be) recorded. Illumina suggests using a multichannel pipette to load up to six samples simultaneously; we instead use a single-channel pipette to ensure that loaded samples do not contain any bubbles and that the correct volume is loaded every time (if tips are not secure multichannel pipettes have a tendency to pipette unequal amounts); even using a single-channel pipette the process is quick; 96 samples are loaded in approximately 15 min (for an experienced user). Samples may also be loaded onto the BeadChip using the Tecan robot (automated mode); however given the time associated with setup and cleaning, we find it is quicker to load the samples manually. *Whichever method is used, make sure that you have recorded which sample is in which position and the corresponding barcode*. Figure 1e shows the BeadChip positions; these positions will need to be reported on the sample sheet (*see* Subheading 3.3, **step 1**).
17. Hybridization: Once samples have been loaded onto the BeadChips, and the hybridization chamber inserts are correctly oriented in the hybridization chamber, the hybridization chamber is sealed (*see* **Note 8**) and placed in the hybridization oven. Hybridization chambers should be placed in the oven so that the clamps face the left and right of the oven; two hybridization chambers will fit (one behind the other) on the rocker and chambers can also be stacked (up to four chambers per stack) so that up to eight chambers can fit in a single oven (Illumina oven). Samples are incubated at 48 °C and at a rocking speed of 5 (this is specific for the Illumina oven; this is a slow rocking speed; rocking the platform is *optional*, but recommended) for 16–24 h. We recommend a 20-h incubation (*see* **Note 6**).
18. In preparation for the following day, prepare the XC4 reagent by adding 330 ml 100 % ethanol to the XC4 bottle (for a final volume of 350 ml), shake vigorously for 15 s, and leave the bottle upright on the bench overnight; ensure that the pellet is fully resuspended before use in the morning (vortex if necessary). *Alternative*: If the buffer is not made up at this point, you can make it up the morning of the XStain procedure—after shaking the bottle vigorously, place it on its side on a rocker at room temperature (at a medium speed); the pellet should dissolve within a few hours.
19. Download decode data (DMAP) files for use in image analysis (Subheading 3.2.3, **step 16**); these files designate the bead type for each position on the BeadChip and are necessary to generate data. You cannot image BeadChips without these files.

You will use DMAP Decode File Client software to download decode data files for each of the BeadChips. To search for the correct DMAP files, you will need to know the BeadChip barcode in addition to one other piece of information (such as BeadChip box ID, order number, or sales number; or you could use two of these three pieces of information for download). You can then select the appropriate BeadChips and download the files onto the PC attached to the imaging instrument (iScan or HiScan). Decode data files can take some time to download, particularly if you are downloading a significant number, and can be done ahead of time if you know the barcodes of the BeadChips you will be processing. Often, we download all decode data files associated with a particular order, which may come close to 100 files. Files must be downloaded before the BeadChips expire (which is typically ~6 months from the purchase date); however once files have been downloaded, they are only available for 30 days following this. This should be taken into consideration as files may need to be downloaded a second time if there are any errors during the download process (which may only be identified when the BeadChips are imaged). For more information refer to the Decode File Client User Guide on the Illumina Web site (www.illumina.com). The Decode Data files should be saved in the designated Decode Data folder on the scanner PC to allow upload of the files.

3.2.3 Washes, XStain Procedure, and Scanning (Day 4)

This process takes approximately 6 h (including about 2–3 h on the robot using the automated mode); scanning will take additional time and can be performed manually or automated (in conjunction with an AutoLoader2.x). *See* **Note 4** for general considerations throughout the protocol.

1. Check the glass plates to ensure that they do not have any scratches or chips that would interfere with reagent coverage (*see* **Note 9**). Clean glass plates to remove dirt, dust, and fluff in preparation for flow through chamber assembly: dip each glass plate into a dish of ethanol (70 % ethanol, made fresh) and immediately wipe with a Kimwipe tissue (briefly, just to remove excess ethanol); use a compressed air can to dry the glass slide on each side (hint: hold the air can at a distance of about 5 cm and the slide at an angle and use short bursts to dry the slide without causing it to "frost"; if doing a lot of glass slides we typically use 2–3 air cans and interchange them, so they do not get too cold and cause the slide to "frost"). Place glass slides on a clean Kimwipe tissue so that they do not get any dust/fluff on them.
2. Thaw RA1 (one bottle, 10 ml required for eight BeadChips), XC1, XC2, TEM, STM, and ATM (one tube for 24–48 samples/2–4 BeadChips, two tubes for 96 samples/8 BeadChips;

record barcodes); ensure that the staining temperatures indicated on the STM reagent bottles are all the same; make up 95 % formamide/1 mM EDTA (or thaw if using frozen aliquot).

3. Make sure that the water circulator (attached to the Tecan robot) is filled to the appropriate level and turn it on so that it reaches a temperature that brings the chamber rack to 44 °C (e.g., this may be ~46 °C, depending on your circulator); you will need to test the chamber rack at several positions with the Illumina temperature probe. Ensure that there are no bubbles trapped in the chamber rack. *See* **Note 10** for an alternative approach to the automated protocol if you are familiar with the process.
4. Following the overnight incubation, the hybridization chambers can be removed from the oven and left to cool on the bench for 30 min prior to opening. Fill two glass wash dishes with 200 ml each of PB1 buffer (record barcode) and place a wash rack inside the first container; fill the Multi-Sample BeadChip Alignment Fixture with 150 ml PB1; fill additional containers with PB1 (*optional*), sufficient to cover the assembled flow through chambers (**step 8**); we utilize lids of pipette tip boxes and fill them ~3–4 cm with PB1; you can add more if you find that this does not cover the BeadChips and you can stack the assembled flow through chambers on top of each other if necessary; alternatively Illumina recommends just placing assembled flow through chambers horizontal on the bench (i.e., not submerged in PB1).
5. We particularly recommend wearing laboratory glasses/goggles for this part of the protocol. After the hybridization chambers have been cooled at room temperature for 30 min, remove the BeadChips one at a time and remove the IntelliHyb seal by holding the BeadChip with your thumb and forefinger on the short edges of the chip (or the long edges if this is easier; do not touch the inlets) and pulling the seal off in a diagonal direction away from you (pulling away will ensure that no solution splatters on you). You should start with a corner of the seal near the barcode end of the chip (*see* Fig. 2a) and pull in a slow consistent manner either downwards (which we prefer) or upwards, whichever is most comfortable for you (*see* Fig. 2b); do not start and stop the pulling motion (the motion should be continuous) and be sure not to touch the exposed BeadChip.
6. After the seal has been removed from each BeadChip, place it immediately in the rack within the first PB1 wash container and proceed with removing the seals from all other chips. Ensure that the chip is completely submerged in the PB1 buffer. The wash racks provided by Illumina hold eight BeadChips; if you are processing more than this you can use additional wash containers/racks or process the samples eight at a time (with new PB1 for each set of 8). On occasion, some glue from

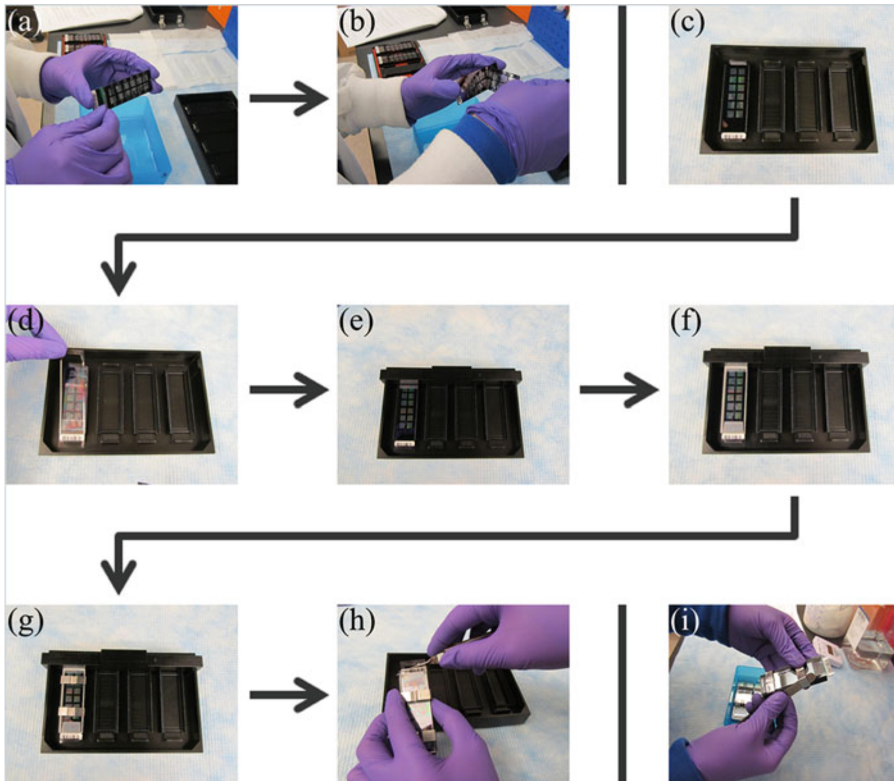

Fig. 2 IntelliHyb seal removal (**a**, **b**), flow through chamber assembly (**c**–**h**) and disassembly (**i**). For ease of visibility, figure shows assembly without immersion in PB1 buffer; during assembly BeadChips should always be submerged in PB1 buffer

the seal sticks to the BeadChip; if this occurs, whilst the chip is submerged in PB1 use a pipette tip to remove the residue by wiping it toward the edge of the chip (do not wipe toward the center or you risk scratching the surface to which your samples are hybridized); use a different tip for each chip (*see* **Note 9**).

7. Wash chips: Once all BeadChips are in the wash rack, move the rack up and down, breaking the surface of the PB1 in a gentle motion for 1 min to wash the chips. Move the rack to the second PB1 container and repeat the washing process for 1 min. Ensure that you check the BeadChips for the remaining residue before proceeding to the assembly, as this will negatively affect the staining procedure; if you see any glue/residue on the BeadChips, remove it as described in **step 6**. Whilst assembling the BeadChips, ensure that all chips remain submerged in PB1 at all times, either in the wash container or the assembly fixture. Once BeadChips are washed, the hybridization chambers should be washed immediately to remove any traces of PB2 in the reservoirs (*see* **Note 11**).

8. Assembly (refer also to Fig. 2, **Note 9**): To assemble the flow through chambers:
   (a) The Multi-Sample BeadChip Alignment Fixture should be prefilled with 150 ml PB1; the fixture allows assembly of up to four chips at one time; use new PB1 for every eight chips (note: for visualization purposes only, Fig. 2 does not show this fixture filled with PB1).
   (b) Place a black frame in the Multi-Sample BeadChip Alignment Fixture (Fig. 2c).
   (c) Place a BeadChip on each of the black frames, oriented so that the barcode on the chip matches the ridges on the alignment fixture (Fig. 2c).
   (d) Place a clear spacer on top of the BeadChip so that it surrounds the alignment fixture grooves; the spacer comes with a white backing; be sure to use the clear spacer (which is flimsier) rather than the white backing. Be sure that this is lying flat and sits around the edges of the BeadChip (Fig. 2d).
   (e) Place the alignment bar onto the alignment fixture; there is a groove in the fixture that matches the bar to ensure correct alignment (Fig. 2e).
   (f) Place a clean glass back plate on top of the clear spacer covering the BeadChip; be sure that the "reservoir" faces toward the BeadChip at the barcode end and that the bottom of the glass plate sits flush with the alignment bar (Fig. 2f). When placing the glass plate over the spacer, ensure that you do not disturb the position of the spacer; it should sit along the edges of the BeadChip and do not allow the glass plate to scratch the BeadChip (bead) surface.
   (g) Holding the glass plate against the alignment bar, place a metal clamp at the top of the assembly and just below the reservoir of the glass plate, so you cannot see any BeadChip stripes between the clamp and the reservoir; place the second clamp toward the bottom of the assembly with one strip showing between the clamp and the alignment bar (Fig. 2g). The clamps should clip onto the assembly if you place them on straight and just push down; we hold the clamp with a thumb and forefinger and apply equal pressure to both sides of the clamp to clip it into place (you will feel/hear if it clicks in correctly and can check by viewing it during **step 8(h)**).
   (h) Remove the alignment bar and lift the assembled flow through chamber out of the buffer. Using small scissors, cut off the ends of the spacer so that they do not interfere with reagent flow through (Fig. 2h). Illumina recommends to place the assembled flow through chambers horizontal

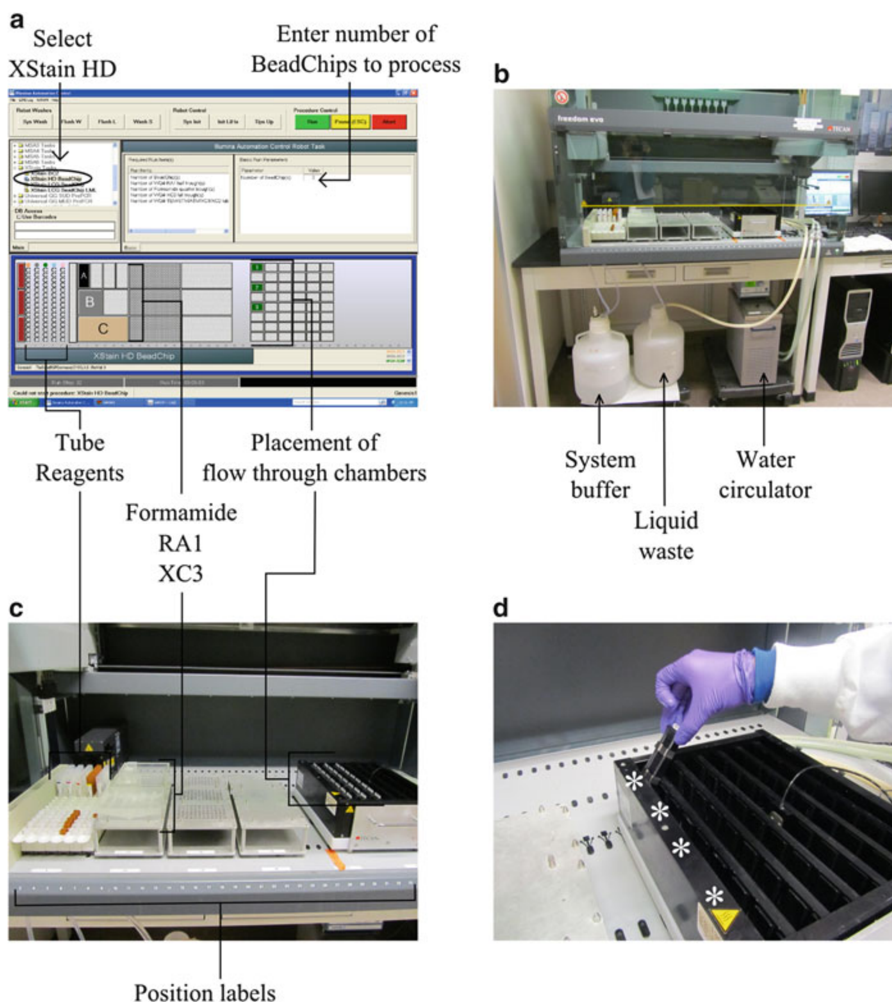

Fig. 3 Tecan (Freedom Evo®) robot setup for automated XStain procedure. (**a**) PC layout for reagents, showing reagent setup for three flow through chambers (BeadChips); (**b**) robotic system showing system buffer and waste containers and water circulator; (**c**) complete reagent setup for 24 samples (showing position labels), tube reagents are placed in positions 2–6 (XC1, XC2, TEM, STM, ATM, six tubes each for 24 samples), 95 % formamide/1 mM EDTA, RA1, and XC3 reservoir frames are placed in positions 8–13 (quarter and half reservoirs are placed to the left of the frame), chamber rack is in position 28–37; (**d**) placement of flow through chambers onto chamber rack, if running only four BeadChips place in the positions indicated by *asterisks*; if running 24 BeadChips, place flow through chambers in every position in the first three rows

on the bench whilst processing the next set; however we prefer to keep them submerged in dishes containing PB1 buffer (there is usually sufficient buffer provided in the kits to do this), particularly if you process a large number of BeadChips simultaneously.

9. Robot setup (if performing the automated protocol, *see* also Fig. 3): Ensure that there is sufficient 1× TE buffer to perform the run (we typically keep the bottle ~1/3 full, but this will

depend also on the positioning of tubing); make more 1× TE if necessary during the run (*see* Fig. 3b for TE buffer, waste, and water circulator setup). We typically perform a tip bleach wash prior to the first use of the day and then a system wash before and after each run (refer to Infinium Assay Lab Set Up and Procedures Guide for more information). Turn on the Tecan (Freedom Evo) robot and click on the Illumina Automation Control icon on the dedicated PC desktop. Login to the program and select XStain Tasks—XStain HD BeadChip from the column on the left. Select the number of chips to be processed in the Basic Run Parameters pane (around the top middle/right of the screen) and you will see the reagent setup at the bottom left corner of the screen; this can be used as a guide to place the reagents in the correct position; a general guide is also given below (a–d); Fig. 3a, c displays the reagent setup. After reagent setup, click run to start the process and login if required. You will be required to enter a temperature for the staining process, which should be printed on the STM reagent tube; if nothing is printed enter 32 °C and click OK. The automated protocol will begin by heating the water circulator to a temperature that allows the chamber rack to reach 44 °C (although this should have already been done in **step 3**; *see* also **Note 10** for an alternative approach). Be sure that the chamber rack is seated in positions 28–37 of the robot bed (there is a chance that this may vary with your setup; ensure that the positions used are defined by Illumina technicians) and that bubbles are not present in the chamber rack by tilting the rack from side to side. The water circulator temperature should be calibrated by Illumina, so if the temperature of the chamber rack is not 44 °C, this may need to be readjusted. The chamber rack temperature should be tested with the temperature probe and the probe should remain on one of the unused positions of the chamber rack for the duration of the protocol. Once the chamber rack temperature reaches 44 °C, the pop-up prompt automatically changes from stating "not to load BeadChips until chamber rack reaches 44 °C" to "load BeadChips *first* and then press OK" (*see* e below). Once OK is clicked, the automated XStain process will begin immediately, so ensure that the chips are loaded before pressing OK. You should watch the robot for the first few steps to ensure that the needles are aligned and dispensing reagents correctly and it is advisable to check intermittently to see if it is still running; if an error occurs during the protocol, you can follow the manual protocol from the point where the error occurred.

(a) Place 95 % formamide/1 mM EDTA in a quarter reservoir (15 ml for 8 chips, 17 ml for 16 chips, 25 ml for 24 chips), place the reservoir in a reservoir frame, and place this toward the back of the robot bed (should start at position 8).

(b) Place RA1 buffer in a half reservoir (10 ml for 8 chips, 20 ml for 16 chips, 30 ml for 24 chips), place the reservoir in a reservoir frame, and place this directly in front of the formamide/EDTA reservoir (should start at position 8).

(c) Place XC3 buffer in a full reservoir (record barcode; 49 ml for 8 chips, 97 ml for 16 chips, 145 ml for 24 chips), and place the reservoir directly in front of the RA1 reservoir (should start at position 8).

(d) Once XStain reagents are thawed (XC1, XC2, TEM, STM, ATM; record barcodes), centrifuge the tubes at 3,000 × *g* for 3 min, remove lids, and place on the robot bed in the designated position/s (from left to right starting at position 2: XC1, XC2, TEM, STM, ATM). For two or four chips (24 or 48 samples), one tube of each reagent will be used; for 8 chips (96 samples), two tubes for each reagent will be placed in front of each other; for 16 chips (192 samples), four tubes for each reagent will be placed in front of each other; for 24 chips (maximum, 288 samples), six tubes for each reagent will be placed in front of each other.

(e) Once the chamber rack reaches 44 °C load the BeadChips (assembled in the flow through chamber) onto it by angling them a little and hooking them over the chamber rack; the flow through chambers should sit flat against the chamber rack. If processing only four chips, they should be placed in the first row and spaced in every second position (starting at the position at the very back of the Tecan, *see* Fig. 3d). If processing 8 BeadChips, they should be placed in the front row; chips 9–16 will be placed in the second row and chips 17–24 will be placed in the third row, spacing out chips if there are only 4 in any particular row.

10. XStain protocol: If performing the manual protocol (with a chamber rack and water circulator) you will follow this staining protocol; this is the same protocol that the robot utilizes, so if there is an error during the automated process, the manual process can be performed from that point. Once XStain reagents are thawed (XC1, XC2, TEM, STM, ATM; record barcodes), centrifuge the tubes at 3,000 × *g* for 3 min; dispense other reagents into disposable reservoirs as they are needed. Once the chamber rack is at 44 °C, load the BeadChips onto the chamber rack, as outlined in **step 9**(e). Using a pipette, you will dispense the following volumes of each reagent into the reservoir of each flow through chamber and allow them to incubate for the appropriate time:

(a) 150 μl RA1, incubate for 30 s. Repeat five times.

(b) 450 μl XC1, incubate for 10 min.

(c) 450 μl XC2, incubate for 10 min.

(d) 200 μl TEM, incubate for 15 min.

(e) 450 μl 95 % formamide/1 mM EDTA, incubate for 1 min. Repeat but incubate for 5 min.

(f) Set chamber rack temperature to 32 °C (or if the STM tube states a different staining temperature, set the chamber rack to this); the water circulator may need to be set at 1–2 °C higher than this to accommodate any heat loss during the circulation process.

(g) 450 μl XC3, incubate for 1 min. Repeat. Wait for the chamber rack temperature to reach 32 °C (or other appropriate temperature) before proceeding.

(h) At correct staining temperature, dispense 250 μl STM, and incubate for 10 min.

(i) 450 μl XC3, incubate for 1 min. Repeat but incubate for 5 min.

(j) 250 μl ATM, incubate for 10 min.

(k) 450 μl XC3, incubate for 1 min. Repeat but incubate for 5 min.

(l) 250 μl STM, incubate for 10 min.

(m) 450 μl XC3, incubate for 1 min. Repeat but incubate for 5 min.

(n) 250 μl ATM, incubate for 10 min.

(o) 450 μl XC3, incubate for 1 min. Repeat but incubate for 5 min.

(p) 250 μl STM, incubate for 10 min.

(q) 450 μl XC3, incubate for 1 min. Repeat but incubate for 5 min.

(r) Wait for an additional 5 min, then remove flow through chambers from the chamber rack, and place horizontally on the bench.

11. In preparation for imaging, you should turn on the iScan or HiScan to stabilize the lasers; this could also be done during the XStain procedure (for details on this *see* **step 16**). Set up a few layers of absorbent paper towel and place a layer of Kimwipes on top of this (this is where the coated BeadChips will briefly drain); repeat this setup and place a tube rack on top (this is where the BeadChips will be placed horizontally; each rack holds 12 chips; be sure that the tube rack fits inside a desiccator). Ensure that a vacuum desiccator is set up; desiccator should be placed in the bottom of the desiccator and a platform on top of this; the top of the desiccator should have a tube attached to the three-way valve which is secured onto a

vacuum (each desiccator will hold 1 rack/12 chips). Fill one Illumina wash dish with 310 ml PB1 (you will fill another with 310 ml XC4 in the next step); these wash dishes are designed to load BeadChips so that they are upright, with the barcode at the top of the dish; it is best to first measure 310 ml of water into the wash dishes and mark where this volume is so that reagents can be poured directly into the wash dishes (reducing potential contamination associated with using additional measuring cylinders). Place the staining rack into the wash dish with PB1; Illumina recommends assembling the staining rack with the handle; however we have modified the protocol slightly, which we find works better and gives more reliable data when processing larger numbers of chips (e.g., 24 chips); our protocol does not utilize the staining rack handle.

12. Wash BeadChips: Using the dismantling tool (or narrow laboratory spatula), remove the metal clamps from the flow through chamber (this tool will avoid chipping the glass plates); you will place the dismantling tool to one side of the flow through chamber between the clamp and the rest of the assembly and pry it apart. Remove the glass back plate and then the clear spacers, being careful not to scratch the BeadChip surface. Remove the BeadChip and place it in the staining rack in the PB1, ensuring that you touch it by the sides and not the BeadChip surface. The BeadChips should remain fully submerged in the PB1 and not be allowed to dry out. Illumina recommends orienting the BeadChips a certain way in reference to the staining rack handle, however our protocol does not require this. Once all BeadChips have been placed in the rack, submerged in the PB1 (up to 24 chips can be accommodated), the chips are washed by lifting the staining rack up and down ten times, breaking the surface of the PB1 each time and then allowing the chips to soak in the PB1 for 5 min (*see* **Note 12**). Whilst the BeadChips are soaking in the PB1, fill another wash dish with 310 ml XC4.
13. Coat BeadChips: Transfer the staining rack to the wash dish containing XC4, although we do not use the staining rack handle, there is still a plastic tab that can be easily handled. Lift the staining rack up and down ten times, breaking the surface of the XC4 each time and then allow the chips to soak for 5 min (*see* **Note 12**). Using forceps, one by one remove the BeadChips from the staining rack in the XC4, once they have been removed from the wash dish, you may find it easier to handle BeadChips with gloved hands rather than forceps, handle by the sides only and close to the barcode end of the chip (do not touch the active surface). Take out each BeadChip and briefly allow it to drain on the pad of paper towel/Kimwipes that was setup in **step 11** by touching just the corner of the chip to the

pad for ~3–5 s, then immediately place the BeadChip horizontally (with barcode facing up) on the tube rack that was set up in **step 11**; repeat for all BeadChips, working quickly. If processing more than 12 BeadChips, two (or more) tube racks will be utilized. Draining of the chips on the absorbent pad to remove any excess XC4 will ensure that no XC4 from chips on the top of the tube rack drips onto chips underneath. If reusing the XC4 (which should be avoided if possible), pour the XC4 back into the original bottle and seal it when finished (*see* **Note 12**).

14. Once all BeadChips are lying horizontal on the tube rack, place the tube rack/s inside the desiccator/s, seal the desiccator, and turn the vacuum on slowly (to prevent any possible dust from being moved around), ensuring that the seal is tight and the lid cannot be lifted. At any time when the vacuum is not turned on, the red plug (which blocks the "air intake" valve) should be in place, it should be removed only when the vacuum is turned on. Dry the BeadChips in the desiccator for 50 min to 1 h (although this can be extended overnight if you are not ready to image). We typically cover the desiccators with a laboratory coat or towel during this time to limit exposure to light, this should especially be done if BeadChips will be left in the desiccator for an extended period of time. Whilst BeadChips are drying, you can wash all necessary equipment/consumables (*see* **Note 11**).
15. Cleaning BeadChips: Once dried (touch the border of the Bead Chip, DO NOT touch the active stripe area), the back of the BeadChip should be cleaned with alcohol to remove any dried XC4 that might prevent the chip from sitting flat in the scanner carriers. We utilize alcohol wipes (70 % ethanol wipes) for this, but if necessary you could also use a lint free tissue moistened with 70 % ethanol or isopropanol (ensure that this is not too wet when wiping the BeadChips or you risk getting alcohol on the bead stripe area). Holding the BeadChips by the sides and at an angle, wipe downwards on the back of the chip with a wipe several times (~5–6) until all XC4 is removed. Be sure not to get ethanol on the sides of the chip as you risk it running onto the bead stripe area. BeadChips should be imaged within 72 h of the staining procedure, they can be stored in a desiccator cabinet until then.
16. Image BeadChips: A comprehensive guide of the imaging platforms can be found on the Illumina Website (www.illumina.com: iScan System User Guide, HiScan®SQ System User Guide). The iScan system should be started 30 min before scanning any chips, to allow the lasers to stabilize.
    (a) To start the iScan system, first turn on the power switch at the back of the iScan Reader (it must have been turned off for at least 2 min prior to this), turn on the AutoLoader2.x

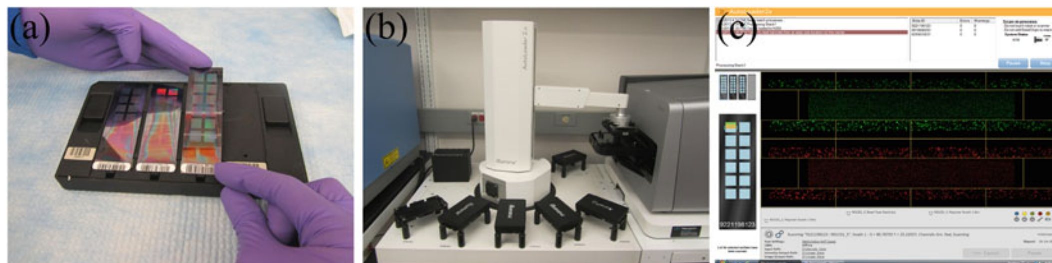

Fig. 4 Imaging BeadChips on the iScan. (**a**) BeadChips being loaded into iScan plate; (**b**) autoloader setup showing plate being loaded into iScan, stacks to the *front left* are "input" stacks, those to the *front right* are "output" stacks; (**c**) imaging of BeadChips: *top left* corner displays number of BeadChips on the plate to be scanned, *left panel* shows progress of scan where *green stripes* indicate successful scanning and *orange stripes* represent sections in process, main image shows *red* and *green* fluorescence emission from beads (probes) on BeadChip

(if using it), then turn on the iScan System computer, and double click on the iScan Control Software (ICS) icon; this will take a few minutes to initialize the iScan reader. If using the HiScanSQ System, you will turn on the computer, temporarily turn off the SQ module, turn on the HiScan Reader, then turn on the SQ module, you will then double click on the ICS icon. The iScan, HiScan, and HiScanSQ all utilize the ICS, and the scanning process outlined below is the same for the three instruments.

(b) The dried, cleaned BeadChips are loaded onto a carrier (up to four BeadChips per carrier) by placing a chip into a slot with the non-barcode end pushed toward the raised stop (*see* Fig. 4a) and the chip aligned within the slot. Gently press on the two "lift" buttons, which will raise pins beneath the BeadChips then release the button so that the chips clip into place. If BeadChips are not lying completely flat, they will not be scanned.

(c) Manual: If you are manually processing the BeadChips (if you have an autoloader but are not using it, you will click "no" to using the autoloader function after opening the ICS), click "start" on the ICS and the iScan reader tray will open; load the BeadChip carrier into the tray, aligning the notches on the carrier with the with the silver beads on the tray, the tray should be placed between these beads and black prongs (each carrier is specific to the iScan Reader and trays should not be switched between instruments); lower the carrier gently onto the tray (do not push down but you can lightly jiggle to make sure that it fits securely); click the "open/close tray" button on the scanner (or click "next" on the ICS) and the tray will close; click "Next" and the barcodes will be scanned and a BeadChip type will

automatically be selected for non-custom chips (but you should verify the correct BeadChip type has been selected); click “Scan” and the scanning process will begin.

(d) Automated: If you are using the AutoLoader2.x (*see* Fig. 4b; can be used in a single-reader or dual-reader mode for one or two scanning instruments), stack multiple carriers onto the input stacks (stack 1 and 2), ensure that the angled corner of the carriers aligns with the angled corner on the stack, then place the lid on the topmost carrier; place a lid on each of the 2 empty output stacks and the error stack (stacks 3, 4, and 5); place a stack extrusion over each stack; upon starting the ICS a window will appear asking if you would like to use the AutoLoader2.x, click “Yes” and the Autoloader2.x will be initialized; press the start icon; at the setup screen you can select either the “Default” or the “Methylation NXT” (recommended) option; click “Next” and the automated scanning process will begin.

(e) The ICS will check for available disk space, load the decode data (*.dmap) files, calibrate the iScan reader, and tilt/align the BeadChips in the carrier to ensure that they can be scanned adequately.

(f) During the scan, you can monitor the instrument for errors (BeadChips that have errors will be displayed in the top right corner), you can view the progress of the scan on the progress indicator. Sections of the BeadChip that are highlighted in blue indicate that they will be scanned, those in dark gray will not be scanned (you have to manually exclude sections that you don’t want scanned), those in orange are being scanned or are registered, those in green were scanned successfully, and those in red failed the scan (*see* Fig. 4c). If necessary, BeadChips or sections of BeadChips can be rescanned, but failure of a section will sometimes indicate that something went wrong in the protocol and often no data can be obtained.

(g) Once the scan is complete, you can transfer the image files to another computer for analysis with GenomeStudio.

3.3 Data Analysis

Given the fairly recent introduction of the Illumina Human Methylation450 BeadChips, there is not currently an established or even common methodology for data analysis, but rather users of this system have been utilizing and modifying several programs to obtain relevant information. Prior to any end-user analysis, data is first analyzed through the GenomeStudio Methylation Module which allows for data normalization, quality control assessment, and differential methylation analysis and provides several graphical overviews of the data, including dendrograms, heat maps, scatter plots, and histograms. Following is an outline of GenomeStudio,

as well as some data analysis options and visualization tools, and a brief summary of some currently available external analytical and quality control software.

3.3.1 GenomeStudio Methylation Module

To run GenomeStudio, you require a 2 core (or more) processor, CPU speed of 2.0 GHz or greater, 8 GB or more of memory, a 250 GB hard drive (or larger), and a Windows XP, Vista, or 7 operating system. The more powerful the computer, the larger the dataset that can be processed. A more comprehensive description of the capabilities of GenomeStudio can be found by searching for "GenomeStudio Methylation Module User Guide" on the Illumina Web site (www.illumina.com).

1. Create samplesheet: Prior to application of the Project Wizard in GenomeStudio (**step 2**), it is recommended to create a Sample Sheet, which allows samples to be easily identified in the graphical interface of GenomeStudio. Although it is possible to run GenomeStudio without creating a Sample Sheet, samples will be labeled as BeadChip barcodes and positions. Additionally, creation of a Sample Sheet allows you to more easily designate "groups," for example affected and unaffected, which can be compared for differential methylation analysis. If you do not generate a Sample Sheet, you will have to manually add samples to each group. The Sample Sheet template is a .csv file that can be manipulated in Microsoft Excel and can be downloaded from the Illumina Web site (search Infinium HD Methylation Sample Sheet at www.illumina.com); this file must always be saved as a .csv file. It is not necessary to fill in information for every column, however you must enter data for the Sentrix_ID and Sentrix_Position columns. Ensure that there are no spaces or symbols (_ can be used) anywhere in the data you enter or this will cause an error during the Genome Studio Project Wizard pipeline (*see* **Note 13**). We typically copy and paste the Sample IDs, barcodes, and positions from the tracking sheets to ensure that there are no errors caused by retyping.
   (a) The Sample_Name column is whatever you have named your samples. It is not necessary to enter data in this column.
   (b) The Sample_Well and Sample_Plate columns describe where your sample was placed throughout the duration of the experiment, this may be useful if you want to identify plate-by-plate variations. For example you might find that the outside wells or a specific single well always has the same quality control issues, indicating perhaps a problem with a well in the thermal cycler, heat plate, or hybridization oven. It is not necessary to enter data in these columns.

(c) The Sample_Group column allows you to group your samples into one or more groups. If there is no common grouping of samples (e.g., you want to assess variation in weight or height), you can label all samples in the Sample_Group column as "all," for example, or you can leave this column blank. If you want to compare samples that are affected versus unaffected, or before or after treatment with a drug, for example, you can label the Sample_Group column accordingly (e.g., untreated, 2hr_treatment, 6hr_treatment). Additionally, if you have run methylation control samples you can label these as a separate Sample_Group (e.g., 0_meth or 100_meth for 0 % and 100 % methylated control samples, respectively). Some analyses in Genome Studio can only be performed on "Groups"; if you would like to perform similar analyses on "samples," you can give each sample a different group name. You can enter as many or as few Sample_Group labels as you like; if this column is left blank, GenomeStudio will create a "DefaultGroup" label; it is not necessary to enter data in this column.

(d) The Pool_ID column is not used for analysis of Infinium methylation data; it is not necessary to enter data in this column.

(e) The Sentrix_ID is the BeadChip barcode. You must enter data in this column.

(f) The Sentrix_Position is the position of the sample on the BeadChip; designated as R01C01, for example, using a combination of the row number (R01–R06) and column number (C01–C02). You must enter data in this column.

(g) You can enter additional columns if you like, for example if you wanted a secondary group added, or wanted to enter a run date or operator to look for any inconsistencies in the quality control. It is not necessary to enter additional columns.

2. GenomeStudio Project Wizard: Open GenomeStudio by double clicking on the desktop icon (or open through the program list) and toward the bottom half of the page on the left you will see a "New Project" window; click "Methylation" to begin the Project Wizard.

(a) A pop-up window will ask which assay you would like to use; select "Infinium," and then click "Next" on the following page; you will then select "Infinium HD" as the assay type.

(b) You will need to enter a "Projects Repository" location, which is the location at which you want your project to be stored. You will also enter a "Project Name"; we recommend you make this as descriptive as possible and we like

to include whether the data is normalized or not in the title. Once a project name has been entered, you can click "Next"; you cannot proceed without inputting a Project Name.

(c) You will need to select a "Repository," which is the location where your BeadChip image folders are currently stored, when browsing for this be sure that you select the folder that contains one or more BeadChip image folders, do not select an actual image folder (these are labeled by the 10-digit barcode corresponding to the BeadChip) as you will be unable to analyze the results. Image files are typically large and are not necessary for GenomeStudio analysis, you will require the .idat, .sdf, and .cfg files only; typically we create "partial image folders" that contain just the required information for easier and quicker transfer of data, each of these folders must still be labeled as the BeadChip barcode or you will be unable to analyze the data. Once you have chosen a repository folder, all folders that are labeled with a BeadChip barcode will be displayed in the panel on the left hand side of the window. Select all of the BeadChips that you would like to analyze and click the "Triple Arrow" button (use the Shift or Ctrl keys to select multiple BeadChips, or transfer one at a time). If you only wish to analyze select samples on each BeadChip you can click the "None" button toward the bottom of the window and then select specific positions on the BeadChip image (use Ctrl to select more than one region). When you have selected the BeadChips (or BeadChip regions) and clicked the "Triple Arrow" button, the BeadChips selected will be listed in the panel at the right hand side of the screen. Click "Next" to continue.

(d) The next screen allows you to group samples. If you have generated a Sample Sheet (**step 1**), you can click on the icon that looks like a folder next to a document (the fourth button down) and the groups that were inputted into the Sample Sheet will automatically be generated. If you did not create a Sample Sheet, you can click on the button that looks like a blue folder (top button) and create a Group Name, if you want multiple groups, create more than one folder; whilst the Group folder is highlighted, select a BeadChip (or multiple) and select a region/position(s) (or all regions) and click the "Arrow" button (second from top); if you highlight BeadChips or Sections, and click the "Triple Arrow" button, a group folder will be generated for each BeadChip position highlighted (this can be useful if you want to perform certain GenomeStudio analyses on samples rather than groups); clicking the reverse button

toward the bottom of the screen will select all positions that were not previously selected on a BeadChip. If you make a mistake during this process, you can delete either Group Folders or Samples by highlighting them and clicking on the "X" button (sixth from the top), however be aware that if you delete groups inputted using a Sample Sheet an error will occur if you try to import another Sample Sheet and you will have to start the Project Wizard from the beginning. For each CpG site, GenomeStudio will create an average beta value based on all samples within a Group, however you will also see the individual sample values. You must enter a Groupset name at the top of the screen; typically we do not utilize the Groupset title at all and just enter "All" as a generic title, however if you are performing different types of analyses that are part of a larger project you may wish to put a descriptive title here; this Groupset title is never listed anywhere in your Project Name and we therefore find it more beneficial to have a descriptive Project Name. A Groupset must be entered or you will not be able to proceed; click "Next."

(e) The final screen allows you to choose analysis type and parameters. The analysis type is selected as "Methylation" by default; we always keep this default option; if you would like to perform a differential analysis ("Diff Methylation"), you can always do this after the project has been created and this will reduce the memory requirements needed for project creation (allowing for analysis of more samples). You need to enter a "Name" for the analysis, which you can set yourself or click the "Default" button; the Default option labels the analysis based on the Groupset and the Parameters defined, so if you choose this option, do so after setting the Parameters. We typically set the Parameters by selecting "controls" under the Normalization drop down arrow; this normalizes the intensity data based on control probes that have no underlying CpG sites in their sequence; we also use a background subtraction (by clicking the box) to minimize the variation in background signals between arrays, the background value is the fifth percentile of the negative control bead types, which is subtracted from probe intensities; the "Content Descriptor" is a *.bpm file, which is a beadpool manifest that contains the CpG site-to-bead-type mapping and file annotations, the current Content Descriptor is "HumanMethylation 450_15017482_v1.2.bpm", which can be downloaded from the Illumina Web site (www.illumina.com). Click "Finish" and the project will be created; this may take several hours, depending on the number of samples being analyzed (*see* **Note 14**).

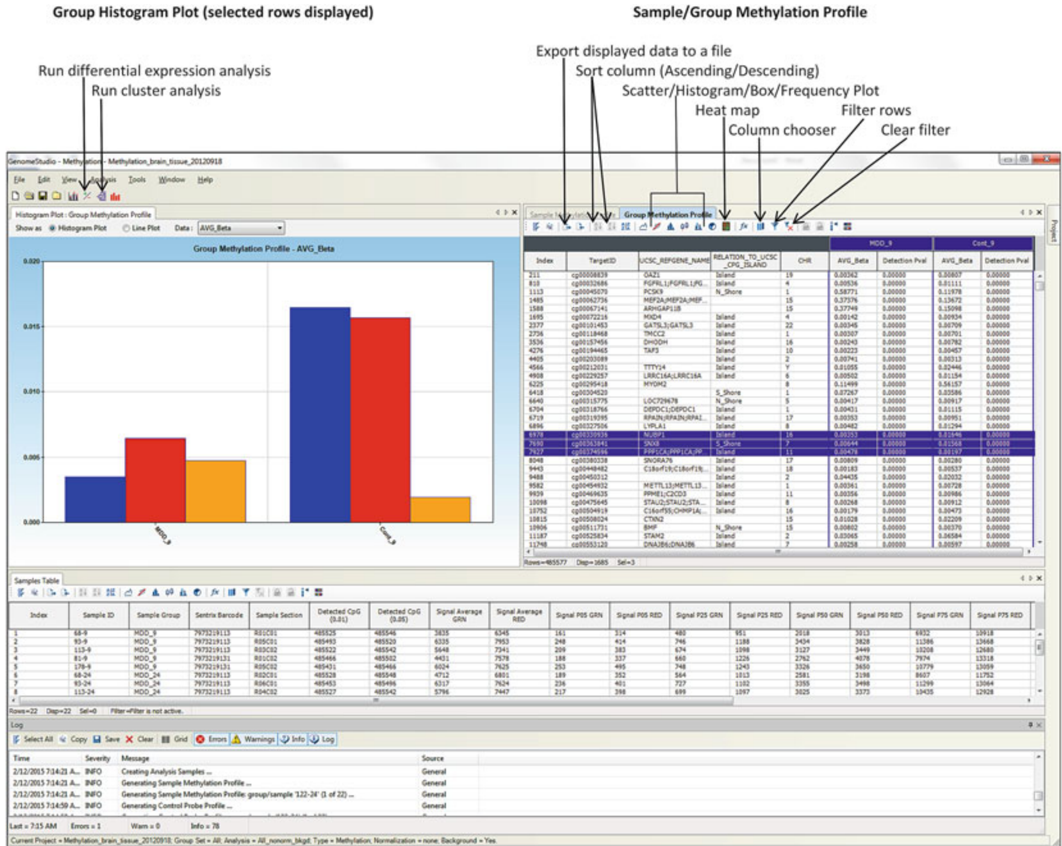

Fig. 5 Analysis view in GenomeStudio, showing most relevant buttons for analysis of samples

3. Project layout and basic tools: When a project is first created, the viewing window will be set to default, this can be adjusted by adding additional tables/graphs to view, changing the tabs that are shown and moving windows to different tabs. To view additional windows click on the "Windows" drop down tab and select the windows you want shown. To add a new tab, right click on the tab header/s shown and click either New Horizontal Tab Group or New Vertical Tab Group, you can also move windows to the next or previous tab group by right clicking on the tab header. For analysis purposes, you should ensure that at least the Sample Methylation Profile and Group Methylation Profile (if applicable) can be viewed; refer to Fig. 5 for the location of analysis buttons discussed in this section.
   (a) Sample Methylation Profile and Group Methylation Profile: This shows data for individual samples or groups, respectively; above the tables you can see a panel of buttons that can be used to view and analyze the data (*see* Fig. 5). Hovering over each of these buttons will display their name allowing you to select the correct one. The default shows columns including the Index (1-485,577), the Target ID

(corresponding to a specific CpG site, SNP or control), AVG_Beta (given for each sample/group, on a scale of 0 (no methylation) to 1 (full methylation); this is an average taken across all beads of the same type and in the case of groups is an average of all samples) and Intensity (given for each sample/group, shows average intensity of the probe).

(b) Column Chooser Tool: By clicking on the "Column Chooser" button, you can choose additional columns to add. We typically like to show all available columns, as much of this data is useful for annotation and CpG site description, including chromosomal location, gene names, island location, and distance from transcription start site, to name a few. We also include the "Detection Pval" as a subcolumn for each sample, which indicates the likelihood that the target sequence signal was distinguishable from the background, we exclude any beta value whose detection *p*-value is >0.01 (as proposed in [8]). Using the process indicated in **step 8**, only data that is shown in the displayed table will be exported for secondary analysis, so it is important you include as much information as you need.

(c) Sort Tool: Data can be sorted in Ascending order (A–Z, increasing numbers) or in Descending order (Z–A, decreasing numbers) using the Sort Tools. Highlight the Column that you would like the data to be sorted upon (e.g., CpG site, AVG_Beta of a particular sample/group, UCSC_REFGENE_NAME) and then click the Sort column button. You can also Sort by multiple columns.

(d) Filter Tool: Both the Sample and Group Methylation Profile Tables can be "filtered," by either selecting rows to be displayed or using the filter button. You are able to filter so that only selected rows are shown, these can be selected manually (using the Shift or Ctrl keys) or by using one of the Visualization Tools (for example, **step 6**(a), below), you will need to right click on a selected row and click "show only selected rows." You can also filter by clicking on the Filter rows button and entering a string of commands. For example, by selecting Group 1 under the Column panel and AVG_Beta under the Sub Column panel, the ">" Operation and entering 0.5, and then repeating this for Group2 with the "AND" Action selected (Filter will be: Group1.AVG_Beta > 0.5 AND Group2. AVG_Beta > 0.5), you can display only CpG sites that have at least 50 % methylation in both groups. If you have applied a particular Filter to the Group Methylation Profile Table, for example, you can easily apply the same filter to the Sample Methylation Profile Table. On the Group tab, you can click the Filter rows button and in the dialog box, under Filter Loading and Saving, click Save and give the

filter a name; then go the Sample tab, click the Filter rows button, and in the dialog box under Filter Loading and Saving, select the filter from the dropdown menu; then click Load. Similarly, these saved filters can be applied across different projects. These filters can be cleared at any time by clicking "Clear All" at the bottom of the Filter Tool dialog box, or by clicking on the Clear Filter button.

4. Viewing controls dashboard: Click the "Analysis" tab and select "View Controls Dashboard"; for a comprehensive explanation of the controls dashboard, refer to the GenomeStudio Methylation Module User Guide. There are several Sample Independent ((a) through (d), below) as well as Sample Dependent ((e) through (h), below) Controls. I have included approximate intensity values that we typically observe. Please note however that this may vary widely depending on your project and the imaging instrument used, these should be used as a basic guideline only. It is important that all of your samples show similar intensities at each control, if a particular sample consistently shows low-intensity values; for example, there is likely an issue with that sample and it may need to be excluded from analysis. If you have concerns about your data you should contact Illumina Technical Support.
   (a) ***Staining***: Checks efficiency of staining procedure, independent of the hybridization and extension steps; intensity values should be high (~30,000+ for the green channel, ~50,000+ for the red channel).
   (b) ***Extension***: Tests the extension efficiency of each base using a hairpin probe; intensity values should be high (~40,000+ for green, ~60,000+ for red).
   (c) ***Hybridization***: Tests the overall performance of the assay using synthetic targets rather than template DNA; intensity values vary from high (~45,000) to medium (~30,000) to low (~15,000), in the green channel only.
   (d) ***Target removal***: Tests the efficiency of the stripping step following extension; intensity values should be low (~1,000 for green, ~3,000 for red).
   (e) ***Bisulfite conversion***: Assesses the efficiency of the bisulfite conversion reaction by checking for converted Infinium I probes (cytosine to uracil; C1-C6) and non-converted probes (cytosines, U1-U6); intensity of converted probes should be high (~15,000 for C1-3 in green channel, ~25,000 for C4-6 in red channel), and unconverted probes (U1-6) should show low or no intensity. Bisulfite conversion is also assessed using Infinium II probes; intensity of converted probes should be high (~25,000 for probes 1–4 in the red channel only).

(f) ***Specificity***: Assesses stringency of probes by ensuring that the template hybridizes to "perfect match" bead types but not "mismatch" bead types; intensity for Specificity I (Infinium I probes) should be high (~15,000 for PM1–3 in green channel, ~25,000 for PM4-6 in red channel), and mismatched probes (MM1-6) should show low or no intensity; intensity for Specificity II (Infinium II probes) should be high (~25,000 for probes 1–3 in red channel only).

(g) ***Non-polymorphic***: Tests the overall performance of an assay by assessing base intensity in a non-polymorphic region of the genome; intensity should be high (~15,000+ for green, ~25,000+ for red).

(h) ***Negative***: Probes are randomly permutated and should not bind anywhere in the genome; intensity should be low (~250 for green, ~350 for red).

5. Checking for sample errors: There are some quality control measures that may help to ensure that the correct sample has been used.

   (a) The HumanMethylation450 BeadChips contains 65 SNPs, which can be used for identification purposes. If samples have been previously genotyped using one of the Illumina SNP platforms, there should be significant overlap with the SNPs on the HumanMethylation450 BeadChip. SNPs on the HumanMethylation450 BeadChips show beta values of approximately 0.0, 0.5 and 1.0, representing homozygotes, heterozygotes and homozygotes, respectively (0 and 1 do not necessarily represent the minor and major alleles, respectively, this is dependent upon the bases added during the extension reaction). SNP data from the HumanMethylation450 BeadChips should correspond with SNP data from other genotyping platforms, if not samples may have been mixed up. Also, if the beta values for several SNPs for a particular sample are typically higher or lower than the other samples (e.g., 0.17 instead of 0.03 or 0.78 instead of 0.94), this may indicate some level of contamination and data should be interpreted cautiously or excluded.

   (b) Although not definitive, you can utilize GenomeStudio to approximate the sex of each sample. In the Sample Methylation Profile Table select the Chromosome column (you may need to add this column using the Column Chooser) and order them by clicking on the "Sort descending" button, all CpG sites contained on the Y chromosomes will be at the top of the list. Select all Y chromosome probes (using the Shift key), then right click and select "Show only selected rows." Click on the Histogram Plot button. When the Histogram Plot is displayed, click off

the "Autoscale," shown beneath the graph and set Min *X* and *Y* values to 0 and Max values to 1 (*X*) and 100 (*Y*, # occurrences may go higher than this for females but you will still see the pattern), click the "Update" button. Under the Column panel to the right click on the first sample, then under the Sub Column panel click on "AVG_Beta"; whilst a sample is selected, you can use the down/up arrows to move to each sample in the list. As stated, this is not a definitive identification of sex; however it allows you to look for "patterns," such that if a supposedly female sample looks like a male sample, you should consider discarding the sample, in some cases the sex is not clear. If there are a high number of occurrences with low AVG_Beta (i.e., the bar graph is shifted to the left) the sample is female (as females do not have a Y chromosome to show high levels of methylation); *see* Fig. 6a. If the graph appears to be more spread out, or there are more occurrences with higher AVG_Beta values (i.e., graph is shifted to the right), the sample is male (*see* Fig. 6b). After you have determined sex for each sample, you can click the cancel filter button on the Sample Methylation Profile Table to once again display all CpG sites.

6. Data visualization: Once a project has been created, the data can be visualized and basic analysis performed using Genome Studio. For location of data visualization tools, refer to Fig. 5.
   (a) Scatter Plot: This can be used to compare two samples (from the Sample Methylation Profile tab) or two groups (from the Group Methylation Profile tab). Click the Scatter Plot button to open the Plot Columns dialog box. Under the Sub Columns panel click AVG_Beta. Under the Columns panel click on the first sample or group to be compared and click the "y axis" button, click on the second sample or group to be compared and click the "x axis" button. Enter a Title for the Scatter Plot, as well as Labels if required and click OK. You can view the plot in linear or logarithmic scale and the r^2 value is displayed below the plot. You can also display CpG sites that show a twofold (or other) difference in methylation levels by entering the fold change you want defined in the "N-Fold" box. Once defined, you can right click on the region outside of the defined fold change and Select "Fold Change >2.0" (for example) so that only CpG sites showing a difference equal to or greater than the defined fold change will be displayed in the Methylation Profile Table. If you are interested in identifying where a certain CpG site is, for example, you can use the Tools drop down menu to select Find Items and search for a CpG site, island, or gene.

Fig. 6 Determination of sex using methylation of CpG sites on chromosome Y, showing characteristic patterns for female (**a**) and male (**b**) samples

(b) Histogram plot: This can be generated in GenomeStudio to give a visual representation of methylation levels for samples or groups. The Histogram Plot will display only data that is shown in the currently selected Table (Sample or Group Methylation Profile). Although you can utilize the Histogram Plot button, this will display only the number of occurrences at which an AVG_beta level is reached (as used in **step 5**(a), above), and does not provide detailed information. An alternative to this is that the Histogram Plot can be viewed in a separate Window that is displayed

as part of the Project (you need to select this option from the Window drop down menu at the top of the page; this can be done for the Group Methylation Profile only; shown in Fig. 5, panel on left). If you would like to view this for individual samples, you could create a new project that places each sample in its own single group. On the Group Methylation Profile Table, you can select several CpG sites to view (for example you may display all that show a twofold or greater difference in methylation levels across groups; derived in a, above) or single CpG sites, to see how they vary across all groups. If you right click on the Histogram Window and select Properties, you can alter the appearance of the graph, for example by adding a legend, *X* or *Y* axis heading or changing the colors of the displayed CpG sites; you can also export the graph image by right clicking on the Histogram Plot Window.

(c) Heat Map: You can click the Heat Map symbol on the tool bar to view a heat map for either samples or groups, which allows you to visualize and compare methylation levels of all samples/groups for each CpG site selected. After clicking the Heat Map button, a dialog box will open where you can choose the Sub Column to display (AVG_Beta) as well as the row label (either Target or Probe ID); enter a Title and click OK to generate the Heat Map. The Heat Map will only show data that was contained within the selected Table (Sample or Group Methylation Profile), as such you can view a selection of CpG sites (e.g., those that show twofold or greater, derived in a, above) or all CpG sites. Using the Tools drop-down menu, you can cluster the Heat Map based on rows (CpG sites) or columns (samples/groups) and you can generate a presentation image.

(d) Cluster Analysis Dendrograms: This can be used to cluster samples or groups based on similarities in methylation levels. GenomeStudio utilizes the nesting with average linkage methodology to cluster samples into the dendrogram and offers four different clustering metrics (recommending you utilize multiple clustering methods). After clicking the Run Cluster Analysis button, you can choose the samples or groups that you want clustered (or you can cluster by genes) as well as the metric to be used and a dendrogram is generated; the distance between samples on the *X* axis indicates their similarity in terms of methylation levels (with samples that are close together being more similar than those that are distant). This can be useful as a quality control measure (i.e., you should check that samples on the same chip do not cluster together) as well as to identify patterns, such as whether all case samples and all control samples cluster together, for example.

7. Differential methylation analysis: Differential methylation analysis can be used to generate a DiffScore that indicates whether AVG_Beta values are significantly different across samples (if each sample is allocated as a different Group) or groups, when compared to a reference sample/group. The DiffScore can be generated utilizing an Illumina Custom Model, the Mann-Whitney model, or the *T*-Test Model. A differential methylation analysis can be performed during project creation (if this method is selected during the Project Wizard, we do not recommend this) or after a project has been created. Under the Analysis drop down menu at the top of the page, select Run Differential Methylation Analysis, you can save any changes made to the current analysis or save them as a different analysis. You need to set your analysis parameters as you did during the Project Wizard, but will need to input a Reference (Ref) Group and an Error Model to use. Multiple testing correction using the Benjamini and Hochberg False Discovery Rate (FDR) [21] can be applied (additional details are provided in the GenomeStudio Gene Expression Module User Guide, available at www.illumina.com). A Diff. methylation table (which displays the DiffScore for each CpG site in all groups but the reference group), as well as a histogram plot for the differential methylation will be generated. The DiffScore generated is a transformation of a *p*-value that provides directionality:

$$\text{Diffscore} = 10 * \text{sgn}(\mu\text{cond} - \mu\text{ref}) * |\log(\text{pdiff})|$$

where sgn(μcond – μref) refers to the sign, given as either positive or negative, for beta value differences in the conditional and reference groups (i.e., positive indicates the beta value of the conditional group is larger than that for the reference group or hypermethylation, whilst negative indicates the beta value of the conditional group is smaller than that of the reference group or hypomethylation); |log(pdiff)| is the absolute value of the log of pdiff, pdiff is 1 minus the differential *p*-value, which takes into account the difference between reference and conditional beta values as well as the standard deviation of the data points for each group. *P*-values can be calculated from the DiffScore using the equation p = 10´(DiffScore*sgn(μcond – μref)/10); a DiffScore of ±13 indicates a *p*-value of 0.05, a DiffScore of ±20 indicates a *p*-value of 0.01 and a DiffScore of ±30 indicates a *p*-value of 0.001.

8. Exporting data: The simplest way to export data is to select the tab of the table to be exported (Sample or Group Methylation Profile), ensure that all Columns and Rows of interest are showing (i.e., remove filters if necessary) and click the Export File button. The displayed table will be exported, as is, as a *.txt file, which can be manipulated in Microsoft Excel, as well as other programs. We utilize this file for secondary analysis of data.

3.3.2 Additional Considerations and Analyses

Although GenomeStudio provides an excellent graphical overview of the data, it is limited in the types of analyses that can be done. Also, due to the limitations of this platform, additional quality control measures should be performed. A brief description of some of these analyses is given below; for full details refer to the publications referenced.

1. Normalization: One of the limitations of the Illumina Methylation450 Assay is the use of two different probe designs, Infinium I and Infinium II, which results in widely divergent beta values. Infinium II probes typically have a lower dynamic range, show bias, and are less reproducible than Infinium I probes [11]. Several publications have attempted to address this issue and correct for the bias. Some of these methods utilize conversion of beta values to *M*-values, which is a Logit transformation that reduces heteroscedasticity for highly methylated or unmethylated CpG sites [22]. Dedeurwaerder et al. proposed a relatively simple-to-implement peak-based correction method, which essentially rescales the beta values such that the methylated and unmethylated "peaks" derived from the Infinium I and II probes match; this approach is based on the assumption that the methylation density profiles are bimodal [11]. Touleimat and colleagues [12] found the efficiency of this method to be sensitive to variations in the shape of the methylation density curves and as such proposed an alternative approach. They suggest a Subset Quantile Normalization (SQN) approach and developed a pipeline to estimate the quality of a dataset after data extraction, filter probes to eliminate signal variation not related to DNA methylation differences or the biological context of the study, correct for adjustment of color balance and background level and correct for the Infinium I/Infinium II shift in beta values [12]. Each of the preprocessing steps is conducted in R, using Bioconductor, and adapting the methylumi [17] and lumi [16] packages. Independently, Maksimovic et al. developed a Subset-quantile Within Array Normalization (SWAN) method, which is available in the *minfi* Bioconductor package [18] and determines an average quantile distribution followed by adjustment of the intensities of the two different probe types within these subsets [13]. Teschendorff et al. instead proposed the Beta MIxture Quantile dilation (BMIQ) normalization algorithm to reduce technical variance, type 2 bias and type 1 enrichment bias [9]. BMIQ fits a 3-state (unmethylated, hemimethylated, methylated) beta mixture model to the type I and type II probes separately and transforms the type II probes to quantiles for normalization; this is an assumption free model [9].
2. Probe ambiguity: Zhang and colleagues identified ambiguous genome alignment of a substantial number of probes (~140,000, 29 %) on the Illumina HumanMethylation450k

BeadChips, using Bowtie 2 [15] and allowing for two mismatches [14]. The authors suggest that probes which may align ambiguously to two or more locations on the human genome can be removed from analysis, thus reducing multiple-testing issues and allowing focus on more reliable biological signals [14]. Similarly, Chen et al. observed significant enrichment of cross-reactive probes hybridizing to the sex chromosomes with more than 94 % sequence identity, and concluded that 6 % of the array probes could therefore generate spurious signals [23]. They propose that 47 bases should be the minimum number of bases used as a criteria to identify cross-reactivity; they provide a list of CpG sites that meet this criteria [23]. However, potential ambiguity identified by these papers, will not necessarily transfer to experimental conditions, and as such removal of such probes from data analysis may cause some important biological signals to be missed. We would instead, at the very least, advise caution in interpreting data from probes that show likely ambiguous binding throughout the human genome. If a biologically relevant finding ensues from analysis of such probes, we would recommend assessing DNA methylation levels using an independent platform, such as pyrosequencing.

3. Consideration of SNPs: It is possible that SNPs (which may be population specific) present in the human genome may influence the binding of template DNA to the probe and there is a portion of probes that are known to overlap the location of known SNPs, some even query CpG sites at which a SNP has been reported. Chen and colleagues identified ~14 % of probes, which could potentially contain a polymorphism either at the "extended" base or the site before it, based on the 1000 Genomes database [23]. Many of these polymorphisms however may be rare and could be population or even family specific, and as such these polymorphisms may not have a significant impact on all studies. Similarly, they found that almost 50 % of the probes had a sequence that overlapped at least one SNP, although once again many of these are rare [23]. Although known SNPs within the probe sequence can be viewed (and exported) using GenomeStudio, by displaying the columns "PROBE_SNPS_10" (SNPs that fall within ten bases of the 3′ end of the probe) and "PROBE_SNPS" (SNPs that fall elsewhere in the probe), it is important to note that these may not be up to date. Also, on their Web site (www.illumina.com), Illumina provides a comprehensive list of underlying SNPs, including minor allele frequencies and their probe location relative to the CpG site. Although there does not yet appear to be an established methodology for filtering of SNPs, several methods have been employed, including removal of probes containing a SNP within 5 bp of the CpG site or

a >10 bp repeat element (using the Illumina Infinium27 k BeadChip) [24]; excluding probes with a SNP within 10 bp of the CpG site (using Illumina GoldenGate 1,505 panel [25] or using Illumina Infinium 27 k and 450 k BeadChips [26]); and excluding probes with a SNP anywhere in the sequence (450 k BeadChip) [27]. In addition, our group is currently developing methodologies to relate probe SNP positions to DNA methylation levels, in order to identify underlying commonalities that may allow us to exclude only probes where a SNP may impart a biological consequence.

4. Additional data analysis: Wang and colleagues recently developed the Illumina Methylation Analyzer (IMA) computational package, which is designed to automate the pipeline for exploratory analysis and summarization of site- and region-level methylation changes [28]. Using beta values derived from GenomeStudio, preprocessing within the IMA software package allows the user to choose several filtering steps, which are optional and user defined. These include filtering of sites with missing beta values from the X chromosome (or with median detection $p > 0.05$) or probes with SNPs at/near the targeted CpG site, data transformation and normalization [28]. IMA also calculates a methylation index by collecting loci within a specific region and deriving an index of overall region methylation [28]. Differential methylation can be defined based on each specific region, with the user defining the statistical test employed (e.g., Wilcoxon rank-sum test, Student's *t*-test) and allowing for covariates, adjusting for confounding factors, and applying multiple testing correction algorithms [28].
5. Association analysis: Depending on your project design, you may also need to employ/develop your own data analysis pipeline. For example, we often run association analyses to determine the contribution of DNA methylation to continuous traits (such as body mass index or fasting glucose levels) in large pedigrees and utilize the program SOLAR [19] for this. CpGassoc is an R-based program that rapidly tests for association between DNA methylation at thousands of sites and a phenotype of interest [20]. In addition to its utility in data normalization, the *minfi* Bioconductor package in R allows you to identify positions that are differentially methylated between two or more groups [18].

4 Notes

1. Trace amounts of undissolved reagent may be present. CT Conversion Reagent is light sensitive and for the best results should be used immediately after preparation. If necessary, the reagent can be stored overnight at room temperature, for

1 week at 4 °C, or for 1 month at −20 °C; stored solution should be warmed to 37 °C and vortexed prior to use.

2. When plating DNA, it is best to randomly assign samples to the sample plate rather than clumping all case samples together and all control samples together (for example); this will eliminate any bias that might be generated due to processing or batch effects. The Illumina protocol recommends ≥500 ng input DNA and the Zymo EZ and EZ-96 Methylation™ Kits recommend up to 2 μg input DNA, but suggest between 200 and 500 ng input DNA for optimal results. If higher amounts of input DNA are used, incomplete bisulfite conversion is possible and could lead to false-positive results. In our experience, we find that bisulfite conversion of 500 ng DNA provides high-quality data and as such, we recommend 500 ng input DNA for this protocol. Illumina provides an additional protocol for DNA derived from formalin-fixed paraffin-embedded (FFPE) tissue, which may be suitable for methylation analysis using the Infinium protocol. To assess whether FFPE-derived DNA is suitable for analysis, qPCR is performed using the Infinium HD FFPE QC Kit. If DNA is of sufficient quality to run the Infinium methylation assay, ≥250 ng FFPE DNA is used in the bisulfite conversion reaction. Following bisulfite conversion, DNA samples undergo restoration using the Infinium HD FFPE DNA Restore Kit and can then be utilized in the Illumina Infinium Methylation Assay.

3. Storage limitations of bisulfite-converted DNA vary widely from lab to lab, from as little as a few days to a few years for many protocols. Illumina recommends not to store the bisulfite-converted DNA for more than 1 month. The fresher bisulfite-converted DNA is, the more likely it will work in downstream applications. Storage at −80 °C, rather than at −20 °C will reduce degradation. We perform all Illumina Infinium assays directly proceeding bisulfite conversion without storage and have found this to produce high-quality data.

4. Standard laboratory practices should be employed; gloves should be worn at all times and sterile techniques utilized. Refer to the Infinium Assay Lab Setup and Procedures Guide (at www.illumina.com) for more detailed information on good laboratory practices. Reagents and waste should be discarded of appropriately, as either biological waste or chemical waste as necessary. The Illumina HumanMethylation450 DNA Analysis BeadChip Kit is packaged for 24, 48, or 96 samples. To ensure there are adequate reagents (particularly if using the automated mode) and to limit freeze/thaw cycles, samples should be processed together, with all 24 samples (corresponding to two BeadChips) being processed simultaneously for the

24 kit and a minimum of 48 samples (corresponding to four BeadChips) being processed simultaneously for the 48 or 96 kit. If a smaller number of samples are processed, and using the automated mode for any part of the protocol, you will need to purchase additional reagents from Illumina. For large-scale projects we process 192 samples simultaneously (after an initial trial with, for example, 48 samples), which limits freeze/thaw cycles for most reagents and allows for plate balancing in the centrifuge. For the XStain procedure, up to 24 BeadChips (288 samples) can be accommodated at the same time. If processing a small number of samples (e.g., 24), it is feasible to use single channel pipettes, however when processing larger numbers of samples (e.g., 48–96), it is best to use multichannel pipettes to reduce hands on time and ensure incubation times are similar for all samples, it is also fine to use a multichannel pipette for just 24 samples. When using a multichannel pipette, pour reagents into a disposable reservoir/trough and pipette reagents from there to the plate, ensuring that the pipette tips do not touch the samples in any way as to eliminate potential contamination. Also, when using a multichannel pipette always check the volume of reagent before dispensing it; if pipette tips are not completely secure on the multichannel pipette, they may not pipette the correct reagent volume; if volumes do not look equal dispense the reagent back into the trough, make sure that the tip is secure and draw up the reagent again; if the problem occurs again, remove whichever tip is causing the problem and proceed with the pipetting, make sure you pipette reagent into any samples that were missed due to this. When thawing reagents, allow them to equilibrate to room temperature before use. Several steps in this protocol require the use of a cap mat and unless otherwise stated, this can be reused. When reusing the cap mat, ensure that the orientation of the mat is the same each time, to avoid contamination. Well positions (for example A1) are printed on the cap mat and these should always be aligned to the well positions on the plate. If you are unsure of which orientation you have put the cap mat in, it should be discarded and a new one used.

5. The purpose of the NaOH is to ensure that the DNA is denatured as it disrupts the hydrogen bonding between the strands. However, be sure not to overdenature your samples, as this may fragment the DNA, ensure that this incubation is no longer than 10 min. We start a timer after the addition of NaOH to the first wells to ensure the time does not exceed 10 min. Although the original base pairing of the DNA no longer exists due to the bisulfite conversion, limited nonspecific base pairing may still be present at room temperature, hence the need for denaturation with NaOH.

6. If performing a large project, you should be consistent with incubation times throughout the project. For ease of reference and to ensure this is always the case; we always use the midpoint of the suggested incubation period such that amplification occurs for 22 h (37 °C) and hybridization occurs for 20 h (48 °C).
7. Following centrifugation you should clearly see a cell pellet, which will be tinged blue; if you do not see a cell pellet at this point, something has gone wrong and you should not proceed with the protocol (you can repeat the initial stages of the protocol at a much cheaper price than repeating the entire protocol). If you do not discard the supernatant immediately following centrifugation, you may risk dislodging the pellet; in this case you should recentrifuge the plate/s for an additional 20 min at 4 °C, 3,000 × *g* before removing the supernatant. To ensure best results, be careful not to allow supernatant from wells to go into other wells (so be sure your inversion is quick), this will avoid any potential source of contamination. In our experience, you can tap the plate very firmly to remove as much of the supernatant as possible, we have never had an occasion where the pellet has become dislodged.
8. It is imperative that hybridization chambers are completely sealed during the hybridization process, if they are not completely sealed the BeadChips will dry out and will not yield any usable data. To ensure that hybridization chambers are sealed and will always seal, it is best to label them, record which chambers are used for which BeadChips, and always keep the same lid with the same bases (so you can easily track if there is a problem). When clamping the lid onto the base, snap two clamps that are diagonally across from each other shut, followed by the other two clamps; alternatively if you shut both clamps on one side of the chamber, make sure that you use one hand to keep the lid in place when you snap the clamps in place and then keep the other hand in place whilst snapping the other two clamps shut (i.e., always keep one hand on the lid to hold it in place). Prior to sealing the chamber, we have found it useful to "wiggle" the lid a little, if seated flat on the base and gasket, it should move just a little and this should let you know that it is flat. If the hybridization chamber is particularly difficult to close, it is likely not sitting flat on the gasket (e.g., it may have lifted onto the edge of the base), so unclip it and start again. After sealing the hybridization chamber, you should make a visual inspection to ensure that the lid is sitting on the gasket, rather than lifted onto the edge of the base.
9. During the XStain procedure, reagents are added to a reservoir, which is created when the BeadChips are assembled into a flow through chamber. It is imperative that the reservoir does

not leak and that there is nothing in the flow through chamber to inhibit reagents flowing over the entire BeadChip. The glass plates have a "cut out" side that faces the BeadChip to create this reservoir, if large chips on this inner side would result in the leaking of reagents, you should not use the glass plate. Cleaning of the glass plates also ensures that there is no dust/fluff stuck to the glass plates, which may interfere with the flow through of reagents. On occasion, glue from the IntelliHyb seal may stick to the BeadChips, it is imperative that this is gently removed (so as not to scratch the BeadChip surface). If any residue remains on the BeadChip, this may allow for a slight gap at the edge of the flow through chamber through which reagents might leak. Whilst assembling the flow through chambers, be sure that there is no obvious fluff/hair/debris in the PB1 solution in the assembly fixture as if this is trapped between the glass plate and the BeadChip it can interfere with flow through of reagents. Also be sure that the spacer sits along the edge of the BeadChip and that it does not shift during assembly to cover any portion of the sample region of the BeadChip. The ends of the spacer must be removed (by cutting) following flow through chamber assembly as they would interfere with the flow through of reagents, when cutting these ends, ensure the spacer does not shift under the glass plate.

10. If you will be using the Tecan (Freedom Evo) robot for the automated XStain procedure and you are familiar with the setup of your reagents, you can initiate the protocol in order to heat the water circulator automatically. Once the software has been initiated, follow the prompts until the prompt says "Do not load BeadChips, adjusting chamber rack to 44 °C, waiting…." After this occurs, you can continue with processing the BeadChips. The protocol will NOT start until you agree that the BeadChips are in place, so if you choose this method you do not have to keep checking on the robot to ensure the protocol does not start. Once the chamber rack reaches temperature, the pop up prompt changes to "the chamber rack has reached 44 °C, load BeadChips and click OK"; DO NOT click OK until the BeadChips are fully loaded. Please note if you select this method, you will not be able to view the setup of reagents and will need to use a detailed protocol to guide you.
11. To ensure high-quality results, correct washing of materials used throughout the procedure is necessary. Following hybridization, the hybridization chambers should be washed as soon as possible; the rubber gaskets, chamber inserts, and hybridization chamber should all be washed with DI water, making sure to thoroughly rinse the reservoirs where the PB2 buffer was dispensed. Glass wash dishes used for washing of BeadChips

prior to flow through chamber assembly as well as the plastic wash dishes used for washing with PB1 and coating of XC4 should be rinsed with DI water (note that XC4 reagent is very sticky/slippery and this dish will need to be washed well). The chamber rack on the Tecan and all reservoirs used on the Tecan should be rinsed with DI water. The black frames, metal clamps, and Multi-Sample BeadChip Alignment Fixture and Bar should be rinsed with DI water. The glass black plates should be rinsed first with a 1 % solution of Alconox Powder detergent (made using DI water), wiping each plate with a Kimwipe, and then with running DI water; the glass plates should be dried on a bench at an angle, we prop them against a black metal frame to get an appropriate angle for drying; they are cleaned with ethanol and dried with compressed air, as outlined in the protocol, immediately before use. In addition to this, approximately every 5–7 uses the glass plates should be soaked in a 10 % bleach solution (in a fume hood) for 1 h, transferred to the Backplate Tecan Storage Rack submerged in DI water, and then rinsed extensively by dipping the rack up and down 20 times, soaking for 5 min and then repeating this wash process (i.e., dipping and soaking) four additional times. Refer to the Infinium Assay Lab Setup and Procedures Guide (at www.illumina.com) for more detailed information.

12. It is important that BeadChips are washed and coated evenly, we have modified the protocol a little to allow for this. Illumina utilizes a procedure that removes the rack from the XC4 whilst still containing the BeadChips; however in our experience, when processing 24 BeadChips we find that the XC4 forms a film between chips, which causes uneven coating. Our protocol instead keeps the BeadChips submerged in XC4 and removes them one at a time; this removal process should be performed as quickly as possible. During the washing and coating steps, ensure that the BeadChips are not touching each other. If the tops of the BeadChips are touching, move the staining rack back and forth until they separate to allow for reagents (PB1 or XC4) to circulate freely between the BeadChips; if necessary you can add some additional PB1 or XC4 to keep the chips from touching. Do not leave the BeadChips soaking in PB1 for more than 30 min, so ensure that disassembly of the flow through chambers is performed efficiently. The XC4 must be used within 10 min of filling the dish and whilst the chips are not in there it should be covered to prevent dust from getting in. If necessary, for lower throughput users, the XC4 can be reused in subsequent experiments (up to six times over a 2-week period for up to eight BeadChips);

however if possible reuse of XC4 should be avoided. If reusing XC4, ensure that the XC4 is poured *immediately* back into its bottle and the lid sealed following the coating step to prevent evaporation of the ethanol within the reagent. To prevent wicking and uneven drying, the BeadChips should not touch the edge of the tube rack, or each other while drying.

13. On occasion, when we proceed through the GenomeStudio pipeline, an error occurs due to a problem with the Sample Sheet, often this is due to spaces in the document but sometimes this occurs for no apparent reason. When this occurs, we first use the "find" (or "find and replace") option in excel to determine if any spaces are present in the Sample Sheet; if using the find and replace, you can leave the "replace" field empty and it will just remove all spaces. If no spaces are found or this does not correct the error then you can generate a new Sample Sheet, however if you have copy and pasted data, the same problem will probably reoccur. The final option is to identify which row(s) of the Sample Sheet are causing the error. We do this by splitting the Sample Sheet in two (by deleting ~ half the sample rows each time) and saving it as a different Sample Sheet. We try to proceed through the GenomeStudio pipeline with each of the new Sample Sheets, if no error occurs then the sample rows present in that Sample Sheet are OK; however if an error occurs then there is a problem with one of the rows on that Sample Sheet. We continue this process until the row(s) is identified and then we go back to the original sample sheet and retype the information from that row in manually. In our experience, this has always solved the problem, but can be a time-consuming process if you are dealing with a large dataset.
14. Using a Dell T5400 workstation with a single 3.0 GHz Xeon X5450 quad core processor, with 16 GB RAM and 3× 1TB hard drives in a Raid-5 array and running Windows XP ×64 operating system, we are able to analyze ~200 samples simultaneously, which takes approximately 2–3 h for project creation. When we have tried to analyze more samples in a single project, we find that the project discontinues progress after several hours. Although no error is displayed you can see that the project has stalled and the program should be shut down. Before shutting the program down, be sure that the progress has indeed stalled; there is a progress bar displayed and the progress bars on this often move very slowly; if no progress has been made in several hours, it is likely that the program has stalled.

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Chapter 11

Investigation of Genomic Methylation Status Using Methylation-Specific and Bisulfite Sequencing Polymerase Chain Reaction

Melanie A. Carless

Abstract

Epigenetic modification plays a central role in the regulation of gene expression and therefore in the development of disease states. In particular, genomic methylation of cytosines within CpG dinucleotides is crucial to development, gene silencing, and chromosome inactivation. Importantly, aberrant methylation profiles of various genes are associated with cancer as well as autoimmune disease, psychiatric and neurodegenerative disorders, diabetes, and heart disease. Various methods are available for the detection and quantification of methylation in a given sample. Most of these methods rely upon bisulfite conversion of DNA, which converts unmethylated cytosines to uracil, while methylated cytosines remain as cytosines. Methylation-specific amplification of DNA can be used to detect methylation at one or more (typically up to about 4) CpG sites by using primers specific to either methylated or unmethylated DNA. Alternatively, amplification of both methylated and unmethylated DNA followed by sequencing can be used to detect methylation status at multiple CpG sites. The following chapter provides protocols for bisulfite conversion of DNA, methylation-specific PCR and bisulfite sequencing PCR.

Key words Methylation, Bisulfite, Polymerase chain reaction (PCR), Methylation-specific PCR (MSP), Bisulfite sequencing PCR (BSP)

1 Introduction

Various mechanisms exist by which gene expression is regulated, including both genetic and epigenetic modifications. In recent years it has become evident that epigenetic modifications are essential for regular cellular function as well as for the development of various disease states. One such epigenetic mechanism is genomic methylation, which occurs at the C(5) position of cytosine in cytosine-guanine dinucleotides (CpG) and is regulated by DNA methyltransferases [1–3]. In normal cells, CpG dinucleotides are generally methylated throughout the genome, except for in regions known as CpG "islands," which are hypomethylated [3]. CpG islands are defined as regions of greater than 500 bp with a G+C

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content equal to or greater than 55 % and an observed/expected CpG ratio of 0.65 [4]. These islands are usually located in promoter regions or 5′ exonic regions of genes and about 70 % associate with human genes [5, 6].

Methylation of cytosine residues within the DNA affects the binding of transcription factors to that DNA sequence [2]. Thus, when CpG islands within the promoter or 5′ exonic regions become methylated, transcriptional silencing ensues either through blocking of transcriptional activators or recruitment of transcriptional co-repressors by associated methyl-CpG-binding proteins [7]. Intragenic methylation, on the other hand, has been shown to both repress [8] and increase [9, 10] gene expression, and that such cell-specific methylation may regulate alternative intragenic promoters [11]. Further, 3′ associated methylation is relatively common (~12 % of genes) and although the consequences of this are unknown, it is suggested that this may be related to suppression of antisense transcripts, regulation of polyadenylation, or transcription termination [10]. As such, DNA methylation plays a critical role in allele-specific expression, X chromosome inactivation, genomic stability, and embryonic development [2]. In addition to this, silencing of genes critical to normal cell growth and development can have detrimental consequences, leading to the development of various diseases. Aberrant methylation plays an important role in the development of cancer [1, 12] and potentially in autoimmune diseases (lupus, multiple sclerosis) [12], psychiatric and neurodegenerative disorders (bipolar disorder and schizophrenia, autism, Alzheimer's Disease, multiple sclerosis) [13, 14], atherosclerosis [15], and diabetes [16].

The potential importance of genomic methylation in a number of diseases calls for techniques to assess gene methylation status. Early methylation detection techniques included employment of methylation-sensitive restriction enzymes (qualitative) and hydrazine or potassium permanganate DNA modification for genomic sequencing [17]. The introduction of a method that uses sodium bisulfite-mediated conversion of cytosine (not 5-methylcytosine) to uracil [18] has heralded a breakthrough in genomic methylation analysis. This allows methylated and unmethylated DNA to be differentiated as only methylated cytosines will remain as cytosines after conversion. Since then, various assays for the assessment of methylation status within specific regions have been developed, including methylation-specific PCR (MSP), methylation-sensitive single-nucleotide primer extension (MS-SNuPE), combined bisulfite restriction analysis (COBRA), bisulfite sequencing PCR (BSP), spectrometry-related assays, real-time quantitative-based assays, enzymatic regional methylation assay (ERMA), oligonucleotide-based microarrays, and next-generation sequencing technologies; for a review of these methods *see* refs. 19, 20. Global methylation techniques such as high-performance liquid chromatography

(HPLC), restriction landmark genomic scanning (RLGS), differential methylation hybridization (DMH), and bacterial artificial chromosome (BAC) arrays can be employed to examine the amount/ratio of methylated DNA in the whole genome or regions of the genome, such as repetitive regions or CpG islands [19].

This chapter focuses on methylation-specific PCR and bisulfite sequencing PCR to investigate the methylation status of specific loci. Both of these techniques use bisulfite-modified DNA to interrogate one or more CpG sites within a CpG island. Briefly, MSP uses two independent primer sets that span one or more CpG sites that are specific for either methylated or unmethylated cytosine. BSP uses a single primer set that flanks a region containing a CpG island, so that all cytosines within the island can be interrogated for methylation status by sequencing. Following are some commonly used methods for the assessment of methylation at CpG sites.

2 Materials

All reagents used throughout these protocols should be of molecular biology grade where applicable. Where a company is preceded by "e.g.," the reagent may be purchased from other vendors, companies that are not preceded by "e.g." should be used for purchasing of reagents to ensure high-quality results. Sterile techniques should always be used in preparation of reagents to prevent contamination during the protocol. Ensure that water used is DNase/RNase-free.

2.1 Sodium Bisulfite Conversion

1. Microfuge tubes (1.5 ml or similar, need to be amber or kept in dark) (e.g. Fisher Scientific, USA Scientific).
2. Pipette tips (2 μl, 20 μl, 200 μl, 1 ml or similar) (e.g. Fisher Scientific, USA Scientific).
3. 1 μg (or less) DNA.
4. *Optional*: Salmon sperm DNA, only required if you have less than 1 μg DNA (e.g. Sigma-Aldrich).
5. 1.0 M NaOH (e.g. Sigma Aldrich, Fisher Scientific).
6. 10 mM Hydroquinone: this must be made fresh the day of the experiment; light sensitive; toxic (hazard class 6.1) (e.g. Sigma-Aldrich).
7. 3.0 M sodium bisulfite, pH 5.0: this must be made fresh the day of the experiment, adjust pH with NaOH (e.g. Sigma Aldrich).
8. Mineral oil (autoclaved, filtered into screw cap tubes and re-autoclaved) (e.g. Sigma-Aldrich, syringe filters available from Fisher Scientific).

9. Heatblock or waterbath (set to 50 °C, must fit microfuge tubes).
10. Vacuum manifold (a Luer-Lok system can be used instead but the vacuum gives higher reproducibility and DNA quality) (e.g. Qiagen, Promega).
11. Wizard DNA Clean-up system: store at room temperature, resin should be protected from light (Promega, A7280).
12. 80 % Isopropanol (e.g. Sigma-Aldrich, Fisher Scientific).
13. 3 M NaOH (e.g. Sigma-Aldrich, Fisher Scientific).
14. 20 mg/ml glycogen (e.g. Roche).
15. 10 M Ammonium acetate (NH_4Ac; e.g. Sigma-Aldrich).
16. Ethanol (100 and 70 % in DNase/RNase-free water) (e.g. Sigma-Aldrich, Fisher Scientific).
17. *Optional*: methylated and unmethylated DNA controls (e.g. Zymo research, Qiagen, New England Biolabs, Life Technologies, Active Motif, Diagenode).

Alternative: Purchase bisulfite conversion kit (e.g. Zymo Research, Qiagen, New England Biolabs, Life Technologies, Sigma-Aldrich, Active Motif, Chemicon, Diagenode, Epigentek).

2.2 Primer Design

1. Primer design software (e.g. MethPrimer, PerlPrimer, BiSearch, Methyl Primer Express v1.0; details given in Subheading 3.2.3).

2.3 PCR

1. 10× Bisulfite PCR buffer (alternatively use buffer provided with *Taq* polymerase).
   - 1.66 ml 1 M ammonium sulfate (166 mM final) (e.g. Sigma-Aldrich).
   - 6.7 ml 1 M Tris-HCL, pH 8.8 (670 mM final) (e.g. Sigma-Aldrich).
   - 670 μl 1 M $MgCl_2$ (67 mM final) (e.g. Sigma-Aldrich).
   - 69.9 μl 14.3 M β-mercaptoethanol (100 mM final) (toxic, class 6.1, keep tightly sealed) (e.g. Sigma-Aldrich).
   - 900.1 μl DNase/RNase-free water (e.g. Fisher Scientific, Life Technologies).
2. 25 mM dNTPs (store at −20 °C) (e.g. Promega).
3. 10 μM forward and reverse primers (e.g. Integrated DNA Technologies, Fisher Scientific).
   - MSP: 2 primer sets specific to methylated and unmethylated DNA.
   - BSP: 1 primer set that does not discriminate methylated from unmethylated DNA.
4. DNase/RNase-free water (e.g. Fisher Scientific, Life Technologies).

5. *Taq* Polymerase (e.g. Life Technologies, Qiagen, Zymo Research, New England Biolabs).
6. Thermocycler (e.g. Life Technologies, Bio-Rad).
7. PCR tubes or 96-well plate (e.g. Fisher Scientific, USA Scientific).

Alternative: Several companies offer optimized polymerases or kits for the amplification of bisulfite converted DNA (e.g. EpiTect MSP kit by Qiagen, ZymoTaq by Zymo Research, EpiTaq HS by Takara (Clontech)).

2.4 Methods of Detection

2.4.1 Methylation-Specific PCR

1. Analytical Grade Agarose (e.g. Fisher Scientific, Promega, Sigma-Aldrich).
2. 1× TAE: Add 975 ml MilliQ water to 25 ml 40× TAE (or make other appropriate dilution depending on starting concentration of TAE) (e.g. Fisher Scientific, Promega, Sigma-Aldrich).
   - 40× TAE: 1.6 M Tris-acetate, 40 mM EDTA, or can purchase concentrated solution.
3. Conical flask (e.g. Fisher Scientific).
4. 10 mg/ml ethidium bromide solution (mutagenic, hazard class 6.1, store in dark) (e.g. Promega, Sigma-Aldrich).
5. 100 % Glycerol solution OR 6× gel loading dye (if it does not interfere with band size) (e.g. Fisher Scientific).
6. 100 bp molecular weight marker (e.g. New England Biolabs, Qiagen, Promega).
7. Gel tray and combs (7 cm × 10 cm tray and 2× 11 teeth combs is sufficient for the PCR described, wide teeth are required for the combs to hold up to 60 μl sample) (e.g. Fisher Scientific, Bio-Rad).
8. Electrophoresis tank and power source (e.g. Fisher Scientific, Bio-Rad).
9. UV light box and camera (ensure glasses/face shield is worn when using UV light box) (e.g. Fisher Scientific, Bio-Rad, Syngene).

2.4.2 Bisulfite Sequencing PCR

1. 1 % (or 2 %) agarose gel, gel tray and combs, electrophoresis tank and power source, UV light box (as described in Subheading 2.4.1).
2. Scalpel/razor for cutting (e.g. Fisher Scientific).
3. Gel extraction/purification kit (e.g. Qiagen, Life Technologies, Promega, Millipore).
4. BigDye® Terminator Mix v3.1, includes 5× Sequencing Buffer (this is specific to the sequencer used, for example the 3730/3730XL BigDye® Terminator Mix v3.1should be used

with the 3730 or 3730XL instrument) (Life Technologies; store at −20 °C in the dark, avoid freeze/thaw more than 5–10 times, keep thawed reagents on ice).

5. 10 μM forward and reverse primers (same as those used in Subheading 2.3).
6. DNase/RNase-free water (e.g. Fisher Scientific, Life Technologies).
7. 125 mM EDTA (e.g. Sigma-Aldrich).
8. Ethanol (100 % and 70 % in DNase/RNase-free water) (e.g. Fisher Scientific, Sigma-Aldrich).
9. Hi-Di™ formamide (store at −20 °C) (Life Technologies).
10. 96-well plate (Life Technologies).
11. Polymer (Pop-7™ for 3730, others available for different instruments; store at 4 °C) (Life Technologies).
12. 10× Running Buffer (store at 4 °C) (Life Technologies).
13. 3730 (or 310, 370, 373, 377, 3100, 3130) genetic analyzer and required accessories (capillary, plate tray, septa seal) (Life Technologies).

3 Methods

3.1 Sodium Bisulfite Conversion

Sodium bisulfite converts unmethylated cytosines into uracil so that following PCR amplification all unmethylated cytosines within a sequence will be represented by a thymine. Methylated cytosines will remain as cytosines following PCR amplification. Bisulfite conversion will only take place on single-stranded DNA and as such, the samples are first denatured. Sodium bisulfite is added to sulfonate cytosines at a low pH, this is followed by spontaneous deamination into a sulfonated uracil molecule, which is then desulfonated resulting in a uracil (Fig. 1). The radical scavenger hydroquinone is also used in the reaction, to inhibit bisulfite oxidation [21]. Methylated cytosines are protected from sulfonation and maintain a cytosine structure. Bisulfite converted DNA can be stored at −20 °C for up to 1 month or at −70 °C for up to 3 months (although less storage time is preferable).

Various kits are available for bisulfite conversion and are often used as some shorten the procedure time (as little as 3–5 h). These kits are available from companies such as Qiagen, Life Technologies and Sigma-Aldrich. Zymo Research, Chemicon, and Active Motif provide kits which require longer preparation times (10–16 h). Such kits claim to have up to >99 % conversion efficiency (of non-methylated cytosine residues to uracil), 99 % protection (of methylated cytosines) and >80 % DNA recovery. The amount of starting DNA varies from 200 pg to 2 μg, depending on the kit. If a kit is

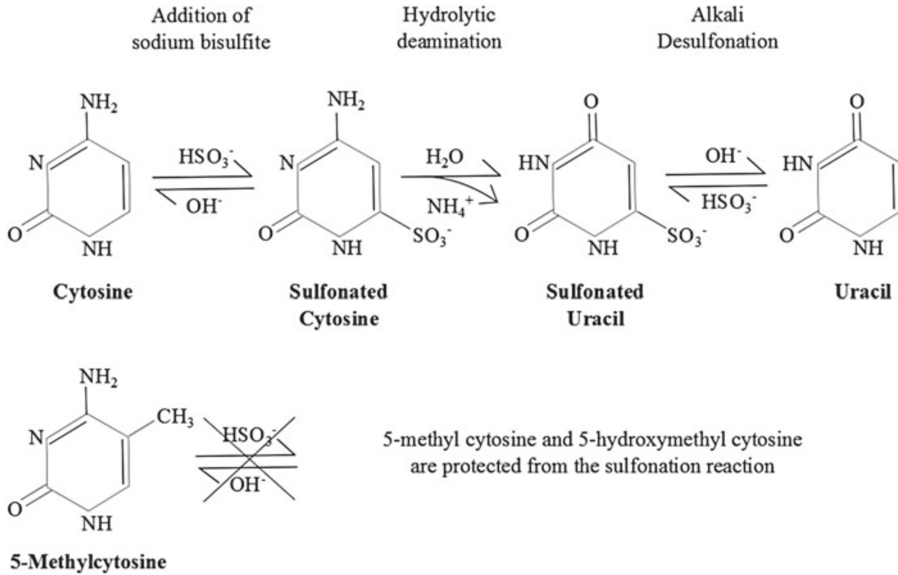

Fig. 1 Bisulfite conversion process

being used for bisulfite conversion, the manufacturers' instructions should be followed. Alternatively, a standard protocol can be used for bisulfite conversion of methylated DNA, this takes about 17 h. The following protocol is a modification of that provided by Frommer and colleagues [18].

1. Denature 1–2 μg DNA (in 40 μl DNase/RNase-free water) with 10 μl 1.0 M NaOH (final concentration 0.2 M) at 37 °C for 10–30 min (*see* **Note 1**).
2. Add 30 μl 10 mM hydroquinone and 520 μl 3.0 M sodium bisulfite (pH 5.0), vortex to mix and briefly centrifuge. Keep samples in dark (use amber tubes or foil).
3. Add 50–100 μl sterile mineral oil (to prevent evaporation) and incubate at 50 °C for 16 h (*see* **Note 2**). Remove the mineral oil prior to purification, this can be done by rolling the entire sample on Parafilm. The oil will stick to the parafilm and the aqueous sample will remain in droplet form that can be pipetted and placed in a microcentrifuge tube.
4. Purify the sample using the Wizard DNA Clean-up system according to the protocol provided with the kit. Due to the large volume, two columns will be used per sample.
   - Briefly, 1 ml of clean-up resin will be added to 300 μl of sample, mixed and added to a mini-column attached to a vacuum manifold, which is used to draw the mix through. The resin is washed with 2 ml 80 % isopropanol.

Extract the DNA by incubating resin with 50 μl prewarmed (65–70 °C) DNase/RNase-free water for 1 min and elute by centrifugation at 10,000 × *g* for 20 s.

5. Add 5.5 μl 3 M NaOH and incubate at room temperature for 10 min.
6. Add 1 μl 20 mg/ml glycogen, 33 μl 10 M ammonium acetate and 268.5 μl (3 volumes) cold ethanol.
7. Precipitate DNA at −20 °C for 2 h (or overnight) then centrifuge at 10,000 × *g* for 30 min (*see* **Note 3**).
8. Remove supernatant and wash pellet with 70 % ethanol and centrifuge at 10,000 × *g* for 20 min.
9. Remove supernatant, air-dry pellet, and resuspend in 20 μl DNase/RNase-free water. At this point you should have two tubes of purified DNA (generated by using two columns of the Wizard clean-up kit). Typically 2 μl is required per PCR reaction, although 1 μl may also be sufficient. DNA should be stored at −20 °C for no more than 3 months (*see* **Note 4**) and freeze/thaw cycles should be avoided, therefore it is best to aliquot samples.

3.2 Primer Design

Methylation-specific PCR and bisulfite sequencing PCR rely heavily upon complete bisulfite conversion of DNA. Primer design has to be carefully executed to account for the conversion of DNA as well as the degradation of DNA that accompanies this conversion. A number of free software programs are available for the design of matched methylated and unmethylated MSP primer sets and for BSP primer sets. It is also possible to design primers without the use of these programs but in any case there are important considerations involved in the design of primer sets.

3.2.1 Methylation-Specific PCR Primer Considerations

Methylation-specific PCR works on the premise that methylated DNA and unmethylated DNA will differ slightly in their constitution after bisulfite conversion. All unmethylated cytosines will be converted to uracil after bisulfite treatment and primers should therefore be designed with thymines in place of cytosines. However, methylated cytosines will remain as cytosines and therefore the methylated primer set will include cytosines at CpG sites. For example, if a DNA sequence is unmethylated all cytosines will be converted to uracil and a corresponding primer should be designed that incorporates thymine at all cytosine positions. However, if that same DNA sequence was methylated at one or more CpG sites then only the unmethylated cytosines will be converted to uracil and the corresponding primer will incorporate cytosines at the CpG sites. The following table shows examples of these sequences.

	Unmethylated DNA	Methylated DNA
Initial sequence	AAC TGA CGT ACT ACG	AAC TGA **Cm**GT ACT A**Cm**G
Converted sequence	AAU TGA UGT AUT AUG	AAU TGA **C**GT AUT A**C**G
Primer sequence	AAT TGA TGT ATT ATG	AAT TGA **C**GT ATT A**C**G

Note that the primer sequence designed for unmethylated DNA is not complementary to the methylated sequence and therefore will not bind. As such, an unmethylated primer set will amplify unmethylated DNA but not methylated DNA and a methylated primer set will only amplify methylated DNA. In this way, if a sample of DNA undergoes two separate PCR amplifications for the methylated and unmethylated primer sets, the presence of an amplified product in one of these reactions will indicate the methylation status of the DNA.

1. As with all PCR, primer specificity is an important issue. Primers should therefore be 20–35 bp in length with an optimum length of 25–30 bp. To keep a similar annealing temperature to standard PCR reactions, the primer is generally longer as GC content is low. All primers that are designed should be interrogated for specificity. It is recommended to assess each of the four primers using methBLAST (http://medgen.ugent.be/methBLAST/), which is based on the NCBI tool BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) but aligns sequences to the human (or rat or mouse) genome based on conversion of predicted methylation sites [22]. It tests for alignment to bisulfite converted unmethylated DNA, bisulfite converted methylated DNA, and their reverse complements.
2. The primer sequence must contain at least one (and up to three or four) CpG sites. Some investigators consider it best to incorporate as many CpG sites into the primer as possible, allowing for good discrimination between methylated and unmethylated DNA. If a CpG island is small (i.e. spanning 30 bp), primers can be designed to cover the entire island. Although this gives information on the methylation status of multiple CpG sites, there are potential problems. If a DNA strand is only partially methylated (i.e. only some CpG sites are methylated) this approach could present problems. Partial methylation and also methylation of only one DNA strand are major drawbacks of MSP. For this reason BSP is often the method of choice, although it is more time-consuming. Some prefer to use MSP but try to design primers that incorporate only one CpG site to avoid issues with partial methylation. If the primers contain only one CpG site, this should be located at the very 3′ end so that discrimination between the methylated and unmethylated DNA is maximal. For example:

Methylated primer	AAT GTT GGA GTA TAG TTT ATG ATT GT**C**
Unmethylated primer	AAT GTT GGA GTA TAG TTT ATG ATT GT**T**

3. Investigation of a heterogeneous population can be performed using MSP. If some cells show methylation and others do not, both methylated and unmethylated primer sets will amplify and a ratio can be determined. This does require full methylation of all CpG sites within the primer set though.
4. It is ideal to run concurrent amplifications with methylated and unmethylated primers and they should therefore be designed to have similar annealing temperatures. However, since the methylated and unmethylated primer sets will have different GC contents, to keep a similar annealing temperature the primers may need to be designed at slightly different positions or be of slightly different lengths. This is true for both the forward and reverse primers. In this case, it is imperative that the methylated and unmethylated primer sets cover all of the same CpG sites, so that the same sites are tested for methylation status. For example:

Methylated primer	GAT **C**GT AGA TTG T**C**G ATG TTG A**C**G TTA
Unmethylated primer	T **T**GT AGA TTG T**T**G ATG TTG A**T**G TTA GGT GA

5. It is important to include multiple non-CpG cytosines in the primer to ensure that only bisulfite converted DNA is amplified. If these are not incorporated, it is possible to amplify DNA that may not have been converted by the sodium bisulfite, which could overestimate the abundance of methylated DNA. For Example:

	No non-CpG sites	Including non-CpG
Unconverted methylated DNA	AAT **C**GT AGG T**C**G	A**C**T **C**GT AG**C** TCG
Unconverted unmethylated DNA	AAT **C**GT AGG T**C**G	A**C**T **C**GT AG**C** TCG
Converted methylated DNA	AAT **C**GT AGG T**C**G	A**T**T **C**GT AG**T** TCG
Converted unmethylated DNA	AAT **T**GT AGG T**T**G	A**T**T **T**GT AG**T** TCG

If there are no non-CpG cytosines the primer designed for the methylated DNA will detect all unconverted DNA as well as the converted methylated DNA and hence indicate that a sample may be methylated when it is not. However, when non-CpG cytosines are included in the primer set, primers will not amplify unconverted DNA.

6. Amplicon size should ideally be between 100 and 500 bp. It is possible to analyze fragments up to about 1 kb, but this may require further optimization. The process of bisulfite conversion is harsh on DNA (50 °C for 16 h) and it therefore becomes degraded. This means that there will be less DNA fragments of an appropriate size to cover the entire amplicon if it is large. Therefore, if possible maintain a smaller amplicon size.
7. A nested PCR can be performed to increase the sensitivity of detection, and is often the method of choice, particularly if DNA is quite degraded after the bisulfite conversion. Nested-MSP involves dual PCR amplification whereby the initial PCR amplifies all methylated and nonmethylated DNA and a second PCR is used to differentiate the two (using the initial PCR as a template). Design of the first primer set is not specific to methylation status and should follow the guidelines of BSP primer design (Subheading 3.2.2). Following this, MSP primer sets should be designed within the amplicon generated in the first PCR that are specific to either methylated or unmethylated DNA.

3.2.2 Bisulfite Sequencing PCR Primer Considerations

Bisulfite sequencing PCR is based on the indiscriminant amplification of a section of methylated or unmethylated DNA, which contains CpG sites within the amplicon but not the primer sequence. It requires only one set of primers to amplify both methylated and unmethylated DNA, which can then be distinguished by subsequent sequencing methods. This method is ideal for the detection of methylation at multiple CpG sites, even if only partial methylation is present in a CpG island.

1. It is important that the primers contain no CpG sites. As bisulfite modification of CpG sites will result in differential nucleotides for methylated and unmethylated DNA, incorporation of a CpG site in the primer will bias amplification of the DNA. If it is unavoidable to include a CpG within the primer sequence, then the primer sequence should be degenerate and have a "Y" at the cytosine site (for detection of C or T), this site should preferably be at the 5′ end. This will allow for amplification of both methylated and unmethylated DNA indiscriminantly. For example:

Methylated DNA	A**Cm**G TCC ATG CAT TGA
Unmethylated DNA	A**C**G TCC ATG CAT TGA
Primer sequence	A**Y**G TTT ATG TAT TGA

2. Primers must be specific. Once primers are designed they should be tested for alignment to a bisulfite converted genome that could be methylated or unmethylated (methBLAST). *See* **step 1** in Subheading 3.2.1 for more information.

3. There should be multiple non-CpG cytosines within the primer sequence to ensure that only bisulfite converted DNA is amplified. If unconverted DNA is also amplified, this will result in a heterogeneous amplicon. If bisulfite converted and nonconverted DNA are sequenced together, all unmethylated cytosine residues will display a thymine and a cytosine. Depending on the ratio of converted/nonconverted DNA this may appear as a heterozygote or as a homozygote, whichever the case the sequence may be difficult to read and analyze. If this occurs at a CpG site, the results may be difficult to interpret.
4. It is best to try to include the entire CpG island within the amplicon as this will be most informative. However, as with MSP, amplicon size is important. Therefore, primers should be designed so that the majority of the CpG island (at least 2/3) is covered. Amplicons up to about 1 kb can be analyzed, sometimes this will require a second round of amplification using the same primer set and 1–2 μl of the first round PCR product as template. Islands which are larger than this may require multiple primers to be designed. Designing primers within a CpG island can be difficult as there may not be a long run of nucleotides without a CpG site. In this case, degenerate primers may need to be used (*see* **step 1**).

3.2.3 Primer Design Software

Both MSP and BSP have advantages and disadvantages associated with their use. Choosing one of these methods depends strongly upon the type of experiment being performed. It may even be necessary or preferable to try multiple methods for detection of methylation status. Whichever method is chosen by an investigator, good primer design is crucial. The previous two sections have described some of the major considerations and this information may be sufficient for manual primer design. However, various programs exist that design matched methylated and unmethylated primer sets for MSP or primers for BSP. Some of these are listed in this section. Each of these websites has tutorials or help options, in particular MethPrimer (recommended for MSP) and PerlPrimer (recommended for BSP) are very user friendly.

1. MethPrimer: http://www.urogene.org/methprimer/ [23]. Designs primers for MSP and BSP. Access is also available to databases containing information on PCR primers for popular DNA methylation analyses, methylated cytosine occurrence, genes affected by promoter CpG island methylation and software tools for handling generated data. A moderated discussion forum is also available through this website.
2. PerlPrimer: http://perlprimer.sourceforge.net/ [24]. Designs primers for BSP and allows the user to input PCR conditions to give an accurate prediction of primer melting temperature

for maximum yield of PCR product. This application is also suitable for standard PCR and QPCR and sequencing.

3. BiSearch: http://bisearch.enzim.hu/ [25]. Designs primers for MSP and BSP as well as standard PCR. Also allows the user to search various genomes for primer specificity.
4. Methyl Primer Express Software v1.0: Available from Life Technologies for purchase (http://www.lifetechnologies.com—search products for Methyl Primer Express). Designs primers for MSP and BSP.

3.3 Amplification of Bisulfite-Converted DNA

Methylation-specific PCR is performed to directly determine methylation status of one or more CpG sites. It requires amplification of a bisulfite-converted DNA sample using two sets of primers—one specific for DNA methylated at the CpG site/s (referred to as "M") and one specific for DNA unmethylated at the CpG site/s (referred to as "U"). When preparing the PCR reactions, ensure that you include two positive controls (if possible include a sample that you know is methylated and one that is not for the gene/CpG island of interest), a no template negative control (water) and an unconverted DNA negative control (this will show certain specificity problems with your primer). Bisulfite sequencing PCR typically amplifies a region flanking a CpG island and therefore one set of primers are used. If the island is particularly large, more than one set of primers may be used. In either case, the methylation status of the CpG sites is detected with additional methods (sequencing; cloning and sequencing).

As with all PCR, optimization is required for both MSP (methylated and unmethylated primer sets) and BSP for each gene of interest. Typically, optimization may include performing a magnesium titration, a primer titration (forward and reverse primer concentrations could be adjusted and do not need to be equal), adjusting the annealing temperature and/or altering the *Taq* polymerase and buffer used. The following protocol is a modification of that provided by Herman and colleagues [26]. Note that this is only one example, PCR conditions will be dependent on your primers selected. If your gene/CpG island of interest has been amplified before, you may find optimized protocols within journals or in databases such as that provided on the MethPrimer website (*see* Subheading 3.2.3, **step 1**). Also, if a nested PCR approach is required, two separate PCR amplifications will need to be performed and both reactions will need to be optimized. Each PCR reaction should follow standard PCR guidelines for optimization.

1. Prepare two separate bulk mixes ensuring that you have sufficient bulk mix for all samples and controls. The following table gives an example of a bulk mix prepared for six samples and four controls. Remember, for MSP this has to be made for both a methylated primer set and an unmethylated primer set.

Note that at this point, no *Taq* polymerase is included (*see* **Note 5**), due to the need for a hot start reaction and the final volume is 39 µl (although concentrations are based on a 50 µl reaction volume).

Reagent	Final concentration	Methylated/unmethylated bulk mix	
		1×	10.5×
10× Bisulfite PCR buffer	1×	5 µl	52.5 µl
25 mM dNTPs	1.25 mM	2.5 µl	26.25 µl
10 µM Forward primer (either U, M, or BSP)	800 nM	4.0 µl	42.0 µl
10 µM Reverse primer (either U, M, or BSP)	800 nM	4.0 µl	42.0 µl
DNase/RNase-free water		23.5 µl	246.75 µl
Total		39.0 µl	409.5 µl

2. Add 39 µl of bulk mix to 1 µl of sample (or control) and denature the sample by heating to 95 °C for 5 min (*see* **Note 5**).
3. While samples are being denatured, make up a *Taq* polymerase bulk mix containing 1.25U *Taq* in 10 µl water per sample. (*See* **Note 5**).
4. After 5 min at 95 °C add 10 µl of water containing 1.25U *Taq* while keeping the temperature at 95 °C.
5. Perform the amplification using the following thermocycling conditions: 35 cycles of denaturation at 95 °C for 30 s, annealing at 30 s (usually 50–60 °C, depending on primer T_m), extension at 72 °C for 30 s; followed by a final extension at 72 °C for 5 min. For larger amplicons (such as 1 kb), extension can occur for 1 min and if amplification is not optimal, annealing time can also be extended to 1 min.

3.4 Methods of Detection

3.4.1 Methylation-Specific PCR

Because MSP uses two sets of primers that amplify DNA with CpG sites that are either methylated or unmethylated, the PCR products can be directly visualized on a gel. Many published protocols use polyacrylamide-based gels but often agarose gels give results that are of equal quality or better. If the size of the amplicon is less than 80 bp, polyacrylamide may be the best method, otherwise agarose is recommended as it is cheaper and easier to handle. As such, this is the method I have included in this chapter; the following protocol is sufficient for detection of methylated and unmethylated primer sets for six samples, two positive controls and two negative controls. If more samples are run a larger gel will need to be made, in this case adjust weights/volumes accordingly. If a polyacrylamide

gel is required or preferred, it should be a 6–8 % nondenaturing gel and run at 10 V for 1 or more hours.

1. Add 70 ml 1× TAE to 1.4 g of agarose (2 % gel) in a conical flask and heat in microwave until agarose is well dissolved (about 2 min, ensure it does not boil over).
2. When cooled, add 2.5 μl 10 mg/ml ethidium bromide (0.5 μg/ml final, wear gloves), mix gently.
3. Pour agarose solution into gel tray and insert comb, allow gel to set before loading samples.
4. While gel is setting, add 3 μl of 100 % glycerol solution to each sample (total volume 53 μl) or 10 μl 6× loading dye (total volume 60 μl).
5. Remove comb from gel when set: load 5 μl 100 bp molecular weight marker (with loading dye) in the first lane; load all samples (containing glycerol) for methylated and unmethylated primer sets, positive and negative controls (6× loading dye can be used in place of glycerol if the dye size is not similar to that of your expected band).
6. Place gel into electrophoresis tank and run the gel at 80–90 V for about 30 min or until you can clearly see your bands of interest using the UV light box.
7. Visualize PCR fragments with a UV light box and capture an image:
   - Methylated positive control: product should only be present with the M primer set.
   - Unmethylated positive control: product should only be present with the U primer set.
   - No template and nonconverted negative controls: no bands should be present.
   - A band present in the M primer set indicates the sample is methylated.
   - A band present in the U primer set indicates the sample is unmethylated.
   - A band present in both primer sets indicated a heterogeneous population (provided there is no contamination and complete conversion, as shown by the negative controls).
   - No bands present in the sample likely indicates mixed CpG methylation within the primer (BSP could be more suitable), or bad sample preparation.

3.4.2 Bisulfite Sequencing PCR

Samples that have undergone bisulfite treatment and amplification using primers that are not specific for methylation status can be sequenced. The sequencing results will indicate methylation status

of each CpG dinucleotide in the amplicon. In most cases direct sequencing is sufficient, however if problems occur (eg. difficult primer optimization for sequencing, heterogenous population) it is possible to clone the amplified product and then sequence DNA from 8 to 10 clones. As direct sequencing is easier, cheaper, and less time-consuming, it is the preferred method for analysis and is the protocol given here. As with all sequencing, a forward and a reverse reaction should be carried out independently for each sample, usually using the same primers that were designed for your PCR. An alternative methodology is to perform pyrosequencing utilizing a PyroMark system (Qiagen), which gives more accurate quantification but is also more costly.

1. Electrophorese the samples on a 1–2 % agarose gel (*see* Subheading 3.4.1). Ensure that your negative controls are free from contamination.
2. Using a scalpel or razor, cut the band of interest out of the gel (for each sample, including positive controls) making sure to cut out as little agarose as possible. There should only be a small margin of agarose surrounding the band.
3. Purify the agarose gel using a DNA purification column (available from various companies), according to the manufacturer's instructions (*see* **Note 6**). Typical elution volumes should be 20–30 μl in DNase/RNase-free water. Run 5 μl of sample on an agarose gel to ensure DNA is still present.
4. *Optional*: If a heterogenous population is present it is possible to clone your DNA, and then sequence 8–10 individual clones.
5. Prepare a bulk mix for your sequencing reaction (prepare one using the forward primer and one using the reverse primer), this should be sufficient for each of your samples and the two positive controls:

Reagent	1× Forward	1× Reverse	8.5×
5× Running buffer	2 μl	2 μl	17 μl
BigDye® terminator ready reaction Mix v3.1	4.0 μl	4.0 μl	34 μl
10 μM Forward primer 10 μM Reverse primer	0.32 μl	 0.32 μl	2.72 μl
Total	6.32 μl	6.32 μl	53.72 μl

6. Add 6.32 μl of your bulk mix to 3.68 μl of your purified DNA (*see* **Note 7**).
7. Run the sequencing reaction at 96 °C for 1 min; followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min, if necessary the products can be stored at 4 °C or −20 °C.

8. Add 2.5 µl 125 mM EDTA to each tube and then add 25 µl 100 % ethanol and invert several times to mix. Leave at room temperature in the dark for 15 min to precipitate the DNA.
9. Centrifuge at 1,600–2,000 × *g* for 45 min at 4 °C.
10. Remove supernatant by decanting onto a paper towel (tap tubes upside down on paper towel to remove residual ethanol).
11. Add 30 µl 70 % ethanol to each tube and centrifuge at 1,600–2,000 × *g* for 15 min at 4 °C.
12. Remove supernatant by decanting (as in **step 10**) and dry the tubes. Tubes can be air-dried for 15 min to 1 h or dried in a speed vacuum for 5 min without heat. It is important to protect the pellet from light during this drying stage. At this point products can be stored at −20 °C in the dark if sequencing is to be done at a later date.
13. Add 5 µl Hi-Di™ formamide to the samples and proceed immediately with capillary electrophoresis using the Life Technologies (formerly Applied Biosystems) 3730 genetic analyzer (or similar instrument suitable for sequencing). The 3730 instrument requires samples to be in a 96-well or 384-well plate. Ensure that fresh (every 2–3 days) 1× running buffer and DNase/RNase free water are in the appropriate trays when using the genetic analyzer.
14. Review your sequencing results using SeqScape software (v2.5) or Sequence Scanner Software (v1.0; free software, limited capabilities). You can differentiate methylated cytosines (C) from unmethylated cytosines (T), as shown in Fig. 2.

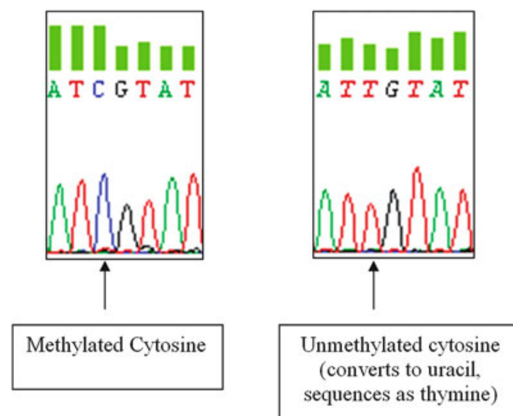

Fig. 2 Methylated versus unmethylated cytosine sequence

4 Notes

1. If you have less than 1 μg DNA you can add 1 μg salmon sperm DNA as a carrier. Although more than 1–2 μg DNA could be used (some protocols suggest 4–10 μg), incomplete bisulfite conversion (due to a higher concentration of DNA) is possible and could lead to false positive results. If this is a concern, it is recommended to limit the amount of DNA to 1 μg and perform replicate conversions if more DNA is required. DNA needs to be fully denatured in NaOH but to prevent degradation limit incubation time to no more than 30 min.
2. Avoid incubating samples for longer than 16 h as methylated cytosines will undergo deamination to uracil, leading to false negative results. Many bisulfite conversion kits use lower incubation times.
3. Although incubation at −20 °C is recommended, several studies have shown that this is unnecessary and precipitation at room temperature should be sufficient, particularly if using ammonium acetate.
4. The storage limitations of bisulfite converted DNA vary widely from lab to lab, from as little as a few days to a few years. The fresher bisulfite converted DNA is, the more likely it will work in downstream applications. Storage at −80 °C, rather than at −20 °C will reduce degradation.
5. *Taq* polymerase can be purchased from a number of companies and most will be successful in amplification. This PCR requires a hot start, if using a *Taq* polymerase such as Platinum *Taq* from Life Technologies then this protocol can be followed. However various *Taq* polymerases such as HotStar *Taq* (Qiagen), ZymoTaq (Zymo), and JumpStart *Taq* (Sigma-Aldrich) are designed to be active only when a particular reaction temperature (eg. 70 °C) is reached. If using a polymerase such as this, a bulk mix can be made up including the *Taq* polymerase and water up to 49 μl; **steps 3** and **4** in this protocol can then be omitted (5 min at 95 °C is still required). If there is difficulty in amplification, JumpStart (or JumpStart Red) *Taq* polymerase is recommended. Note that each of the polymerases will come with a 10× reaction buffer. This is likely to be more suitable for the PCR reaction than the one provided in this protocol and will also require optimization. I would recommend first trying the PCR with the provided buffer using a magnesium titration (1–4 mM) and annealing temperature optimization, the amount of primer (200nM final) and dNTPs (200 μM) could also be lowered in this case.
6. Some gel extraction/purification kits may require a specific grade of agarose such as low-melt agarose and often work best

when low percentage gel is used (e.g. 1 %). Also, some kits may only be suitable for fragments larger than 100 bp (e.g. Life Technologies), while others are suitable for smaller fragments (70 bp for Qiagen). It is important to check the details of a kit before choosing one, including reagents and equipment needed.

7. For sequencing reactions, as little as 0.5 μl of BigDye can be used in a 10 μl reaction. Optimization of this protocol for each gene can reduce the amount of BigDye used and increase the amount of DNA used in the sequencing reaction if DNA quantity is low. If using smaller volumes of BigDye more 5× sequencing buffer may be required. This can be made in the laboratory with 2 ml 2 M Tris (pH 9.0), 100 μl 1 M $MgCl_2$, 7.9 ml DNase/RNase-free water (final concentration 400 mM Tris, 10 mM $MgCl_2$).

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Chapter 12

In Vitro and In Vivo Assays for Studying Histone Ubiquitination and Deubiquitination

Heui-Yun Joo, Qian Dai, Amanda E. Jones, Ling Zhai, and Hengbin Wang

Abstract

Posttranslational histone modifications play important roles in regulating chromatin structure and function (Rando, Curr Opin Genet Dev 22:148–155, 2012; Zentner and Henikoff, Nat Struct Mol Biol 20:259–266, 2013). One example of such modifications is histone ubiquitination, which occurs predominately on H2A and H2B. Recent studies have highlighted important regulatory roles of H2A ubiquitination in Polycomb group protein-mediated gene silencing and DNA damage repair (de Napoles et al., Dev Cell 7:663–676, 2004; Wang et al., Nature 431:873–878, 2004; Doil et al., Cell 136:435–446, 2009; Gatti et al., Cell Cycle 11:2538–2544, 2012; Mattiroli et al., Cell 150:1182–1195, 2012; Stewart et al., Cell 136:420–434, 2009; Bergink et al., Genes Dev 20:1343–1352, 2006; Facchino et al., J Neurosci 30:10096–10111, 2010; Ginjala et al., Mol Cell Biol 31:1972–1982, 2011; Ismail et al., J Cell Biol 191:45–60, 2010), H2B ubiquitination in transcription initiation and elongation (Xiao et al., Mol Cell Biol 25:637–651, 2005; Kao et al., Genes Dev 18:184–195, 2004; Pavri et al., Cell 125:703–717, 2006; Kim et al., Cell 137:459–471, 2009), pre-mRNA splicing (Jung et al. Genome Res 22:1026–1035, 2012; Shieh et al., BMC Genomics 12:627, 2011; Zhang et al., Genes Dev 27:1581–1595, 2013), nucleosome stabilities (Fleming et al., Mol Cell 31:57–66, 2008; Chandrasekharan et al., Proc Natl Acad Sci U S A 106:16686–16691, 2009), H3 methylation (Sun and Allis, Nature 418:104–108, 2002; Briggs et al., Nature 418:498, 2002; Dover et al., J Biol Chem 277:28368–28371, 2002; Ng et al., J Biol Chem 277:34655–34657, 2002), and DNA methylation (Sridhar et al., Nature 447:735–738, 2007). Here we describe methods for in vitro histone ubiquitination and deubiquitination assays. We also describe approaches to investigate the in vivo function of putative histone ubiquitin ligase(s) and deubiquitinase(s). These experimental procedures are largely based on our studies in mammalian cells. These methods should provide useful tools for studying this bulky histone modification.

Key words Chromatin, Histone ubiquitination, Histone deubiquitination, In vitro assay, In vivo assay

1 Introduction

The organization and function of chromatin, the physiological template for all nuclear processes involving genetic information, are largely dictated by posttranslational histone modifications [1, 2]. These modifications, which include acetylation, methylation,

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phosphorylation, ubiquitination, and sumoylation, are believed to mark an individual nucleosome or a short stretch of nucleosome arrays, with a specific "code," which is recognized by downstream regulatory proteins and elicits specific cellular responses [1, 2]. The extensive efforts recently made to identify and characterize the enzymes involved in histone acetylation, methylation, and phosphorylation, together with the development of antibodies which can specifically recognize acetylated, methylated, and phosphorylated histones, have brought significant insight into the subcellular distribution, dynamics of global and locus-specific levels, and biological functions of these modifications [1, 2]. Compared with other histone modifications, ubiquitination is unique in that the bulky ubiquitin protein is two-thirds the mass of an individual histone [3, 4]. Initial attempts to generate antibodies that recognize specific ubiquitinated histones prove to be difficult and only an anti-ubiquitinated H2A (uH2A) antibody has been described [5]. Therefore, a combination of genetic and immunoblot approaches have been developed for studying histone ubiquitination both in vitro and in vivo [6–8]. However, an antibody that specifically recognizes ubiquitinated H2B (uH2B) has recently been generated [9]. These antibodies, together with the application of high-throughput sequencing technologies and the identification of histone ubiquitin ligases and deubiquitinases, have revealed multiple functions of histone ubiquitination in transcription regulation, pre-RNA processing, DNA damage repair, nucleosomes stability, cell cycle progression, recombination, etc. [6, 10–32].

Of the four core histones, H2A and H2B are ubiquitinated in a wide range of organisms and cell types [3, 4]. Ubiquitination of H2B is highly conserved and has been reported in budding yeast, *Arabidopsis*, *Drosophila*, and mammals [3, 4]. The abundance of uH2B varies from organism to organism, which can account for up to 10 % of total cellular H2B [33]. Rad6, a multifunctional ubiquitin conjugating E2 enzyme, was identified as the E2 enzyme for histone H2B and the specific activity of Rad6 for histone H2B is conferred by a Ring-finger containing protein Bre1 [33–35]. Research interest in H2B ubiquitination was boosted by the finding that H2B ubiquitination regulates K4 and K79 methylation of histone H3 in a unidirectional, trans-histone fashion [28–31]. This trans-histone regulation appears to be conserved and was recently recapitulated using in vitro reconstituted nucleosome templates, suggesting that H2B ubiquitinated nucleosomes have intrinsic features that facilitate downstream histone methylation [22, 36–38]. Interestingly, H2B deubiquitination in *Arabidopsis thaliana* was found to regulate DNA methylation and heterochromatin gene silencing [32]. Unlike H2B, ubiquitination of H2A has not been detected in budding yeast or *C. elegans*, although H2A ubiquitination might exist in *Arabidopsis* [10, 32, 33, 39]. The ubiquitin ligase for histone H2A was identified as the Polycomb group

protein Ring2, indicating that H2A ubiquitination may be an epigenetic mechanism that is acquired later during evolution [6, 10]. In addition to H2A and H2B, histones H3, H4, H2A.Z, and H1 have also been reported as substrates for ubiquitin modification [4]. Recent research has given significant insight into the function of these histone ubiquitinations. Cul4/DDB/ROC1 ubiquitinates both histone H3 and H4 and ubiquitination of these histones destabilizes nucleosomes and facilitates downstream DNA damage repair processes [7]. Consistent with the role in destabilizing nucleosomes, Cul4-associated ubiquitin ligase ubiquitinates histone H3 that has already been acetylated at K56 and this ubiquitination destabilizes the association between Asf1 and H3-H4 tetramer, enabling the transfer of H3-H4 tetramer to downstream histone chaperones during nucleosome assembly [40]. H3 is also ubiquitinated by Uhrf1 at K23 and this ubiquitination is involved in coupling DNA methylation to replication [41]. H4K31 ubiquitination mediated by Cul4-associated ubiquitin ligase is proposed to cooperate with linker histone H1.2 during transcription activation [42]. The enzyme responsible for H1 ubiquitination was identified as TAF_{II} 250 and ubiquitination of histone H1 has been linked to gene repression in the Dorsal pathway in *Drosophila* [43].

Similar to ubiquitination of other proteins, histone ubiquitination occurs through a series of enzymatic reactions (Fig. 1). First, ubiquitin is activated by the E1 ubiquitin activating enzyme

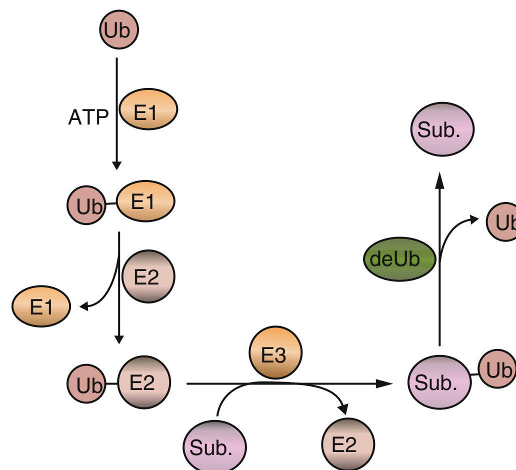

Fig. 1 Ubiquitination and deubiquitination reactions**.** For protein ubiquitination, ubiquitin is first activated by E1 ubiquitin activating enzyme in an ATP-dependent formation of ubiquitin-E1 thiol ester. Ubiquitin is then transferred to E2 ubiquitin conjugating enzymes, again through the formation of adenylate intermediate. Finally, the ubiquitin E3 ligase brings together the E2-ubiquitin complex and the substrates and mediates the isopeptide bond formation. Ubiquitin conjugated to target proteins can be cleaved by specific deubiquitinases and results in the release of intact ubiquitin

through an ATP-dependent formation of ubiquitin-E1 thiol ester via an ubiquitin adenylate intermediate. Second, E1 transfers activated ubiquitin to E2 ubiquitin conjugating enzymes through the formation of a new ubiquitin adenylate intermediate. Finally, the ubiquitin E3 ligase conjugates ubiquitin to the ε-N of lysine residues in the target protein through an isopeptide bond. Since the substrate specificity is primarily determined by the ubiquitin E3 ligase, a great deal of effort has been made to identify the ubiquitin ligases for different substrates [44, 45]. Like other histone modifications, histone ubiquitination is a reversible process [46, 47]. Ubiquitin conjugated to target proteins can be cleaved by thiol-specific proteases known as deubiquitinating enzymes or deubiquitinases [46, 47]. Recent studies have identified multiple deubiquitinases that target to specific histones and interestingly, characterization of these histone deubiqutinases has revealed additional functions of histone ubiquitination in multiple processes [25, 32, 48–53]. In the following sections, we describe experimental procedures for in vitro histone ubiquitination and deubiquitination assays. We also describe experimental procedures for studying the in vivo function of putative histone ubiquitin ligases and deubiquitinases. These procedures are mainly based on our studies in mammalian cells. Detailed experimental protocols for studying histone ubiquitination in budding yeast can be found in recent reviews [8, 54].

2 Materials

2.1 *In Vitro Histone Ubiquitination Assay*

1. Substrates. Histone octamers, mononucleosomes, and oligonucleosomes prepared from HeLa S3 cells [55]. All materials should be dialyzed against histone storage buffer [10 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 10 mM KCl, 10 % Glycerol, 0.2 mM PMSF] and stored at −80 °C (*see* **Note 1**).
2. Reaction buffer. Prepare reaction buffer from stock solutions immediately before use. 1 M Tris–HCl (pH 7.5), 1 M $MgCl_2$, 100 mM NaF, 100 mM ATP, 10 μM Okadaic acid, 0.05 M DTT.
3. Other reagents. E1 ubiquitin activating enzyme (Calbiochem), E2 ubiquitin activating enzyme (ubc5c), Flag-ubiquitin (Sigma), anti-Flag M2 antibody (Sigma), HeLa nuclear fractions or putative histone ubiquitin ligases.
4. Equipment. SDS-PAGE apparatus, semi-dry transfer unit, and power supply.

2.2 *In Vitro Histone Deubiquitination Assay*

1. Substrate preparation.

   Cells: HeLa cells, yeast T85 strain [51], and yeast T85 strain expressing human Flag-H2B [25, 52].

Constructs: pcDNA3-Flag-H2A (neomycin resistant), pcDNA3-Flag-H2B (neomycin resistant), and pcDNA3-HA-ubiquitin (hygromycin resistant).

Buffers: Buffer A: 0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 10 mM MES-KOH (pH 6.5), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 % Triton X-100, 1 mM DTT, 0.1 mM PMSF.

Buffer B: 10 mM Tris–HCl (pH 8.0), 100 mM NaCl, 5 mM $CaCl_2$, 2 mM $MgCl_2$, 0.3 M sucrose, 1 mM DTT, 0.1 mM PMSF.

Nucleosome extraction buffer: 20 mM Tris–HCl (pH 7.9), 10 mM EDTA, 0.5 M KCl, 1 mM DTT, 0.1 mM PMSF.

Denaturing buffer: 20 mM Tris–HCl (pH 7.4), 50 mM NaCl, 0.5 % Nonidet P-40 (NP-40), 0.5 % sodium deoxycholate, 0.5 % SDS, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF.

Elution buffer: 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1 mM PMSF.

TAE buffer: 40 mM Tris-acetate, 1 mM EDTA.

Buffer P: 5 mM Hepes-KOH (pH 7.5), 40 mM KCl, 10 mM potassium phosphate (pH 7.5) 0.01 mM $CaCl_2$, 10 % glycerol, 1 mM DTT, 0.1 mM PMSF.

Histone unfolding buffer: Freshly prepared. 6 M guanidinium chloride, 20 mM Tris–HCl (pH 7.5), 5 mM DTT.

Histone refolding buffer: 2 M NaCl, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 5 mM 2-ME.

2× HC Buffer: 300 mM HEPES-KOH pH 7.6, 2 mM EDTA, 100 mM KCl, 20 % glycerol, 2 mM DTT, protease inhibitors.

1× HC Buffer: 150 mM HEPES-KOH pH 7.6, 1 mM EDTA, 50 mM or 250 mM KCl, 10 % glycerol, 1 mM DTT, protease inhibitors.

2 M and 5 M NaCl; 10 mM Tris–HCl (pH 7.5); 50 % Sucrose; 5 % Native PAGE.

Other reagents: recombinant histones [56], neomycin, hygromycin, micrococcal nuclease [200 U/ml, 40 % protein, balance primarily sodium acetate, Sigma, prepared in 5 mM NaH_2PO_4 (pH 7.0), 2.5 μM $CaCl_2$, *see* **Note 2**], Anti-Flag M2 affinity gel (*Sigma*), Flag peptide (custom synthesized), anti-Flag M2 antibody (*Sigma*), anti-HA antibody, HA peptide (custom synthesized), YPD medium, Trp minus medium, Effectene (*Qiagen*).

2. 5× Reaction buffer. 500 mM Tris–HCl (pH 8.0), 5 mM EDTA, 0.5 mM PMSF, 5 mM DTT, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A.

3. Equipment. HPLC (*GE Healthcare Life Sciences*), 1 ml Hydroxyapatite column (*Bio-Rad*), Seperdex-200 gel filtration column (*GE Healthcare Life Sciences*), SDS-PAGE apparatus, semi-dry transfer unit, power supply, and benchtop microcentrifuge.

2.3 In Vivo Histone Ubiquitination and Deubiquitination Assay

1. HeLa cells or other mammalian cell lines.
2. Constructs. pcDNA3-Flag-H2A (neomycin resistant), pcDNA3-Flag-H2B (neomycin resistant), and pcDNA3-HA-ubiquitin (hygromycin resistant).
3. Other reagents. neomycin, hygromycin, anti-Flag M2 affinity gel (*Sigma*), Flag peptide (custom synthesized), anti-HA antibody, Effectene (*Qiagen*), Lipofectamine 2000 (*Invitrogen*), denaturing buffer (*see* Subheading 2.2, **item 1**), anti-uH2B antibody (Millipore, 05-1312), anti-uH2A antibody (Millipore, 05-678).
4. Equipment. SDS-PAGE apparatus, semi-dry transfer unit, and power supply.

3 Methods

3.1 In Vitro Histone Ubiquitination Assay

1. Mix histone octamers (5 μg), mononucleosomes (5 μg), and oligonucleosomes (5 μg) with protein fractions or a putative histone ubiquitin ligase in a 32.5 μl reaction system containing 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 2 mM NaF, 2 mM ATP, 10 μM Okadaic acid, 0.6 mM DTT, 0.1 μg E1, 0.2 μg E2 (ubc5c), 1 μg Flag-ubiquitin (Sigma).
2. Incubate the reaction mixture at 37 °C for 1 h. Stop the reaction by mixing with SDS sample loading buffer.
3. Resolve the reaction product in a 15 % or 8–15 % SDS-PAGE and transfer proteins to a nitrocellulose membrane.
4. Perform a standard Western blotting assay with anti-Flag antibody. Ubiquitinated histones migrate around the size of individual histones plus Flag-ubiquitin (8 kDa, Fig. 2a).
5. Negative control assays: omit E1, E2, substrate, E3 (protein fraction or the putative H2A ubiquitin ligase) individually from the ubiquitin ligase reaction and perform parallel experiments. The signal for ubiquitinated histones should be abolished (Fig. 2b).

3.2 In Vitro Histone Deubiquitination Assay

3.2.1 Purification of Native uH2A-Containing Mononucleosomes

1. Linearize pcDNA3-Flag-H2A and pcDNA3-HA-ubiquitin with *Mfe1* and recover the linearized plasmid with the Wizard SV Gel and PCR Clean-up System (*Promega*). Cotransfect these two plasmids into HeLa cells with Effectene (*Qiagen*) following the manufacturer's instruction. 48 h after transfection, split the cells (1:3) and add neomycin and hygromycin to a final concentration of 0.5 mg/ml and 0.3 mg/ml, respectively. Continue culture until individual clones form. Test the expression of Flag-H2A and HA-ubiquitin in each clone by Western blot assay with anti-Flag and HA antibodies. Stable cells with high expression of both epitope tagged proteins will be used for the following experiment (*see* **Note 3**).

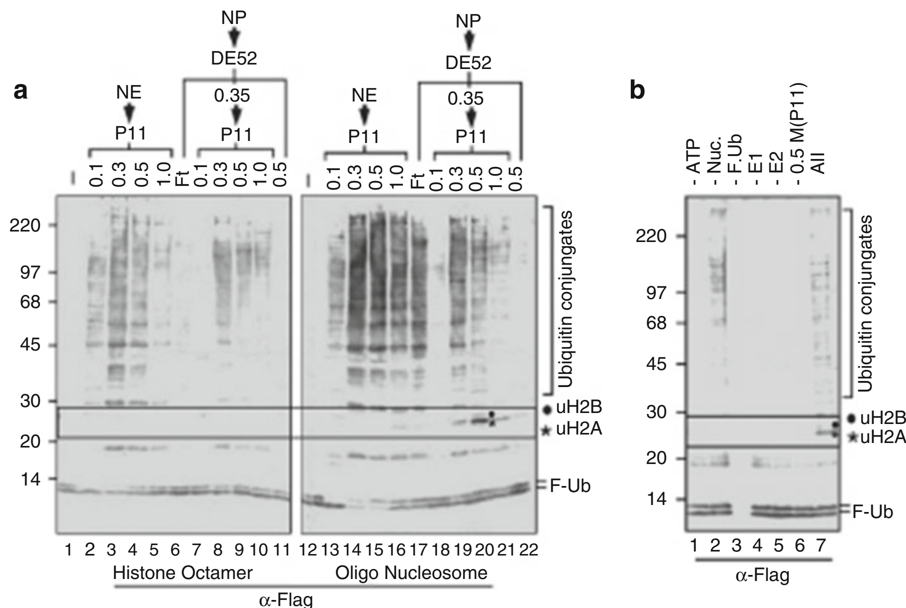

Fig. 2 In vitro histone ubiquitination assay. (**a**) Ubiquitin ligase assay using HeLa nuclear proteins fractionated on DE52 and P11 columns. Numbers on *top* of the panels indicate the salt concentration (M) for step elution. NE and NP represent nuclear extracts and nuclear pellet, respectively. *Left* and *right panels* use histone octamer and oligonucleosome substrates, respectively. (**b**) The ligase activity depends on the presence of ATP, E1, E2, ubiquitin, nucleosomal histones, and proteins present in the 0.5 M P11 nuclear pellet fraction. Reproduced with permission from Wang et al. [6]

2. Collect stable cells from two to three 15 cm plates (yield 7–10 mg of mononucleosomes) and wash twice with cold PBS. Resuspend cells in 1 ml buffer A and incubate on ice for 15 min. Collect nuclei by centrifugation at 1,200 × *g* for 10 min (benchtop microcentrifuge) and wash one more time with buffer A.

3. Wash nuclei with buffer B once and resuspend the nuclei in 0.4 ml buffer B. Add 2 μl micrococcal nuclease (200 U/ml) and incubate at 37 °C for 30 min (*see* **Note 4**). Mix well every 10 min. After digestion, wash nuclei once with buffer B.

4. Resuspend digested nuclei in 0.4 ml nucleosome extraction buffer and incubate on ice for 15 min. Centrifuge at 16,100 × *g* for 10 min (benchtop microcentrifuge) and save the supernatant as nucleosomes. Repeat the extraction until no protein comes out.

5. Combine the extracted nucleosomes and measure protein concentration by Bradford assay (*Bio-Rad*). Dilute nucleosomes to a final concentration of 0.25 mg/ml by adding drops of histone storage buffer while vortexing vigorously. To avoid precipitation, 0.05 % NP-40 should be added to histone

storage buffer prior to dilution. After dilution, dialyze samples against histone storage buffer for at least 4 h (*see* **Note 5**).

6. Wash 100 μl anti-Flag M2 affinity gel with histone storage buffer three times and incubate with nucleosome preparation at 4 °C for 2 h. After incubation, wash resin with histone storage buffer three times. Elute proteins bound to the resin with 0.2 mg/ml Flag peptide (in histone storage buffer) at 4 °C for 30 min. Repeat the elution step 2–3 times until no proteins are eluted out. Dialyze the eluted nucleosomes against histone storage buffer at 4 °C for 2 h.
7. Incubate the dialyzed anti-Flag elute nucleosomes with anti-HA antibody that has been cross-linked to protein A at 4 °C for 2 h. Wash resin with histone storage buffer three times. Elute proteins bound to the anti-HA resin with 0.8 mg/ml HA peptide (in histone storage buffer containing 0.5 M NaCl). Dialyze the eluted nucleosomes against histone storage buffer and store at 4 °C. Purified mononucleosomes should be used within 2 weeks.

3.2.2 Reconstitution of Ubiquitinated Histone-Containing Mononucleosomes

1. DNA fragments used for nucleosome reconstitution are amplified in a PCR reaction with the pXP-10 plasmid as template and 5′-Bio-TGTTACTCAGAATGGCAA-3′ and 5′-TGATTACGAATTCGAGCT-3′ as primers (*see* **Note 6**). Resolve the PCR products in a 1 % agarose gel and purify the DNA fragments with the Wizard SV Gel and PCR Clean-up System (*Promega*).
2. Recombinant H3, H4, H2A, and H2B are purified from *E. Coli* by chromatography and stored at −80 °C after lyophilization [56].
3. To purify histones containing uH2A, stable cells expressing Flag-H2A (two to three 15 cm plates, *see* Subheading 3.2.1) are dissolved in 0.5 ml denaturing buffer with the aid of sonication. After centrifugation at 16,100 × *g* (benchtop microcentrifuge) for 10 min, the supernatant is incubated with 100 μl anti-Flag M2 affinity gel at 4 °C for 2 h with rotation. After washing with denaturing buffer and elution buffer three times each, proteins bound to resin are eluted with 0.2 mg/ml Flag peptide (in elution buffer) at room temperature for 20 min. Repeat the elution steps until no proteins are eluted out. Dialyze samples against water overnight and lyophilize the samples. Store at −80 °C.
4. Histones containing uH2B are purified from yeast strain T85 under denaturing conditions and proteins are eluted in elution buffer (*see* **Note 7**). Dialyze samples against water overnight and lyophilize the samples. Store at −80 °C.
5. To further purify uH2A and uH2B, resolve the anti-Flag eluate on a 15 % SDS-PAGE. In order to obtain enough samples,

multiple lanes can be loaded. uH2A and uH2B migrate around 25 kDa while uH2B migrates a little higher than uH2A. Excise gel strips containing uH2A and uH2B, based on their relative positions with protein size markers. As controls, we also excise gel strips containing H2A and H2B. Place gel strips in 15 ml Falcon tubes and wash extensively with water. Transfer gel strips to a dialysis tube (with molecular weight cutoff of 6–8,000 Da) and immerse in elution buffer. Place the dialysis tube in TAE buffer. Apply 60 V to elute proteins from gel slices at 4 °C (*see* **Note 8**). Load sample on a 1 ml hydroxyapatite column and elute proteins with buffer P containing 1 M KCl. Dialyze fractions containing uH2A and uH2B against water at 4 °C for overnight. Lyophilize and store at −80 °C.

6. Histone octamer refolding. Dissolve recombinant histones, uH2A, uH2B, H2A, and H2B into histone unfolding buffer at a concentration of 1 mg/ml and incubate at 4 °C for 1 h. Mix the histones at equal molar ratios as judged by Coomassie blue staining. Dialyze the mixture against histone refolding buffer for 24 h with at least three changes of dialysis buffer. Load the samples onto a Superdex-200 gel filtration column (*GE Healthcare Life Sciences*). Concentrate fractions containing histone octamers (Amicon Ultra, Millipore). Samples can be stored at 4 °C up to 2 months.
7. Nucleosome reconstitution. Dilute DNA with 5 M NaCl to a final concentration of 2 M NaCl. Add appropriate amount of octamers (*see* **Note 9**). Incubate for 30 min at room temperature, then add an equal volume of 10 mM Tris–HCl (pH 7.5), and incubate for 1 h at room temperature. Sequentially dilute the mixture to reduce the concentration of NaCl from 1.0 M → 0.8 M → 0.67 M → 0.2 M → 0.1 M by adding 10 mM Tris–HCl (pH 7.5). Incubate for 1 h at room temperature after each dilution.
8. Resolve a small aliquot of the reconstituted nucleosomes in a 5 % native PAGE. Stain the gel with ethidium bromide and visualize under UV light. Successfully reconstituted mononucleosomes migrate around 700 bp while naked DNA migrates around 200 bp (Fig. 3, *see* **Note 10**).

3.2.3 Purification of Native Ubiquitinated H2B-Containing Mononucleosomes

1. Inoculate 25 ml yeast liquid culture in YPD (T85 strain) or Trp minus medium (T85 expressing human H2B) and grow at 27–30 °C for overnight. Use the 25 ml overnight culture to inoculate 1 liter liquid culture (*see* **Note 11).**
2. Harvest yeast cells and wash cell pellets with 2× HC buffer two times. Aliquot yeast cell suspension into 1.5 ml tubes and collect cell pellet by centrifugation at 16,100 × *g* (benchtop microcentrifuge) for 10 min. Freeze cell pellets at −80 °C until use (*see* **Note 12**).
3. Thaw and resuspend yeast pellet in 800 μl 1× HC buffer containing 250 mM KCl and transfer to a screw cap tube containing

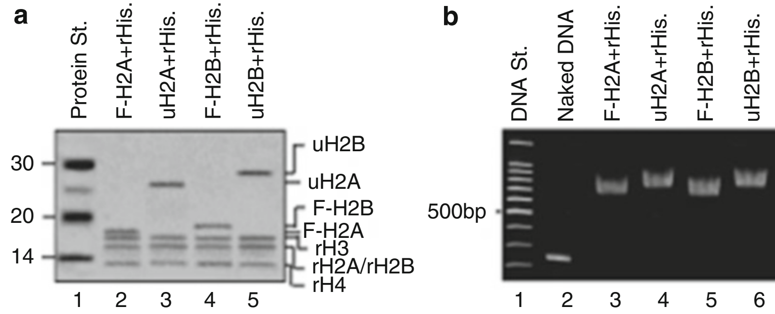

Fig. 3 Nucleosome reconstitution with uH2A and uH2B**.** (**a**) A Coomassie blue-stained gel containing different histone octamers as indicated on the top of the panel. The identity of each protein is indicated on the right side of the panel. (**b**) An ethidium bromide-stained 5 % native polyacrylamide gel containing different reconstituted nucleosomes and naked DNA that was used to reconstitute nucleosomes. Reproduced with permission from Joo et al. [53]

about 200 μl acid washed glass beads. Lyse cells using Fast Prep bead beater or by vortexing rigorously.

4. Transfer the supernatant into 15 ml conical tube and sonicate to produce mononucleosomes (the sonication condition is experimentally determined).
5. Centrifuge supernatant at 16,100 × *g* (benchtop microcentrifuge) for 10 min at 4 °C to collect supernatant. Incubate supernatant with anti-Flag resins equilibrant with 1× HC buffer (250 mM KCl) at 4 °C for 3 h.
6. Wash beads with 1× HC buffer (250 mM) three times. Elute nucleosomes with 0.5 μg/μl FLAG peptide in 1× HC buffer (50 mM KCl) (*see* **Note 13).**
7. Dialyze nucleosomes against histone storage buffer, aliquot, and store at −80 °C. Core histones are obtained from purified nucleosomes by a small scale hydroxyapatite column as described previously [53, 57].
8. Determine the amount of substrates that are needed for in vitro histone deubiquitination assay by performing a standard anti-Flag Western blot assay with different amounts of nucleosomes or core histones.

3.2.4 Histone Deubiquitination Reaction

1. Mix 1.5 μg uH2A-containing mononucleosomes or 0.8 μg of reconstituted uH2A- or uH2B-containing mononucleosomes or the minimum amount of native uH2B-containing mononucleosomes (determined in experiments in Subheading 3.2.3) with protein fractions or putative histone deubiquitinase(s) in 1× deubiquitination reaction buffer. Set up reactions for 5 time points (0, 10, 20, 40, and 60 min).
2. Incubate at 37 °C for the appropriate time. Stop each reaction by mixing with SDS sample loading buffer. Resolve reaction

mixture in a 15 % SDS-PAGE and transfer proteins to a nitrocellulose membrane.

3. Perform a standard Western blot assay with the anti-HA antibody (for uH2A-containing mononucleosomes or reconstituted uH2A- or uH2B-containing mononucleosomes) or anti-Flag (for native uH2B-containing mononucleosomes). The histone deubiquitinase activity is reflected by the decreased levels of ubiquitinated histones and increased release of intact ubiquitin (Fig. 4a, b, *see* **Note 14**).

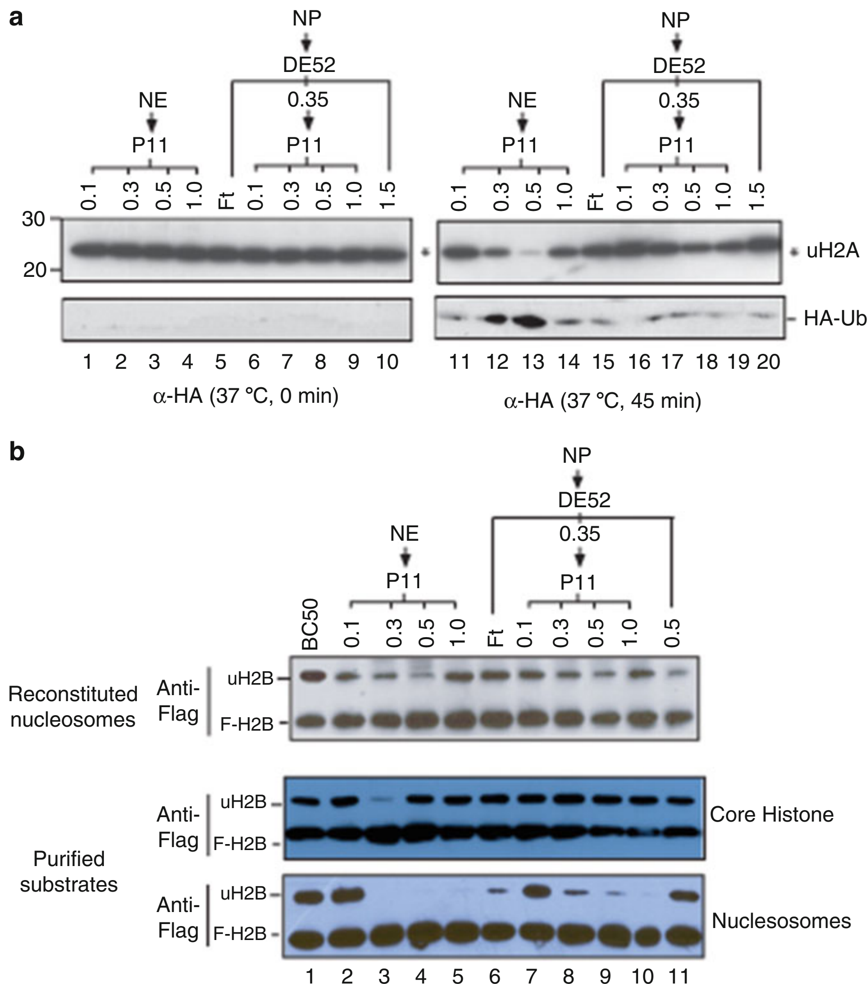

Fig. 4 Histone deubiquitination assay of HeLa nuclear proteins fractionated on DE52 and P11 columns. (**a**) In vitro histone H2A deubiquitination assay. *Left* and *right panels* show Western blot analysis of the reaction mixture after 0 and 45 min incubation at 37 °C. Reproduced with permission from Joo et al. [53]. (**b**) In vitro H2B deubiquitination assay with reconstituted (*top panel*) and native purified uH2B-containing nucleosomes (*bottom panel*) or core histones (*middle panel*) as substrates. Numbers on *top* of the panels indicate the salt concentration (M) for step elution. NE and NP represent nuclear extracts and nuclear pellet, respectively. Reproduced with permission from Zhang et al. [25]

3.3 *In Vivo* Assays for Histone Ubiquitination and Deubiquitination

3.3.1 Transfection and Immunoprecipitation Under Denaturing Conditions

1. Linearize pcDNA3-Flag-H2A and pcDNA3-Flag-H2B with *Mfe1* and recover the linearized plasmid with the Wizard SV Gel and PCR Clean-up System (*Promega*). Transfect these plasmids into HeLa cells with Effectene (*Qiagen*) following the manufacturer's instruction. 48 h after transfection, split cells (1:3) and add neomycin at a final concentration of 0.5 mg/ml. Continue culture until individual clones form. Test the expression of Flag-H2A and Flag-H2B in individual clones by Western blot assay with the anti-Flag antibody. Choose cell lines with high expression of Flag-H2B and low expression of H2A for further experiments.
2. Seed stable cells in a 6 well plate at a density of 1×10^5 cells/well and incubate at 5 % CO_2, 37 °C for overnight.
3. Transfect siRNA against the putative histone ligase or deubiquitinase with Lipofectamine 2000 and incubate for 24 h at 37 °C. Transfect HA-ubiquitin into cells with Effectene and incubate for another 24 h at 37 °C.
4. Harvest cells and wash once with PBS. Dissolve cell pellet in denaturing buffer (add protease inhibitors prior to use) with the aid of sonication. Centrifuge at $16{,}100 \times g$ at 4 °C for 10 min (benchtop microcentrifuge), take one-tenth of the supernatant as input. Incubate the remaining supernatant with anti-Flag M2 affinity gel at 4 °C for 2 h.
5. Wash resin three times with denaturing buffer and elute the bound proteins with 0.2 mg/ml Flag peptide (in denaturing buffer). Resolve the eluate in SDS-PAGE and transfer proteins to a nitrocellulose membrane.
6. After blocking, cut the nitrocellulose membrane into two parts around the size of the molecular weight marker of 20 kDa. Blot the top half of the membrane with anti-HA antibody and the bottom half with anti-Flag antibody. Perform a standard Western blot assay (*see* **Note 15**) (Fig. 5a).

3.3.2 Using the Anti-uH2A and uH2B Antibodies

1. Seed mammalian cells (HeLa or other cell lines) in a 6 well plate at a density of 1×10^5 cells/well and incubate at 5 % CO_2, 37 °C for overnight.
2. Transfect siRNA against the putative histone ligase or deubiquitinase with Lipofectamine 2000 and incubate for 24 h at 37 °C. Transfect siRNA again if the knockdown efficiency is low. Incubate for additional 24 h.
3. Harvest cells and wash once with PBS. Dissolve cell pellet in denaturing buffer (add protease inhibitors prior to use) with the aid of sonication. Centrifuge at $16{,}100 \times g$ (benchtop microcentrifuge) at 4 °C for 10 min.
4. Collect supernatants and resolve equal amount of total proteins in a 6.5–15 % gradient SDS-PAGE followed by standard Western blot procedures.

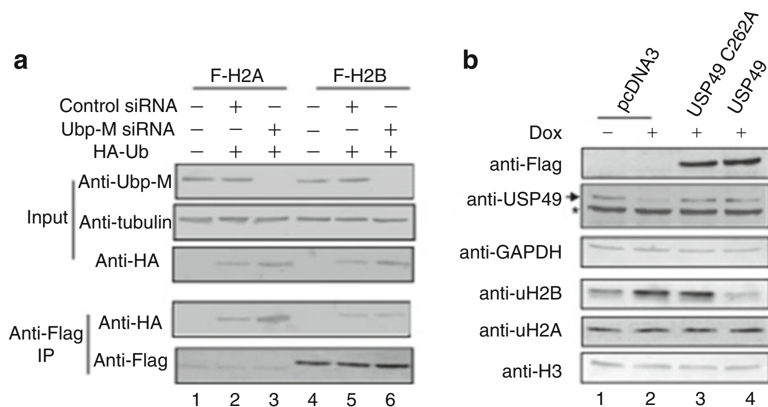

Fig. 5 In vivo histone deubiquitination assay. (**a**) In vivo histone H2A deubiquitination assay. HeLa stable cell lines expressing F-H2A (*lanes 1–3*) and F-H2B (*lanes 4–6*) were transfected with the indicated combinations of siRNA and plasmid. Western blot analysis was performed with total cellular proteins (*top three panels*) and anti-Flag immunoprecipitates under denaturing conditions (*bottom two panels*). Reproduced with permission from Joo et al. [53]. (**b**) In vivo histone H2B deubiquitination assay. Dox-induced USP49 knockdown results in a specific increase of uH2B level and does not affect uH2A levels (*lanes 1 and 2*). Expression of wild type but not C262A mutant USP49 restores histone H2B ubiquitination levels induced by USP49 knockdown (*lanes 3* and *4*). USP49 was marked with *arrow head* and nonspecific bands were marked with *asterisks*. Antibodies used are indicated on the *left* side of the panels

5. Cut nitrocellulose membranes around 30 kDa and incubate bottom part of the membrane with anti-uH2A and/or uH2B antibodies and top part of the membrane with antibodies against specific histone deubiquitinases or ubiquitin ligases. uH2A is detected at 25 kDa while uH2B is slightly higher than uH2A (Fig. 5b).

4 Notes

1. Oligonucleosomes can be stored at 4 °C for at least 1 month without significant change in their ability to serve as substrates for histone ubiquitin ligase reactions. Mononuclesomes are less stable and precipitation can occur even after overnight storage at 4 °C. Therefore, it is recommended to store nucleosomes, especially mononucleosomes, in small aliquots at −80 °C to avoid multiple rounds of freeze and thaw. We also found that the presence of histone H1 slightly reduces the ability of nucleosomes to serve as substrates for some histone ubiquitin ligases. Therefore, it is recommended to use oligonucleosomes without H1 as substrates for histone ubiquitination reactions. In addition, we found that the ability of

nucleosomes to serve as substrates for histone ubiquitination varies from batch to batch.

2. Micrococcal nuclease from Sigma is less active but allows precisely controlled digestion. Digestion with Micrococcal nuclease from Worthington biochemical (Freehold, NJ) is hard to control and therefore it is not recommended for use in this experiment.

3. We routinely obtain 5–6 clones that can be used for further experiments from cotransfection of two 6-well plates. Clones that only express one epitope-tagged protein can be used for the experiments in Subheadings 3.2.2 and 3.3. Sequential transfection with one plasmid and then use of the stable clones for the second transfection is time-consuming and less efficient. This is likely due to the reduced ability of transfected cells to accept the second plasmid and therefore this approach is not recommended.

4. Digestion of nuclei (from two to three 15 cm plates) for 30 min yields primarily mononucleosomes. To obtain oligonucleosomes, we first optimize the conditions of digestion by comparing different time points. Bulk nuclei are digested to give nucleosomes ranging from 1 to 15 nucleosomes. We separate mono- and oligo-nucleosomes by a 5 ml 5–30 % sucrose gradient, which is prepared by loading layers of increasing concentrations of sucrose (mixing the 5 % and 30 % sucrose solution at different ratios). Histone octamers containing uH2A are prepared by a hydroapaptide column [55].

5. To observe the release of intact ubiquitin, uH2A-containg mononucleosomes, which are purified from anti-Flag and anti-HA tandem immunoprecipitation, are required. However, as a readout of the histone deubiquitination activity, the reduction of signal from ubiquitinated histones can be observed in bulk nucleosome preparations. Therefore, it is recommended to first confirm that the reduction of uH2A signal is indeed due to the presence of a specific deubiquitinase using double immunoprecipitation-prepared nucleosomes and once confirmed, the reduction of uH2A signal can be used as an indicator for the histone deubiquitination activity.

6. PCR program: 94 °C, 4 min, 1 cycle.

94 °C, 30 s	
50 °C, 30 s	40 Cycles
72 °C, 30 s	
72 °C, 7 min, 1 cycle	

Expected size of PCR product: 232 bp.

7. The level of uH2B in budding yeast depends on cell density and culture conditions. We routinely culture T85 yeast strains in YPD medium and use OD600 of 0.3–0.5 to prepare uH2B.
8. We wash gel strips three times in a 15-ml tube for 30 min with rotation. The gel strips are then transferred to a dialysis tubing with molecular weight cutoff of 6–8,000 Da. To further reduce the contamination with SDS, we use elution buffer to elute proteins and TAE buffer for electrophoresis.
9. For every freshly prepared DNA and/or histone octamers, we first calculate the molar ratio of DNA and histone octamers. We then use equal molar ratio and ratios slight higher or lower to reconstitute nucleosomes in a small scale. The quality of reconstituted nucleosomes is checked in a 5 % native PAGE to determine the best ratio of DNA and histone octamers for large scale reconstitution.
10. Pre-run the 5 % native PAGE (59:1, acrylamide : bisacrylamide) for 1 h at 4 °C at 150 V. Mix reconstituted nucleosomes with 50 % sucrose at ratio of 10:1 (volume) and load the sample.
11. Yeast cells grow fast at 30 °C with gentle shaking; however, it can still grow at slightly lower temperatures without agitation. Growth rate should be determined experimentally as uH2B level is maximal when OD600 is between approximately 0.3–0.5.
12. Freeze helps rupture the cells and improves protein yields. Therefore, even if the yeast cells will be used in the same day, freezing and thawing are still recommended.
13. Switching the salt from 50 mM KCl to 50 mM NaCl improves the yield.
14. It is noted that native purified mononucleosomes are better substrates than reconstituted nucleosomes. This is possibly due to the binding of SDS to uH2A or uH2B during denaturing purification. In some cases, it is difficult to see the increase of intact ubiquitin with the anti-HA antibody. In order to see the released intact ubiquitin, we use the anti-ubiquitin (FK2) antibody.
15. It is recommended to cut the membrane to probe each half separately. If the entire membrane is probed with the anti-Flag antibody, the ubiquitinated histones cannot be seen due to the large amount of nonubiquitinated histones. Additionally, probing the top half of the membrane with anti-HA antibody gives less background.

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Chapter 13

Immunostaining Analysis of Tissue Cultured Cells and Tissue Sections Using Phospho-Histone H3 (Serine 10) Antibody

Jaya Padmanabhan

Abstract

Post-translational modifications of histones play an important role in regulation of gene expression through condensation and decondensation of chromatin structure. These modifications include acetylation, methylation, phosphorylation and ubiquitination. Phosphorylation on histones is associated with cellular responses such as DNA damage, transcription, chromatin compaction and mitosis or meiosis. One of the most extensively studied modifications of histones is the Serine 10 phosphorylation on histone H3 N-terminal tail. This specific phosphorylation on Histone H3 has been associated with condensation and transcriptional inactivation of mitotic chromosomes, but recent studies have suggested a role for this specific phosphorylation in chromatin relaxation and activation of transcription in interphase cells. Co-immunostaining analysis of cells using antibodies specific to serine 10P-Histone H3 together with those to cell cycle specific markers will allow us to determine the nature of phosphorylation in a cell cycle-specific manner. In a complex system, such as tissue specimens, analysis using P-Histone H3 and a cell type specific antibody will allow identification of specific cells that are affected by this histone modification. This is of particular interest in the field of cancer biology or neurobiology where identification or quantification of the transcriptionally active or mitotic cells will enable one to evaluate the progression of the disease development. The protocol described here provides details on how co-immunostaining and analysis can be performed in tissue cultured cells or tissue sections.

Key words Histone, Phosphorylation, Immunofluorescence, Immunohistochemistry

1 Introduction

Protein phosphorylation is one of the several posttranslational modifications that affect the activity and function of a protein and this affects the signaling cascades that is associated with that specific protein [20]. Many of these signal transduction pathways are initiated at the cell surface and transported to the nucleus where the transcription is under tight control. The chromatin fiber in the nucleus is composed of repetitive units called nucleosomes that are comprised of core histones. These include H2A, H2B,

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H3, and H4. Chromatin organization is altered by modifications of the histones by acetylation, phosphorylation, methylation, and ubiquitination [21, 22]. Histone acetylation has been extensively correlated with transcriptional activation. H3 phosphorylation at serine 10 has been shown to be associated with transcriptional activation as well as chromosome condensation during mitosis and meiosis [1, 2, 20, 23–32]. During cell division, phosphorylation is first detected in pericentromeric heterochromatin and is associated with the whole chromosome in mitosis. This phosphorylation is also important for progression of cells from metaphase to anaphase. During interphase Histone H3 ser10 phosphorylation is associated with activation of several genes involved in signal transduction. Dephosphorylation of H3 begins in anaphase and ends in early telophase.

Association of phosphorylated histones with different mitotic machinery can be analyzed by immunocytochemistry [25, 27, 33, 34] and the detailed protocol for this is provided here. In brief, the cells or tissue sections are fixed, which is followed by permeabilization and nonspecific blocking, which can be achieved in a single step. This is followed by incubation of cells with P-Histone H3 antibody alone or in combination with any other specific antibody of interest, and the staining is visualized by fluorescence or bright-field methods.

2 Materials

1. Chamber slides for tissue culture (Fisher Scientific).
2. Fisherfinest cover glasses (Fisher Scientific).
3. Colorfrost plus microscope slides (Fisher Scientific).
4. Fluorogel mounting media (Fisher Scientific).
5. Anti phospho-histone H3 (serine 10) rabbit polyclonal antibody (Cell Signaling Technology, Inc. Danvers, MA).
6. Anti-alpha-tubulin mouse monoclonal antibody (Sigma-Aldrich, St. Louis, MO).
7. Anti-neuron specific nuclear (NeuN) antibody (Millipore, Bedford, MA).
8. Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrogen Corporation, Carlsbad, CA).
9. Alexa Fluor 594 goat anti-rabbit IgG (H+L) (Invitrogen Corporation, Carlsbad, CA).
10. Biotinylated anti-rabbit IgG (H+L) (Vector Laboratories, Inc. Burlingame, CA).
11. GIBCO goat serum (Invitrogen Corporation, Carlsbad, CA).
12. Poly L-Lysine (Sigma-Aldrich, St. Louis, MO).

13. Bovine serum albumin (BSA) (fraction V) (Sigma-Aldrich, St. Louis, MO).
14. bisBenzimide H 33342 trihydrochloride (Hoechst 33342) (Sigma-Aldrich, St. Louis, MO).
15. VECTASTAIN Elite ABC kit (Vector Laboratories, Inc. Burlingame).
16. DAB Peroxidase substrate kit (Vector Laboratories, Inc. Burlingame).
17. Ethanol (EtOH), 100 and 95 %.
18. Xylene.
19. Nembutal for anesthetizing the mice is obtained from the animal facility at the University.
20. Cytoseal XYL: Xylene-based mounting media (Fisher Scientific).

2.1 Solutions

1. Phosphate buffered saline (PBS):

   1× PBS: Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 800 ml of distilled H_2O and adjust the pH to 7.4.

   10× PBS: Dissolve 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4 in 800 ml water. Adjust the pH to 7.4 and bring up the volume to 1 l with distilled water.
2. Tris-buffered saline (TBS):

   1× TBS: Dissolve 3.03 g Tris base (25 mM), and 8.766 g NaCl (150 mM) in distilled water. Adjust pH to 7.4 and bring up the volume to 1 l with distilled water.

   10× TBS: Dissolve 30.3 g Tris base and 87.66 g NaCl in 800 ml distilled water. Adjust pH to 7.4 and bring up the volume to 1 l with distilled water.
3. 10 % Triton X-100: Mix 5 ml Triton X-100 with 45 ml distilled water by end-over-end mixing. Triton X-100 is really viscous and will take time to get a complete homogenous solution. Vortexing and vigorous shaking will result in foaming of the solution. The prepared 10 % solution can be diluted further with required buffers to make solutions that contain 0.2 % Triton X-100.
4. Tris-buffered saline containing 0.2 % Triton X-100 (TBST): Mix 1 ml of 10 % Triton X-100 with 49 ml 1× TBS.
5. 10 % sodium azide: Dissolve 1 g sodium azide in 10 ml of water and store at room temperature (*see* **Note 1**).
6. Blocking solution (1 % BSA/TBST): Dissolve 0.5 g bovine serum albumin (BSA) in 50 ml of 1× Tris-buffered saline (TBS) containing 0.2 % Triton X-100 (TBST). Add 0.1 ml of 10 % sodium azide to prevent bacterial growth, filter-sterilize and keep at 4 °C.

7. Alternative blocking solution: 10 % normal goat serum in TBST: Dilute 10 ml normal goat serum with 90 ml of TBS containing 0.2 % Triton X-100 to make 100 ml solution.
8. bisBenzimide H 33342 trihydrochloride (Hoechst 33342): Hoechst is dissolved in distilled water at a concentration of 10 mg/ml and aliquoted and stored at −20 °C.
9. 4 % Paraformaldehyde (PFA): Add 4 g paraformaldehyde to 80 ml dH_2O at 60 °C, add 1 drop of 2 N NaOH to the solution and stir until the PFA is completely dissolved. Do not let the temperature of the solution shoot above 60 °C. Once dissolved, add 10 ml of 10× PBS and bring up the volume to 100 ml. Filter the solution through a 0.45 μm filter and store at 4 °C (*see* **Note 1**).
10. Saline (0.9 % NaCl): Dissolve 0.9 g NaCl in 100 ml distilled water or 9 g NaCl per liter.
11. Citrate Buffer, pH 6.0.
    (a) Prepare 0.2 M solution of acetic acid by diluting 11.55 ml acetic acid in 1 l dH_2O.
    (b) Prepare 0.2 M solution of sodium acetate by dissolving16.4 g of $C_2H_3O_2Na$ or 27.2 g of $C_2H_3O_2Na.3H_2O$ in 1 l dH_2O. 10 mM Citrate buffer: Mix 4.8 ml of solution A + 45.2 ml of solution B, and dilute to 900 ml with dH_2O, adjust pH to 6 and bring up to 1 l.

2.1.1 Equipment

1. Leica Model SM2000R freezing stage microtome.
2. Zeiss AxioImager upright microscope with AxioVision Rel 4.8 or any fluorescent microscope with appropriate software program for fluorescent and bright-field analysis of cells and tissue sections.

3 Methods

3.1 Immunostaining Analysis Using P-Histone H3 Serine 10 Antibody

1. Culturing cells for Immunostaining analysis: H4 glioma cells are adherent and are cultured in chamber slides for immunocytochemical analysis. The chamber slides are coated with 100 μg/ml poly L-lysine from Sigma for 30 min in the tissue culture hood at room temperature. After the 30 min incubation, the slides are washed twice with distilled water and cells are plated (*see* **Note 2**).
2. Fixation of cells: After treatment of the cells, aspirate the tissue culture medium with great care and fix the cells with 4 % paraformaldehyde (PFA). Add enough PFA to cover the cells (250 μl/well for 8-chamber slides) and incubate for 10 min at room temperature. Remove PFA and carefully wash the cells with 1× PBS three times, 5 min each.

3. Inhibition of non-specific binding: Incubate the cells with blocking solution (1 % BSA/TBST) for 1 h at room temperature, on a platform rocker at very low speed. Alternatively, blocking can be achieved by incubating the cells with 10 % normal goat serum diluted in TBS/0.2 % Triton X-100 for 1 h at room temperature.
4. Primary antibody incubation: Anti-P-Histone H3 (ser10) antibody is used at a concentration of 5 μg/ml diluted in 1 % BSA/TBST (blocking buffer) and cells are incubated overnight at 4 °C, on a platform rocker at low speed.

After the primary antibody incubation, the slides are washed thoroughly with PBS (four times, 5 min each). At this point cells can be stained for analysis by bright-field or fluorescent microscopy.

3.2 For Fluorescent Microscopy

1. Cells are incubated with fluorescent-labeled secondary antibody. The secondary antibody is chosen based on the species where the primary antibody is raised. In this particular case the anti-P-Histone H3 antibody used is raised in rabbit and therefore the secondary will be Alexa fluor 594, anti-rabbit antibody. Alexa fluor 594 is diluted 1–4,000 with blocking buffer and cells are incubated for 2 h at room temperature on a platform rocker, protected from light as the light can quench the fluorescence. The slides can be placed in a dark box or covered with aluminum foil during the incubation period.
2. Instead of using a primary antibody and a fluorescent-labeled secondary antibody (indirect immunofluorescence assay), cells can also be stained using a fluorescent-labeled primary antibody in a single step if a fluorescent conjugated primary antibody (in this case labeled P-Histone H3; direct immunofluorescence) is available.
3. Nuclear stain: At the end of the incubation period cells are washed with PBS twice and incubated with Hoechst for 2 min at room temperature. Hoechst is diluted with PBS and used at a concentration of 1 μg/ml. At the end of the incubation the cells are washed twice more with PBS for 5 min each.
4. Removal of wells and gasket from slides: The wells from the chamber slides are removed by prying it apart, and the rubber gasket is removed using a forceps. The slides are cover-slipped using Fluorogel mounting media.
5. Storage: The slides are placed flat in slide trays and kept at 4 °C protected from light overnight and analyzed for P-Histone H3 positive cells on a Zeiss AxioImager upright microscope using AxioVision Rel 4.8 software.

3.2.1 Double Labeling of Cells Using Two Different Antibodies

If the cells need to be double labeled with a cell-specific marker or an antibody specific for another antigen, then the second primary antibody is chosen in such a way that it is raised in a different host.

For example, an alpha-tubulin antibody raised in mouse can be used along with anti-P-Histone H3 antibody raised in rabbit to determine the phosphorylation of histones and microtubule organization simultaneously in cells.

1. Cells are fixed and blocked as mentioned under Subheading 3.1.
2. The primary antibodies are diluted in 1 % BSA/TBST as follows: 5 μg/ml anti-Ser10-P-Histone H3 rabbit polyclonal and 1:3,000 dilution of anti-alpha tubulin mouse monoclonal antibodies. Cells are incubated with this mixture overnight at 4 °C.
3. Secondary antibody incubation: After washing thoroughly with PBS, the cells are incubated in the dark with secondary antibodies Alexa 594 anti-rabbit (1–4,000) and Alexa 488 anti-mouse (1–1,000) diluted in 1 % BSA/TBST for 1–2 h at room temperature. At the end of the incumation cells are washed twice with PBS by rocking for 5 min each.
4. Nuclear staining using Hoechst: Cells are incubated with Hoechst (1 μg/ml in PBS) for 2 min at room temperature. After two more washes the wells and gasket are removed from the slide.
5. Mounting of slides: The slides are mounted using Fluorogel for analysis with fluorescent microscope. Care should be taken to not allow the slides to get dry in between the staining procedure (*see* **Note 3**).

An example of double immunofluorescence staining in H4 glioma cells using anti-tubulin (1 :3,000 dilution) and anti-Ser10-P-Histone H3 (5 μg/ml) antibodies is shown in Fig. 1. Nuclei are visualized using Hoechst (1 μg/ml) (Fig. 1).

3.2.2 For Bright-Field Analysis

Bright-field staining analysis is performed using VECTASTAIN ABC kit from the Vector laboratories with slight modifications from the manufacturer's protocol. (For bright-field staining we always use only one primary antibody specific for a particular antigen, in this case P-Histone H3, although co-immunostaining can be done using two different primary antibodies raised in two different species of hosts). The steps leading up to the secondary antibody incubation are performed as described under Subheadings 3.1 and 3.2.

1. After primary antibody incubation the cells are washed thoroughly with PBS and incubated for 1 h at room temperature with biotinylated anti-rabbit IgG (H+L) from Vector laboratories diluted 1:200 in blocking buffer.
2. VECTASTAIN ABC reagent: While the cells are being incubated, the VECTASTAIN ABC reagent is prepared as follows: To 5 ml of blocking buffer add one drop of reagent A, mix and add one drop of reagent B and mix again. Incubate this mix at room temperature for 30 min before use.

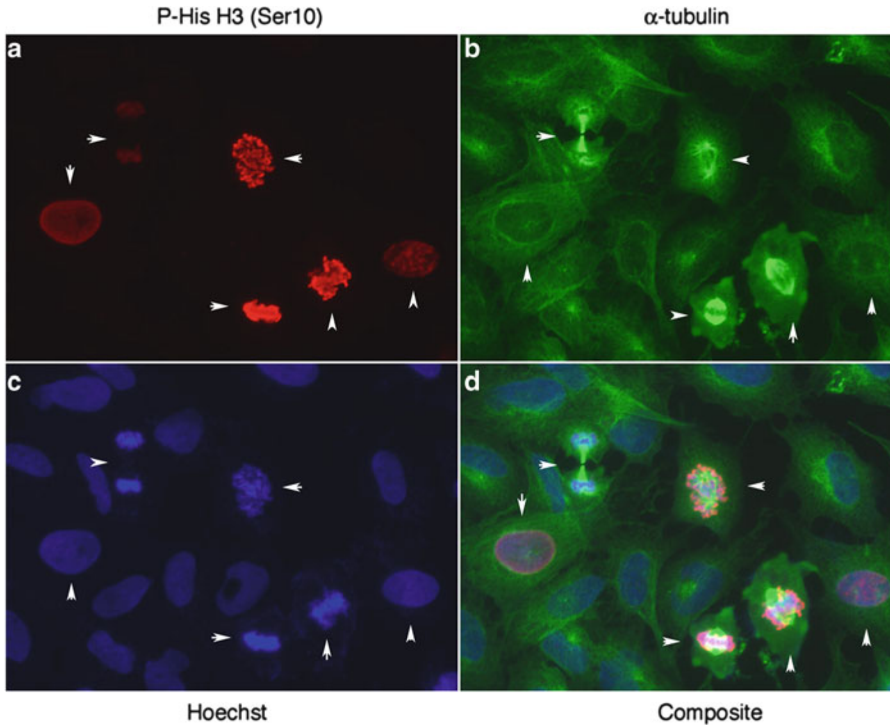

Fig. 1 Co-immunostaining analysis of H4 cells using P-Histone H3 (Serine 10) and alpha-tubulin antibodies: The cells are immunostained using (**a**) P-Histone H3 (Serine 10) antibody, and (**b**) alpha-tubulin antibody. Staining was visualized using Alexa 594 goat anti-rabbit or Alexa 488 goat anti-mouse fluorophores respectively. (**c**) Represents staining of the same cells using Hoechst to analyze nuclei, and (**d**) is the composite image showing all the three stainings. The arrows indicate P-Histone H3 positive cells in different phases of the cell cycle

3. Once the incubation with biotinylated antibody is finished, cells are washed and incubated with the VECTASTAIN ABC reagent for 1 h at room temperature.
4. After this incubation, cells are washed four times, 5 min each and incubated with peroxidase substrate provided with the DAB kit from Vector Laboratories following the manufacturer's protocol. The reaction is light sensitive and works better if performed under reduced light. Once the staining reaches desired intensity, the slides are washed in water to stop further development of the color. The DAB reagent is toxic and care should be taken while using it, and the residual, excess solution should be discarded appropriately (*see* **Note 4**).
5. The wells and gaskets are removed from the slide and mounted using Fluoromount as described under fluorescent microscopy or using xylene-based mounting media as follows.

6. Xylene-based mounting: Prior to mounting with xylene-based mounting media cells are dehydrated:

   Incubate for 2 min in 70 % EtOH.
   Incubate for 2 min in 95 % EtOH.
   Incubate for 2 min in 100 % EtOH twice.
   Incubate for 2 min in 100 % xylene twice.

7. Mount the slides using Cytoseal, a xylene-based mounting medium, for analysis with bright-field microscopy using Nikon E1000 microscope and Image Pro-Plus software program or Zeiss AxioImager fitted with CCD camera and AxioVision Rel 4.8 software.

3.2.3 Staining of Cells Grown in Suspension

Cells grown in suspension can be stained in suspension or after collecting them to a slide by cytocentrifugation (Cytospin). Pellet the cells in suspension by centrifugation at 1,000 rpm for 5 min and fix by resuspending in 4 % paraformaldehyde. After 10 min fixation, cells are washed three times with PBS by centrifuging and aspirating the PBS and replacing with fresh PBS. After the final wash cells are incubated with blocking buffer, and primary and secondary antibodies as mentioned under Subheadings 3.1 and 3.2. The suspension cells are collected on to a slide by Cytospin prior to analysis by fluorescent or bright-field microscopy depending on the staining method used.

3.3 Double Immunohistochemical Analysis of Brain Tissue Sections with Ser10-P-Histone H3 and Neuron Specific NeuN Antibodies

Preparation of tissue for immunostaining:

1. Perfusion: Mice are sedated by Nembutal (10 mg/kg body weight) and perfused with saline (50 ml) until the liver becomes pail. The perfusion is continued with 4 % paraformaldehyde (30 ml) and brains are dissected out carefully. The fixation is continued overnight at 4 °C by immersing in 4 % paraformaldehyde.
2. Cryopreservation of tissue: After the fixation is complete, the brain is cryoprotected by passing sequentially through 10, 20, and 30 % sucrose gradient. The brain is incubated in each sucrose solution until it sinks, after which it is ready for sectioning.
3. Sectioning of brain: The brain to be sectioned is placed carefully on to the freezing stage using tissue freezing medium (Ted Pella, Inc) and allowed to freeze. 25 μm thick sections are made using a freezing stage sliding microtome (Leica model SM2000R). The sections are placed in PBS containing 0.02 % sodium azide until the brain is completely sectioned through.
4. Mounting of tissue sections on slides: The sections are mounted on to frosted slides. To do so, a drop of 30 % ethanol is placed on the slide and each section is transferred into this using a thin paintbrush, allowing the section to slowly spread with the help of the brush. The section is allowed to dry onto the slide.

It takes few hours to overnight depending on the amount of liquid on the slide. Depending on the size of the tissue section, multiple sections can be plated on each slide. Once dry, using rubber cement make an edge around the sections (*see* **Note 5**). This will help to retain the solutions on the slide during staining protocol. Once the rubber cement is dry, rehydrate the tissue section by immersing the slides in PBS in a coplin jar for 5 min. Wash once with PBS. (The staining can be performed using floating sections as well, in which case the mounting will be done at the end of the staining protocol, prior to visualization and analysis).

3.4 Immunostaining Analysis of Brain Sections

1. To inhibit non-specific binding the sections are blocked for 2–3 h with 1 % BSA/TBST containing 0.2 % triton X-100 (same as in staining for cells mentioned above, except the tissue is incubated for longer time period). Blocking can also be achieved by incubating the sections with 10 % normal goat serum in TBST. All the incubations are done in a humid atmosphere to avoid drying of the sections on slides.
2. Incubate the tissue sections with primary antibodies Ser10-P-histone H3 rabbit (5 μg/ml) and anti-NeuN mouse (1:200 dilution) antibodies diluted in blocking buffer (1 % BSA/TBST) overnight in a humidified container, at 4 °C, on a platform rocker set at low speed.
3. Tissue sections are washed with PBS thoroughly and incubated with Alexa Fluor 594 anti-rabbit and Alexa Fluor 488 anti-mouse secondary antibodies for 2–3 h at room temperature, protected from light.
4. At the end of the incubation the slides are washed twice with PBS and incubated with Hoechst diluted in PBS at a concentration of 1 μg/ml for 5 min, protected from light. The slides are washed two more times and mounted using Fluoromount. After drying the slides overnight at 4 °C in dark, analysis is carried out using a Nikon E1000 fluorescent microscope using Genus 2.8 software program or Zeiss AxioImager and AxioVision Rel 4.8 software. An example of brain section stained with NeuN and P-Histone H3 antibodies, and Hoechst is shown in Fig. 2.

3.5 Immunohistochemical Analysis of Paraffin-Embedded Tissue Sections

Removal of paraffin and rehydration of sections: The paraffin-embedded tissue sections are treated as follows for removal of paraffin and for rehydration.

1. The sections are first heated at 60 °C for 1 h.
2. Following this the sections are immersed in xylene for 3 min twice.

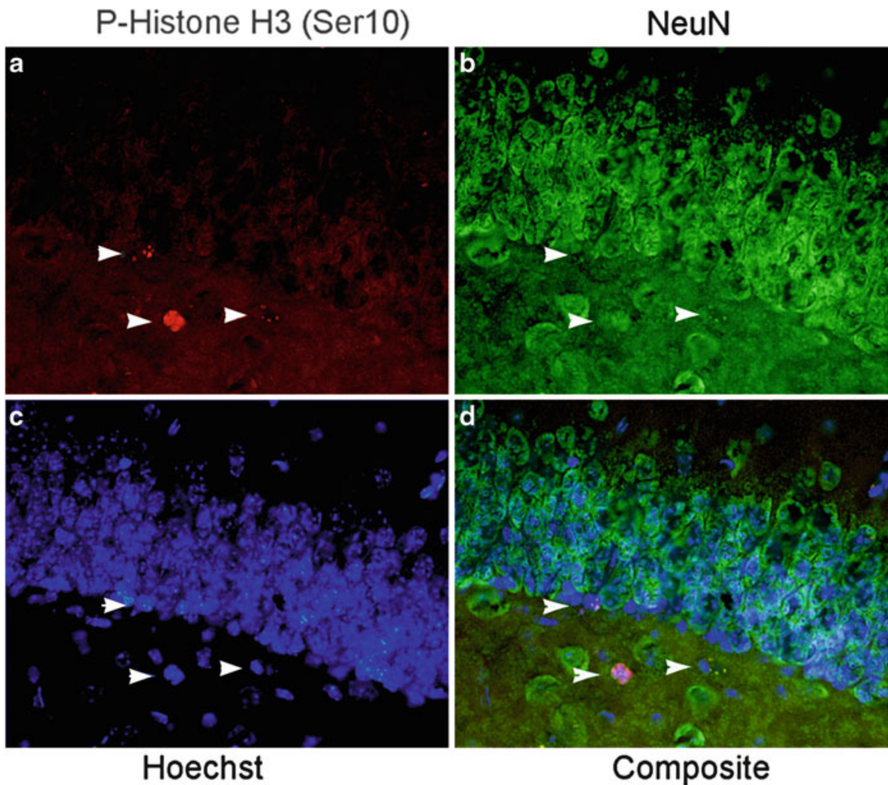

Fig. 2 Immunohistochemical analysis of brain sections using P-Histone H3 (Serine 10) and NeuN antibodies: Brain sections are co-immunostained using (**a**) P-Histone H3 (Serine 10), and (**b**) neuron specific NeuN antibody. Staining was visualized using Alexa 594 goat anti-rabbit or Alexa 488 goat anti-mouse fluorophores respectively. (**c**) Represents the nuclear staining (in pyramidal layer of hippocampus) in sections using Hoechst and (**d**) is a composite image of all the three staining. The arrows indicate P-Histone H3 positive cells in the brain section

3. The sections are rehydrated by sequential passage through 100 % EtOH 2 min twice, 95 % EtOH 2 min once, and 70 % EtOH 2 min once. The sections are then rinsed in distilled water.
4. Antigen retrieval: This is achieved by heating the sections for 10 min at 95 °C in citrate buffer pH 6.0. The sections are then cooled slowly to room temperature and rinsed twice with dH_2O. Antigen retrieval can also be achieved using 10 mM EDTA buffer pH 7.0, and heating in a pressure cooker for 3 min at full pressure [35] or using Tris–EDTA buffer (pH 9.0) and heating in a pressure cooker for 10 min at 125 °C [13].
5. Inhibition of endogenous peroxidase activity: Quenching of Peroxidase activity is achieved by incubating the sections for 20 min in Methanol containing 0.5 % H_2O_2 at room temperature.

6. After rinsing the sections twice with water, they are incubated with blocking buffer and staining continued as mentioned above under Subheading 3.2.2, for bright-field analysis. If preferred double staining can be achieved in bright-field analysis as well.

Double staining for bright-field analysis: Double immunostaining for bright-field analysis can be achieved by performing all the staining steps using the first primary antibody up to the finish, including substrate reaction, followed by blocking and staining using the second primary antibody and repeating the staining steps. The substrate used for developing the second antibody reaction should provide a color that is different from the first substrate. For example, Vector Blue can be used as the substrate for the first primary antibody detection and Nova-red for the second primary antibody. These substrates are from Vector laboratories and can be used following the manufacturer's protocol.

Hematoxylin and eosin (H & E) staining of sections: This staining is performed to identify the various tissue types and the morphological changes. Hematoxylin stains the nucleus blue and eosin stains the cytoplasm pink [36].

4 Notes

1. Sodium azide is toxic and care should be taken to avoid inhalation of the powder by wearing a mask. Paraformaldehyde is a carcinogen and caution should be taken when making the solution. Wear mask and gloves and prepare the solution in a fume hood. Do not overheat as heating the solution above 60 °C will lead to break down of paraformaldehyde. The prepared solution is good for a week at 4 °C.
2. Depending on the experiments (the number of parameters to be tested), adherent cells can be grown in chamber slides with different number of wells 1, 2, 4, 8, or 16. This allows comparative studies under various treatment conditions, such as treatment of cells in each well with different drugs or with varying concentrations of same drug, followed by staining and analysis without disturbing the attached cells. Since these cells are processed on the same slide, the sample-to-sample variation will be minimal.
3. Do not allow the slides (containing cells or tissue sections) to dry during the entire staining procedure. Drying will result in non-specific binding of antibodies and the results will not be reliable. The given concentrations of primary and secondary antibodies are the ones that worked in our hand and they are working very well with different cell types and brain sections.

If problems occur, each user may have to titer the antibody concentration that work well under their analysis conditions.

4. DAB reagent: DAB is considered as a carcinogen and should be handled with caution. Avoid skin contact and wear gloves during the use of DAB. Excess solution should be discarded appropriately, following the protocol provided by the supplier. One option is to mix DAB solution with equal volume of 20 % bleach solution and inactivated DAB can be discarded by pouring down the sink followed by excess running water.

5. Not all type of slides work well when plating sections and it is very important to use the Colorfrost or equivalent ones otherwise sections will not attach very well. If the sections are not dried well on to the slides, during washes they can come off and therefore it is important to let them dry completely. Also care should be taken when spreading the sections onto the slide. Allow the sections to open up in the 30 % EtOH solution on slides and using a thin paintbrush spread the sections. Additionally, sections can be floated in PBS in an appropriate tray and slowly moved to the slide by immersing the slide in the PBS and carefully moving and positioning the sections onto the slide. Paraffin-embedded sections are not recommended for use with fluorescent methods as the section may autofluoresce, thus interfering with the specific staining.

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Chapter 14

Identification and Characterization of Nonhistone Chromatin Proteins: Human Positive Coactivator 4 as a Candidate

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Abstract

The highly dynamic nucleoprotein structure of eukaryotic genome is organized in an ordered fashion, the unit of which is the nucleosome. The nucleosome is composed of core histones and DNA of variable size wrapped around it. Apart from the histone proteins, several nonhistone proteins also interact with the complex consisting of the DNA, the core and linker histones conferring highly regulated fluidity on the chromatin and permitting fine tuning of its functions. The nonhistone proteins are multifunctional and accentuate diverse cellular outcomes. In spite of the technical challenges, the architectural role of the nonhistone proteins altering the topology of the chromatin has been studied extensively. To appreciate the significance of the chromatin for genome function, it is essential to examine the role of the nonhistone proteins in different physiological conditions. Here, taking the example of a highly abundant chromatin protein, PC4 (Positive coactivator 4), we describe strategies for the identification of the chromatin-associated proteins and their structural and functional characterization.

Key words High mobility group proteins, Heterochromatin protein 1, Positive co-activators 4, Histone chaperones, Heterochromatinization

1 Introduction

There are different types of chromatin-associated nonhistone proteins (CAPs) that are differentiated based on the modes of interaction with the components of the chromatin and the varied functional outcomes. The association of nonhistone proteins such as the High Mobility Groups (HMGs) and the linker histones with the chromatin is highly dynamic in nature with the interaction of the HMGs with the chromatin facilitated through core histones. However, the majority of the CAPs show nonspecific DNA binding ability. The linker histones are necessary for the higher order chromatin organization [1]. The H1 histone interacts with nucleosomes and limits their mobility. HP1 (Heterochromatin Protein 1) is a

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well-characterized member of the CAP family with an established role in heterochromatization. HP1 binds to dimethylated and trimethylated lysine 9 of histone H3 which constitute the epigenetic marks for the silenced genes [2, 3]. MENT (Myeloid and erythroid nuclear termination stage-specific protein), a chromatin architectural protein, belongs to the Serpin protein family of serine protease inhibitors which promotes chromatin condensation by bringing the linker DNA entry/exit regions into close proximity [4]. MENT can also promote the formation of protein bridges between chromatin fibers by self-oligomerization [5]. DEK is another nonhistone protein which binds to the DNA based on the secondary structure, but not the underlying sequence, of the DNA [6]. It alters DNA topology by inducing positive supercoils into closed circular DNA. DEK also interacts with histone H2A/H2B dimer that in turn is necessary for the DEK-mediated change in topology of nucleosomal DNA [7].

The human positive coactivator 4(PC4) is a highly abundant, nuclear protein [8, 9]. PC4 is a small protein with a molecular weight of 14 kDa known to be involved in several cellular functions [8–12]. PC4 is highly conserved across species ascertaining its biological significance. In *Homo sapiens*, PC4 consists of 127 amino acids having unstructured N-terminal domain (1–62) and highly structured C-terminal domain (62–127). The N-terminal domain contains two serine-rich acidic regions (SEAC) separated by a lysine-rich (LYS) region [13]. The SEAC region is predicted to undergo phosphorylation. The crystal structure of the PC4 C-terminal domain (at 2.8 Å resolution) revealed a dimeric structure with two ss DNA binding channels running in the opposite direction to each other [14]. PC4 also possesses a double-stranded DNA-binding domain that overlaps with its coactivation domain (22–87). Apart from its first known role in transcription, lately PC4 has been implicated in several other important cellular phenomena including replication, repair, chromatin organization and tumor suppression [10–12, 15–17]. Recently, the role for PC4 in mediating the global chromatin organization has been discovered [15]. PC4 is tethered to the chromatin and exclusively associated with the nucleosomal, but not with nonnucleosomal, fraction, as is evident from fractionated HeLa chromatin using sucrose density gradient [15]. The association of PC4 with chromatin is consistent through the different stages of the cell cycle. Further, PC4 is broadly distributed on metaphase chromosome in a punctuate manner except at the centromeric region [15]. The stable association of PC4 with the chromatin could be through its interaction with different components of the chromatin. Apart from its interaction with the DNA, the general transcription factors or the nonhistone chromatin-associated proteins, PC4 in vitro directly interacts with all the core histone proteins with a preference for histones H3 and H2B. PC4 docks to the globular domains of histones H3 and H2B but not to their tail regions. An interaction

between CenH3, a centromeric-specific histone, and PC4 has not been identified probably explaining the observation that PC4 is not present in the centromere [15]. Through its ability to interact with histones, PC4 mediates the condensation of the chromatin. PC4 can compact H1-stripped HeLa chromatin in vitro. However, a PC4 mutant deficient in histone-interaction ability failed to condense the H1-stripped HeLa chromatin suggesting that PC4 interaction with the core histones is crucial for chromatin condensation [15]. Importantly, the nature of the PC4-mediated chromatin condensation is strikingly distinct from that of the linker histones which favour the solenoid formation. Transient silencing of PC4 in HeLa cells caused decompaction of the chromatin as visualized by atomic force microscopy. Recent results alluded to the role of PC4 in heterochromatinization, thereby regulating the expression of a subset of genes [18]. Stable knockdown of PC4 could be helpful to explore the physiological consequences, thereby shedding light on the importance of ordered chromatin structure for cellular functions. The chapter presents several approaches to validate the association of a protein (here PC4) with the chromatin, thereby modulating the structure and function of the latter.

2 Materials

2.1 Sucrose Gradient Fractionation of the Chromatin Fragments

2.1.1 Cell Culture

1. HeLa cells (ATCC).
2. Dulbecco's Modified Eagle's Medium (DMEM).
3. L-glutamine.
4. Penicillin–streptomycin–amphotericin B (PSA).
5. Fetal Bovine Serum (FBS) (Hyclone).
6. Trypsin–EDTA.
7. 1× PBS.
8. 37 °C CO_2 incubator.

2.1.2 Buffers and Solutions

1. Hypotonic buffer (10 mM Tris–HCl, 10 mM KCl, and 15 mM $MgCl_2$).
2. MNase enzyme.
3. Nuclear digestion buffer (10 % glycerol, 10 mM Tris–HCl pH 8, 3 mM $CaCl_2$, 150 mM NaCl, 0.2 mM PMSF).
4. 10 mM EDTA.
5. Sucrose solution.
6. NTE buffer (10 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EDTA).
7. Phenol–chloroform.
8. Ethanol.

2.1.3 Equipment

1. Beckman centrifuge tube.
2. Beckman ultracentrifuge (SW60Ti rotor).
3. Bio-Rad semidry transfer apparatus.

2.1.4 Affinity of PC4 for the Chromatin

1. HeLa cells (ATCC).
2. Transport Buffer TB (20 mM Hepes pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA, 2 mM DTT).
3. Ice.
4. 0.1 % NP40 (in water).
5. 40 μg/mL digitonin (in water).
6. 10 mM $MnCl_2$.
7. 20 μg/mL DNaseI.
8. Bio-Rad semidry transfer apparatus.
9. Antibodies against PC4, Histone H3 (Lab reagent), H1 (Sigma), and HSC70 (Sigma).

2.2 Interaction of PC4 with Histones

2.2.1 The In Vivo Interaction

1. HeLa cells.
2. Mammalian cell expressing the FLAG-fusion construct of PC4.
3. Lipofectamine (Invitrogen).
4. 1× PBS.
5. Lysis buffer (50 mM Tris–HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1 % Triton X-100).
6. Anti FLAG M2 affinity gel.
7. TBS (50 mM Tris–HCl, with 150 mM NaCl, pH 7.4).
8. 2× sample buffer (125 mM Tris–HCl, pH 6.8, with 4 % SDS, 20 % (v/v) glycerol, and 0.004 % bromophenol blue).
9. Antibodies against individual histones (Lab reagent).

2.2.2 The In Vitro Interaction

1. The individual recombinant histones H2A, H2B, H3, and H4.
2. His_6-PC4 protein.
3. Ni-NTA His•Bind Resin (Novagen).
4. BC buffer (20 mM Tris–HCl pH 7.4, 20 % glycerol, 0.2 mM EDTA, 0.1 % NP40, 2 mM PMSF, and 2 mM β mercaptoethanol).
5. 3 M KCl.
6. 1 M imidazole.
7. 5× SDS loading dye.
8. Antibodies against individual histones (Lab reagent).
9. Microcentrifuge.
10. End-to-end rocker.

2.3 The Distribution of PC4 on the Metaphase Chromosome

1. HeLa cells.
2. 1 mg/mL nocodazole.
3. 3 M KCl.
4. FBS.
5. 1× PBS.
6. Glass slide.
7. Cytobucket rotor.
8. Anti-PC4 antibodies (Lab reagent).
9. Alexa conjugated secondary antibodies (Molecular Probes).
10. Hoescht33258 (Sigma).

2.4 siRNA Mediated Silencing of PC4

1. HeLa cells.
2. DMEM.
3. Oligofectamine.
4. siRNAs targeting PC4 sense, 5′-r[ACAGAGCAGCAGCAG CAGA]dTT-3′; antisense, 5′-r[UCUGCUGCUGCUGCUC UGU]dTT-3′.

Scrambled RNA (sense, 5′-r[GAAAGGCAACGACGGACAC] dTT-3′; antisense, 5′-r[GCGAACACUAACGUACCUCAU] dTT-3′).

2.5 The Establishment and Characterization of the PC4 Stable Knockdown Cell Line

2.5.1 Plasmids

1. pPAX2, pVSV-G, and pRSV-Rev (The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH).
2. shRNAs targeting PC4 containing pGIPZ lentiviral vectors (Open Biosystems).

2.5.2 Cell Culture

1. HEK293T and HEK293 cells (in log phase of growth and at 50 % confluence at the time of transfection or infection).
2. Dulbecco's Modified Eagle's Medium (DMEM).
3. Fetal calf serum (Life Technologies).
4. Penicillin (50 U/mL) and streptomycin.
5. Cell culture incubator—37 °C, 5 % CO_2 and 100 % relative humidity.
6. $CaCl_2$ (2.5 M).
7. Falcon tubes.
8. 0.45 μm filter (Millipore).
9. Freeze vials.
10. Liquid nitrogen.

11. HIV–1 p24 ELISA kit (PerkinElmer).
12. 2× HEPES-buffered saline (140 mM NaCl, 1.5 mM $Na_2HPO_4.2H_2O$, and 50 mM HEPES).
13. Hexadimethrine bromide (Polybrene), 1 mg/mL stock.

2.5.3 The Whole RNA Extraction and RT-PCR

1. TRIzol.
2. Chloroform.
3. DEPC water.
4. Sodium acetate (pH 5.2).
5. Isopropanol.
6. Ethanol.
7. Nuclease-free water.
8. –70 °C freezer.
9. Refrigerated fixed rotor centrifuge.
10. NanoDrop spectrophotometer (Thermo Scientific).
11. Superscript Reverse Transcriptase (RT) enzyme (Invitrogen).
12. SYBR green mix (Bio-Rad).
13. PC4 gene primers: Forward-5′-TGAGACTTCGAGAGCCC-3′; Reverse-5′-TCGCGAACACTAACGTAC-3′.
14. Actin gene primers: Forward-5′-GTGGGGCGCCCCAGGCACCA-3′; Reverse-5′-CTCCTTAATGTCACGCACGATTTC-3′.

2.5.4 The Whole Protein Extraction and Western Blotting

1. SDS PAGE gel.
2. RIPA lysis buffer (50 mM Tris–HCl (pH 7.4), 1 % NP-40, 0.25 % Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL each of aprotinin, leupeptin, pepstatin, 1 mM Na_3VO_4, 1 mM NaF).
3. TNN lysis buffer (50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 0.5 % NP-40, 200 μM PMSF, 0.5 mM dithiothreitol (DTT), 1 μg/mL each of pepstatin and leupeptin).
4. BSA.
5. Ultrospec 2100 Pro UV/visible spectrophotometer (Amersham Biosciences).
6. Loading dye (5× concentration: 250 mM Tris–HCl (pH 6.8), 10 % SDS, 0.5 % bromophenol blue, 50 % glycerol, and 500 mM β-mercaptoethanol).
7. Prestained marker (Invitrogen).
8. Dry bath.
9. Transfer buffer (For 500 mL transfer buffer, dissolve 1.65 g Tris base, 7.20 g glycine in 400 mL water. 100 mL methanol and 1.8 mL 10 % SDS was added to it).

10. Nitrocellulose membrane.
11. Skimmed milk powder (Himedia).
12. Antibodies against PC4 (Lab reagent).
13. HRP conjugated secondary antibodies (Molecular probes).
14. Chemiluminescence kit (Thermo Scientific).
15. X-ray films (Kodak).
16. GBX-Developer-Fixer Kit (Premiere Kodak reagents).

2.6 Chromatin Characterization Following the PC4 Knockdown

2.6.1 DAPI Staining

1. Control and PC4 stable knockdown cells.
2. Glass coverslips.
3. Poly-L-lysine.
4. 1× PBS.
5. DAPI/Hoescht 33258.
6. Glass slide.
7. Carl Zeiss confocal microscope.

2.6.2 MNase Digestion

1. HeLa cells.
2. Hypotonic solution.
3. MNase enzyme.
4. 1 M EDTA.
5. 37 °C water bath.
6. 1 % agarose gel.
7. 123 bp marker.
8. Ethidium bromide.

2.6.3 AFM Visualization of Chromatin

1. HeLa cells.
2. 1× PBS.
3. Buffer A (100 mM NaCl, 0.5 % Triton X-100, 3 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris–HCl, pH 7.5, 1 mM PMSF).
4. 100 mM $CaCl_2$.
5. 4 % paraformaldehyde.
6. MNase.
7. 1 M EDTA.
8. Poly-L-lysine coated coverslips.
9. 37 °C incubator (Thermocon).
10. Refrigerated centrifuge (Eppendorf Centrifuge 5415R).
11. BioScope/BioScope SZ NanoScope IIIa Controller (Veeco Instruments, Santa Barbara, CA).

2.6.4 Epigenetic Marks Analysis

1. Poly-L-lysine coated glass coverslips.
2. 1× PBS.
3. FBS (Hyclone).
4. Triton X-100.
5. Paraformaldehyde.
6. Permeabilizing buffer (0.1 % Triton X-100 in 1× PBS).
7. Blocking buffer (5 % FBS in 1× PBS).
8. 37 °C incubator.
9. Antibody solution (Appropriate antibody dilution in 1 % FBS containing 1× PBS).
10. Wash buffer (1 % FBS in 1× PBS).
11. Histone modification antibodies.
12. Alexa fluorophore conjugated secondary antibodies.
13. Glass slide.
14. Mounting solution (70 % glycerol in PBS).
15. Carl Zeiss confocal laser scanning microscope (Axioskop 2 Plus).

2.7 Visualization of the In Vitro Chromatin Compaction Ability of PC4

2.7.1 The Purification of HeLa Core Histones

1. HeLa cells.
2. Hypotonic buffer (10 mM Tris–HCl, 10 mM KCl, and 15 mM $MgCl_2$).
3. Buffer A (100 mM Potassium phosphate buffer pH 6.7, 0.1 mM EDTA, 10 % glycerol, 0.1 mM PMSF, 0.1 mM DTT, 630 mM NaCl).
4. Dounce's homogenizer (Wheaton) and pestle B.
5. Hydroxyapatite BioGel HTP (Bio-Rad).
6. 10 mM Potassium phosphate buffer pH 6.7.
7. Econoglass column.
8. 5 M NaCl.

2.7.2 Chromatin Assembly on a 100 kb Plasmid

1. Plasmid DNA.
2. HeLa core histones.
3. Hi-buffer (10 mM Tris–HCl, pH 7.5, 2 M NaCl, 1 mM EDTA, 0.05 % NP-40, and 5 mM 2-mercaptoethanol).
4. Dialysis tube.
5. Lo-buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.05 % NP-40, and 5 mM 2-mercaptoethanol).
6. Peristaltic pump.

2.7.3 AFM Visualization of PC4/H1-Mediated Chromatin Compaction

1. The recombinant PC4 and H1 protein.
2. The in vitro assembled chromatin.
3. Fixation buffer (0.3 % glutaraldehyde, 50 mM NaCl, and 5 mM Hepes-K^+ pH 7.5).

4. Mica substrate.
5. 10 mM spermidine.
6. Nitrogen gas.
7. NanoScope IIIa or IV (Digital Instruments); cantilever (OMCL-AC160TS-W2, Olympus).

2.8 Additional Reagents

2.8.1 SDS-PAGE Electrophoresis

1. SDS-PAGE reagents (Acrylamide, Bisacrylamide, 1.5 M Tris–HCl (pH 6.8), 1 M Tris–HCl (pH 8.8), APS, 10 % SDS, TEMED).
2. Bio-Rad gel apparatus (Glass plates, combs, gel running tank, power pack).
3. The SDS running buffer (For 500 mL 5× SDS running buffer: 7.55 g of Tris base, 47 g glycine, and 25 mL 10 % SDS was mixed homogeneously in water and volume was made up to 500 mL).

2.8.2 Agarose Gel Electrophoresis

1. Agarose.
2. 1× TBE (0.09 M Tris borate and 0.002 M EDTA) (For 1 L 2× TBE: 27 g Tris base, 12.75 g boric acid, and 8 mL 0.5 M EDTA were mixed in water and final volume was made up to 1 L.
3. Microwave oven.
4. 6× gel loading dye (For 1×—0.25 % bromophenol blue, 0.25 % xylene cyanol in 40 % Sucrose).
5. 100/123 bp marker.
6. Electrophoresis tank and power pack.
7. Ethidium bromide.
8. Gel tray and combs.
9. Gel documentation system (Bio-Rad).

3 Methods

3.1 Sucrose Gradient Fractionation of Chromatin Fragments

The protocol is useful for determining whether or not a nuclear protein is part of the nucleosomal chromatin. Although several proteins contribute to the nuclear pool, they need not necessarily be associated with the nucleosomes. To examine the association of PC4 with the chromatin, HeLa nuclei are partially digested with the micrococcal nuclease (MNase) followed by the sucrose density gradient fractionation of the resultant chromatin fragments. The presence of the nucleosomal DNA is ascertained by resolving the fractionated nucleosomal fragments on a 1 % agarose gel (Fig. 1a). PC4 is typically present only in the nucleosomal fractions and this could be validated further by the presence of the core histone H3 (Fig. 1b).

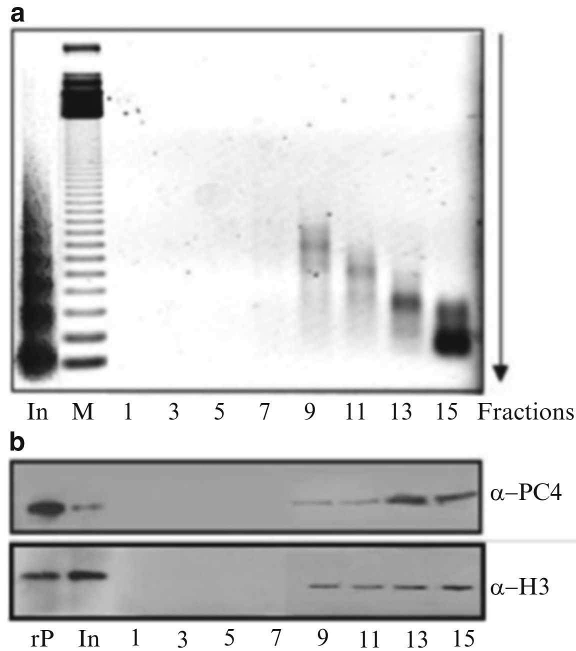

Fig. 1 PC4 cofractionates with HeLa Nucleosomes in sucrose gradient: (**a**) HeLa nuclei were partially digested with micrococcal nuclease (MNase) and fractionated on a 15–40 % sucrose gradient. Individual fractions were deproteinized, and the alternative fractions were resolved on a 1 % agarose gel and visualized by ethidium bromide staining. (**b**) Corresponding fractions were analyzed by western blotting for the presence of PC4 and histone H3 using respective antibodies. *Lanes 1–15* are chromatin fractions. The lane In stands for input control and the lane rP stands for the corresponding recombinant proteins

3.1.1 Preparation of Nuclei and MNase Digestion

1. Hela Cells (50×10^6) were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10 % fetal bovine serum, L-glutamate and penicillin–streptomycin–amphotericin B (PSA) antibiotics cocktail in a CO_2 incubator.
2. Cells were trypsinized using 0.025 % trypsin-EDTA (Sigma) and centrifuged at $134.49 \times g$ for 10 min. Pellet was given wash with 5 mL 1× PBS and centrifuged again.
3. The cells were suspended in hypotonic buffer followed by 10 min of incubation at 4 °C. The suspension was centrifuged at $134.49 \times g$ at 4 °C to yield nuclei.
4. The nuclei were resuspended in nuclei digestion buffer followed by digestion with (0.2 U/μL) micrococcal nuclease (MNase) for 10 and 15 min at room temperature.
5. MNase digestion was stopped by the addition of 10 mM EDTA to the nuclei.

3.1.2 The Sucrose Gradient Fractionation

1. A series of NTE buffer was prepared with increasing concentration of sucrose (15, 20, 25, 30, 35 and 40 %).
2. A 4.8 mL linear sucrose gradient was made in Beckman centrifuge tube (5 mL) and chromatin was loaded gently onto the gradient.
3. The gradient was centrifuged in Beckman ultracentrifuge (SW60Ti rotor) at 109243.8 × g at 4 °C for 14 h.
4. 100 μL aliquot fractions were collected from the top without disturbing the gradient.
5. Alternate fractions were taken and phenol chloroform extraction followed by ethanol precipitation of DNA was performed.
6. Fractions were resolved on a 1 % agarose gel and visualized by ethidium bromide staining (Fig. 1a).

3.1.3 The Detection of PC4 in the Nucleosomal Fractions

1. Fractions with and without nucleosomes were resolved in 12 % SDS-PAGE and western blotting was performed. Briefly, gel was incubated in transfer buffer and transferred to nitrocellulose membrane using Bio-Rad semidry transfer apparatus.
2. Membrane was incubated with anti PC4 antibody after prior blocking of the membrane with 5 % skimmed milk solution in 1× PBS.
3. After appropriate secondary antibody incubation, the membrane was developed using chemiluminescence kit and luminescence were captured on X-ray films (Kodak) (Fig. 1b).

3.2 Affinity of PC4 for the Chromatin

The following protocol enumerates the affinity of a specific protein for the chromatin. The cells are treated with two different types of detergents NP-40 or digitonin of variable strength. NP-40 is a stronger detergent as compared to digitonin [19]. The NP-40 treatment would dissociate proteins that are less strongly associated with the chromatin. The data show that while treatment with digitonin, a weak detergent, failed to dissociate PC4 from the chromatin, only small quantities of the protein are found in the supernatant following treatment with NP-40 (Fig. 2, Panel I) unlike histones H3 and H1 which are absent in the supernatant even after the treatment with NP-40 (Fig. 2, Panels II and III). This observation indicates that the binding of PC4 to chromatin is not as strong as that of the core histones or the linker histone H1, that remain associated with the chromatin following NP-40 treatment. The presence of a cytoplasmic marker (HSC70) only in the supernatant fraction, in treatments with either of the detergent confirms the experimental stringency of the assay (Fig. 2, Panel IV). These data suggest that PC4 is tightly bound to the chromatin although the binding affinity is not as strong as that of the core histones or the linker histone H1. The present protocol can also be used to determine the relative or altered affinity of a

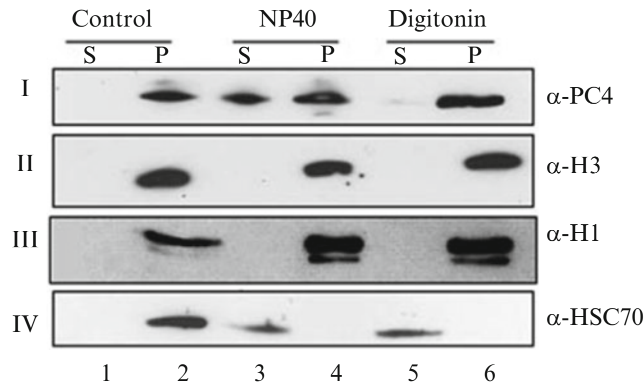

Fig. 2 Relative affinity of PC4 for the chromatin: The cells were incubated in a buffer with 0.1 % NP-40 or 40 μg/mL of digitonin. After the incubation, the supernatants (S) and the remnants of permeabilized cell pellets (P) were analyzed by western blotting using antibodies against PC4 (IV), histone H3 (I), histone H1 (II) and HSC70 (III)

protein for the chromatin at different stages of the cell cycle or physiological conditions.

1. HeLa cells were cultured in DMEM media supplemented with 10 % FBS, L-Glutamate and PSA antibiotics cocktail followed by incubation in CO_2 incubator till confluency is reached to 80 %.
2. The adherent cells were washed three times with ice-cold transport buffer, TB or TB with 0.1 % NP40 or 40 μg/mL digitonin.
3. Supernatants (S) were recovered and preserved.
4. The cell remnants (P) were incubated for 10 min at 37 °C with TB buffer supplemented with 0.1 % NP40, 10 mM $MnCl_2$, 20 μg/mL DNaseI.
5. The supernatants and cell remnants were analyzed by western blotting, using antibodies against PC4.
6. Samples were also analysed by western blotting, using antibodies against histone H3, H1 and HSC70 (cytoplasmic marker) for comparison.

3.3 Interaction of PC4 with Histones

3.3.1 The In Vivo Interaction

The stable association of a protein to the chromatin could occur through any of the diverse components of the chromatin namely, interaction with DNA, general transcription factors, other nonhistone chromatin-associated proteins or the core or linker histones. Several chromatin-associated nonhistone proteins (HMGs, HP1 etc.) are known to interact with core histones. The in vivo or in vitro interactions can be studied by using fusion proteins. The fusion protein could be either transfected into the cells or used for in vitro

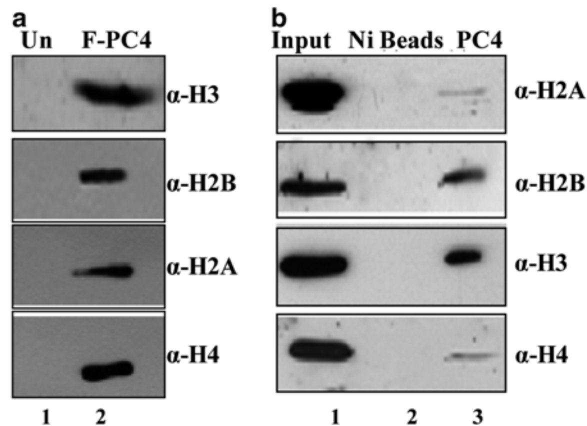

Fig. 3 PC4 interacts with histones in vivo and in vitro. (**a**) HeLa cells were transfected with FLAG-PC4 (F-PC4) mammalian expression construct. The expressed F-PC4 was pulled down by M2-Agarose beads, and the complex was subjected to western blotting analysis using different antibodies as indicated (*lane 2*). *Lane 1*, untransfected control (Un). (**b**) The in vitro interactions were assessed by incubating 1 μg of His_6-PC4 bound to Ni-NTA beads with 200 ng of individual recombinant core histones (H2A, H2B, H3, and H4). The complexes were pulled down and analyzed by western blotting. *Lane 1*, individual histones (Input); *lane 2*, the histones incubated with only Ni-NTA agarose; and *lane 3*, individual histone incubated with Ni-NTA agarose bound to His_6-PC4

pull down experiments. After the pull down, immunoblotting using antibodies against the interacting proteins (here histones) could determine interaction between the proteins (Fig. 3).

1. HeLa cells were cultured in antibiotic-free DMEM media supplemented with 10 % FBS, and L-glutamate followed by incubation in CO_2 incubator till confluency reached 80 %.
2. The adherent cells were then transfected with FLAG-tagged mammalian expression vector of PC4 using Lipofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions.
3. Cells were washed gently with PBS so as to prevent dislodging.
4. The lysis buffer was added to the cells (For 10^6–10^7 cells, 1 mL of lysis buffer should be used).
5. The cells were incubated for 15–30 min on a shaker.
6. Cells were scraped and the cell lysate was centrifuged for 10 min at 12,000 × *g*, 4 °C.
7. The supernatant was transferred to a chilled eppendorf on ice and was used immediately (Cell lysate could be kept at −70 °C for later use).
8. The anti-FLAG M2 affinity resin was aliquoted (as per requirement) and is equilibrated thrice with TBS. (Generally, ~10 μL of packed gel volume binds to >1 μg FLAG fusion protein).

9. Approximately 200–1,000 μL of cell lysate (depending upon the expression level of FLAG fusion protein) was added to the equilibrated resin. The final volume was made up to 1 mL by adding lysis buffer. Cell lysate made from the untransfected cells could be used as negative control.
10. The samples were kept in a shaker for constant agitation for 2 h at 4 °C. Binding step may be extended to overnight in order to increase the binding efficiency.
11. The resin was centrifuged for 30 s–1 min at 5,000–8,200 × *g*. The supernatants were removed with a narrow-end pipette tip.
12. The resin was washed three times with 0.5 mL of TBS. Supernatant should be removed carefully without losing resins.
13. 20 μL of 2× sample buffer was added to each sample and control and heated for 3 min.
14. Sample was centrifuged briefly and supernatant was collected carefully.
15. Supernatant was loaded on to a 12 % SDS PAGE gel and western blotting was carried on using antibodies against histones (H3, H2A, H2B, and H4).

3.3.2 The In Vitro Interaction

1. 10 μL of Ni-NTA beads slurry was aliquoted in 1.5 mL eppendorf tube. Beads were equilibrated by washing thrice with 1 mL BC buffer followed by short spin.
2. 1 μg of His_6-PC4 and 200 ng of recombinant individual histones H2A, H2B, H3, and H4 were added to the beads in a final volume of 200 μL BC buffer containing 150 mM KCl.
3. The sample were supplemented with 30 mM imidazole each and kept in end to end shaker at 4 °C for 2.0 h.
4. The beads were washed five times (1 mL each) with the BC buffer containing 150 mM KCl and 30 mM imidazole.
5. 5× SDS loading dye was added to the final concentration of 1× in the sample.
6. The sample was heated and was used in western blotting using anti H2A, H2B, H3, and H4 polyclonal antibodies.
7. Control experiments were performed with 5 μL of Ni-NTA beads incubated with 200 ng of individual recombinant histones H2A, H2B, H3, and H4 without His_6-PC4 protein in the same buffer.

3.4 The siRNA Mediated Silencing of PC4

The significance of a gene product could be studied by reducing the expression of the gene using the RNAi strategy. Both the transient and stable knockdown strategies of a gene product have been popular in understanding the importance of the protein. While the transient knockdown strategies offer potential early leads, the stable knockdown approach confirms the physiological outcomes.

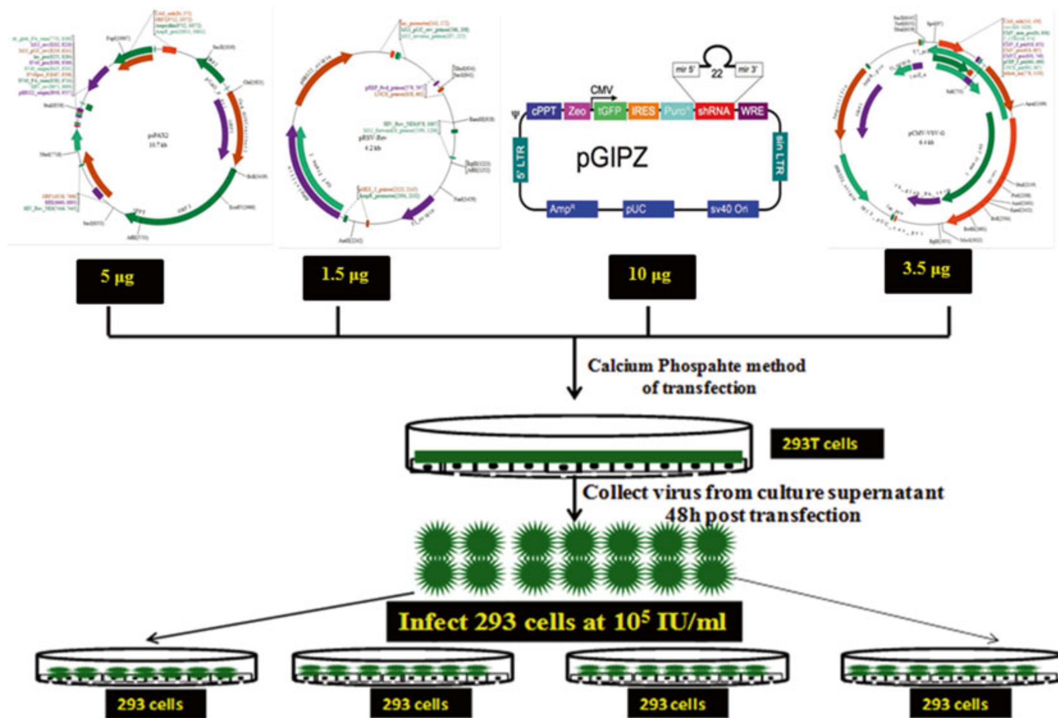

Fig. 4 The Scheme for generation of PC4 knockdown stable cell lines. The shRNA expression plasmid and the helper plasmids were transfected into HEK293T cells. Forty-eight hours following the transfection culture supernatant containing virus was collected and HEK293 cells were infected at 10^5 IU/mL. After 72 h the cells were subjected to antibiotic selection. Plasmid maps psPAX2 and pRSV Rev adapted from source: http://www.biovisualtech.com/bvplasmid/Lentiviral.htm and pCMV VSV-G from: https://www.addgene.org/12260

The shRNAs against a protein are integrated in the genome using a diverse range of delivery systems (the present protocol describes a lentiviral delivery system) (Fig. 4). The stable cell lines are passaged for several generations under the selective antibiotic pressure to ensure stable propagation of the shRNA.

1. Approximately 85,000 HeLa cells per 30 mm dish were seeded 24 h prior to transfection in DMEM complete media.
2. At the time of transfection, complete media was replaced with antibiotics and serum free media.
3. Oligofectamine (Invitrogen) was mixed with siRNAs (sense, 5′-r[ACAGAGCAGCAGCAGCAGA]dTT-3′; antisense, 5′-r[UCUGCUGCUGCUGCUCUGU]dTT-3′) and left for 20 min at room temperature. Scrambled RNA (sense, 5′-r[GAAAGGCAACGACGGACAC]dTT-3′; antisense, 5′-r[GCGAACACUAACGUACCUCAU]dTT-3′) was used as a negative control. The complex was then added to cells.
4. 5–6 h after transfection, older media was discarded and cells are replenished with fresh media containing 10 % FBS.
5. After 24–48 h cells were processed.

3.5 Establishment and Characterization of PC4 Stable Knockdown Cell Line

3.5.1 Preparation of the Lentiviral Particles

1. HEK293T cells were seeded at a density of 2×10^6 cells in a 90 mm dish.
2. 20 μg of lentiviral packaging mix comprising 10 μg of pGIPZ, 2.5 μg of VSV-G, 2.5 μg of RSV-REV and 10 μg pPAX2 was prepared.
3. The lentiviral packaging mix was transfected to adherent HEK293T cells by calcium phosphate transfection method [20].
4. 48 h after transfection, the viral supernatant were collected and spun at $537.98 \times g$ for 5 min at room temperature in a 50 mL falcon tube.
5. The viral supernatant was filtered using a 0.45 μm filter.
6. Small aliquots of 500 μL viral supernatant were made in freeze vials, flash-frozen in liquid nitrogen, and stored at −80 °C.
7. The lentiviral vector stocks were normalized based on p24 antigen content using HIV-1 p24 ELISA kit as per the manufacture's protocol. The infectious titers of the viruses encoding EGFP were determined by flow cytometry.

3.5.2 Transduction of HEK293 Cells with Lentiviral Particles

1. HEK 293 cells were seeded in a 90 mm dish at a density of 2×10^6 cells for each lentiviral construct and incubated overnight at 37 °C in a humidified incubator at 5 % CO_2.
2. Complete DMEM containing polybrene to a final concentration of 10 μg/mL was prepared.
3. Lentiviral particles were added to the medium at a multiplicity of infection (MOI) one to the plate and mixed by gentle swirling and incubated overnight at 37 °C in a humidified incubator at 5 % CO_2.
4. 48 h post-infection, GFP expression was monitored under fluorescence microscope.
5. The older media was removed, cells were washed with 1× PBS and were subsequently split.

3.5.3 Selection of Stable Knockdown Cells by Puromycin Selection

1. The virus transduced cells were seeded in a 60 mm dish at a density of 10^6 cells.
2. The cells were supplemented with complete DMEM medium containing puromycin at a concentration of 1 μg/mL.
3. The medium was replaced every 2 days with complete DMEM medium with puromycin at a concentration of 1 μg/mL.
4. The cells were examined daily for cell death. After stabilization the cells were split, aliquoted and frozen stocks of the stable cell line were prepared.
5. The cells were passaged and assayed for the knockdown efficiency of the shRNAs by western blot or real-time PCR.

3.5.4 Validation of PC4 Knockdown

Whole RNA Extraction and Real-Time PCR

1. PC4 stable knockdown and control cells were cultured in DMEM medium with 10 % FBS in 37 °C CO_2 incubator till 75–80 % confluency.
2. Cells were harvested from the culture dishes by scraping or trypsinization (with 0.025 % trypsin–EDTA).
3. Cell pellet was washed with 1× PBS followed by homogenous resuspension in TRIZOL (Invitrogen) reagent at a ratio of 1 mL per ten million cells.
4. Cell suspension was centrifuged at 19367.3×*g*, 4 °C for 10 min.
5. The supernatant was subjected to chloroform extraction thrice and aqueous layer was precipitated by equal volume of isopropanol.
6. Precipitated pellet was resuspended in 70 μL DEPC treated water and was subjected to precipitation with 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of isopropanol.
7. The mixture was kept at −70 °C for 1 h followed by centrifugation at 22729.73×*g*, 4 °C for 30 min.
8. Pellet was washed with 70 % ethanol, air-dried, and dissolved in nuclease-free water, estimated by NanoDrop spectrophotometer and visualized in 1 % agarose gel for the integrity of RNA.
9. 1 μg of total RNA was used for cDNA synthesis with initial 12 μL reaction volume containing 40 pmoles of oligo-dT and water.
10. The reaction mixture was incubated at 70 °C for 10 min, followed by quick chilling on ice for 10 min.
11. The reaction was subsequently continued by the addition of 0.5 mM dNTP mix, 4 μL 5× first strand synthesis buffer, and 1 μL superscript RT enzyme.
12. Reaction was carried out at 37 °C for 50 min followed by enzyme in activation at 84–90 °C for 15 min.
13. The cDNA was subsequently used for real-time PCR using gene-specific primers and was stored at −20 °C.
14. 1 μL of respective cDNA was used in PCR reaction mix containing specific gene primers (here PC4 and actin gene primers) and 2× SYBR green mix. SYBR green mix contains SYBR green I dye and required PCR reagents like dNTPs, DNA polymerase and compatible buffers.
15. Reaction tube was put in Corbett real-time PCR machine and amplification protocols were followed as provided by manufacturers. PCR conditions were standardized for each set of gene primers used.

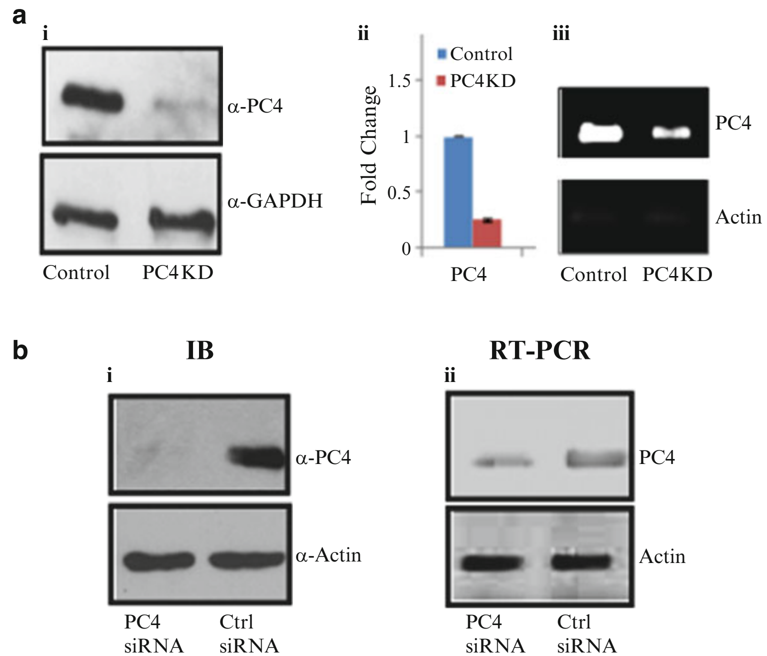

Fig. 5 The validation of PC4 downregulation. (**a**) The PC4 knockdown stable cells (PC4 KD) were validated for the PC4 expression using western blot (IB) (i) and RT-PCR (ii, iii). (**b**) Knockdown of PC4 with siRNA. Silencing of the PC4 expression was achieved using siRNAs either nonspecific (control siRNA) or against PC4 (PC4 siRNA). The alteration in the expression was scored by western blotting (IB) (i) and RT-PCR (ii)

16. Fold expression change was calculated using ΔΔCt method using actin gene primers for normalization. Relative fold change = $2^{-\Delta\Delta Ct}$. Specificity and sensitivity of the primers was ascertained by melt curve analysis.
17. PCR reaction mix was loaded on 1 % agarose gel and amplicon was visualized by UV light in the presence of ethidium bromide (Fig. 5a ii, b ii).

Whole Protein Extraction and Western Blotting

1. Cells were harvested by scraping or trypsinizing following 1× PBS wash.
2. Cell pellet was resuspended in RIPA or TNN lysis buffer (up to 5–10 times packed cell pellet volume). Cell suspension was incubated in ice for 3 h with regular mixing followed by centrifugation at 13449.54 × *g*, 4 °C for 15 min.
3. Supernatant (whole cell lysate) was either used immediately or stored at −80 °C.

4. Bradford protein estimation reagent was used to estimate protein concentration in whole cell lysate using UV/visible spectrophotometer. Known concentrations of BSA were used for plotting standard curve. A linear curve generated by absorbance values of known BSA concentrations was employed to assign concentration values to unknown samples.
5. Equal amount of cell lysates were taken and loading dye was added such that final concentration of dye was 1×. Sample–dye mixture was heated on dry bath (90–95 °C) for 5 min and loaded in a SDS-PAGE gel.
6. After the run, the gel was incubated for a period of 15 min in transfer buffer along with the nitrocellulose membrane of same dimensions as of gel.
7. Proteins were then transferred to the membrane using a semidry western transfer apparatus at 25 V, for appropriate period of time depending upon mobility of the protein on SDS PAGE gel.
8. After transfer, blot was blocked in 5 % skimmed milk solution or 3 % BSA in PBS for overnight at 4 °C or at room temperature for 1 h.
9. The blot was incubated with primary antibody (PC4 or any other) solution (diluted in 2.5 % milk solution or 1 % BSA) for a period of 3 h at 4 °C (or overnight depending upon the affinity of the antibodies used).
10. Blot was subjected to washing with PBS containing 0.05 % Tween 20 for 10 min.
11. Appropriate HRP conjugated secondary antibody solution was then added for a period of 3 h at 4 °C.
12. Blot was again subjected to washing with 0.05 % Tween 20 containing PBS.
13. After washing of blots, the blot was developed using the Pierce Super Signal West Pico chemiluminescence kit, as per the manufacturer's protocol.
14. The blots were exposed in Kodak X-ray films, for different time points and developed using GBX-Developer-Fixer Kit (Fig. 5a i, b i).

The Distribution of PC4 on Metaphase Chromosomes

The protocol is used to ascertain the presence and distribution of a protein on mitotic chromatin (chromosomes).

1. HEK293 cells and HEK293 PC4 Knockdown stable cells were cultured as monolayers on culture flask in DMEM medium supplemented with 10 % FBS. Cells were grown till 60–70 % confluency at 37 °C in CO_2 incubator.
2. Cells were treated with 100 ng/mL Nocodazole for 24 h.

3. Culture flask was shaken gently and the floating cells were collected.
4. Cells were resuspended in 75 mM KCl for 10 min on ice.
5. Swollen cells were then fixed in a fixative solution (3:1 methanol) and the cell suspensions were spread on to a clean microscopic slide.
6. Condensed mitotic metaphase chromosomes were then processed for immunostaining. 1 % FBS in 1× PBS was added onto the slide for 1 h at room temperature.
7. Chromosomes were then probed with polyclonal antibody against PC4 (reconstituted in 1 % FBS in 1× PBS) for overnight at 4 °C.
8. After mild wash with 1× PBS, appropriate secondary antibody conjugated with Alexa dye was incubated for 1 h at room temperature.
9. To stain the chromosomal DNA Hoechst 33258 (Sigma) was used for 20 min at room temperature.
10. Fluorescence was visualized by using confocal laser scanning microscope (Fig. 6).

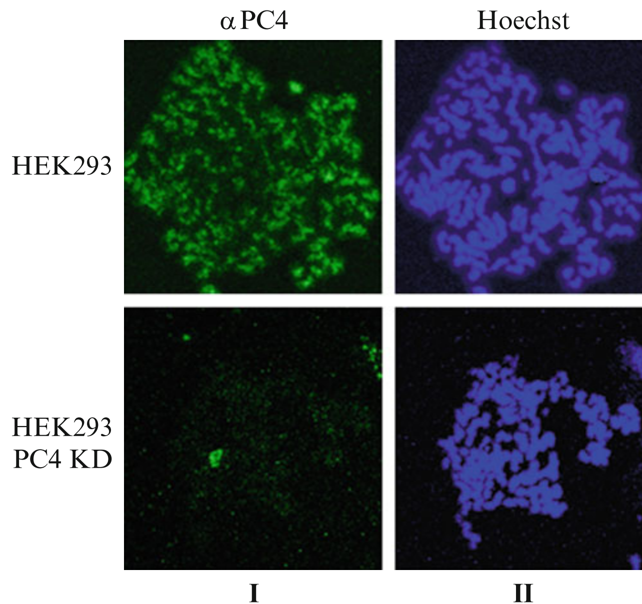

Fig. 6 Distribution of PC4 on mitotic chromosomes Immunostaining to visualize the localization of PC4 in HEK293 and HEK293 PC4 knockdown mitotic chromosome spreads. After the preparation of metaphase spreads chromosomes were stained with anti-PC4 (I) antibodies and counterstained with Hoechst (II)

3.6 Chromatin Characterization Upon Knockdown of PC4

Ordered chromatin structure is critical for proper cellular functioning. A large number of experimental strategies could be used to examine the influence of a nuclear protein on the maintenance of the structural integrity of the chromatin which in turn could modulate its function. The eukaryotic chromatin is typically compartmentalized into a denser heterochromatin tightly packed and a lightly packed euchromatin. A fluorescent dye, 4′,6-diamidino-2-phenylindole (DAPI) could differentiate between the two forms of the chromatin. DAPI stained heterochromatin appears as denser foci compared to the euchromatin region that takes lesser stain. A loss of such demarcation between these two chromatin phases or a significant alteration to the staining pattern is suggestive of the changes in the chromatin structure (Fig. 7). The state of the chromatin is also regulated by a set of epigenetic marks. Changes in such epigenetic marks may reveal altered structure function relation of the chromatin in terms of gene expression (Fig. 8). Compaction of the chromatin is measured by its accessibility to micrococcal nuclease. The chromatin condensed to a smaller extent, as compared to the one more compactly condensed, is more accessible to the enzyme which digests the chromatin to oligonucleosomes of smaller size (Fig. 9). The change in the chromatin structure can also be visualized by atomic force microscopy as discussed in the following section (Fig. 10).

3.6.1 Heterochromatin Foci Analysis: DAPI Staining

1. Cells were fixed in 2 % paraformaldehyde in 1× PBS for 20 min at room temperature.
2. After washing cells twice with 1× PBS, the cells were permeabilized by 1 % Triton X-100 (reconstituted in PBS), followed by blocking in 1 % FBS.
3. In order to visualize the DNA, the cells were stained with 0.1 μg /mL Hoechst 33258 in PBS.

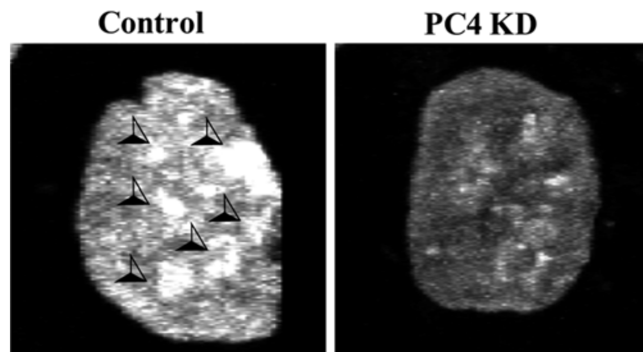

Fig. 7 Alterations in the nuclear architecture upon knocking down PC4. Hoechst staining images of control and PC4 knockdown stable cells (PC4 KD)

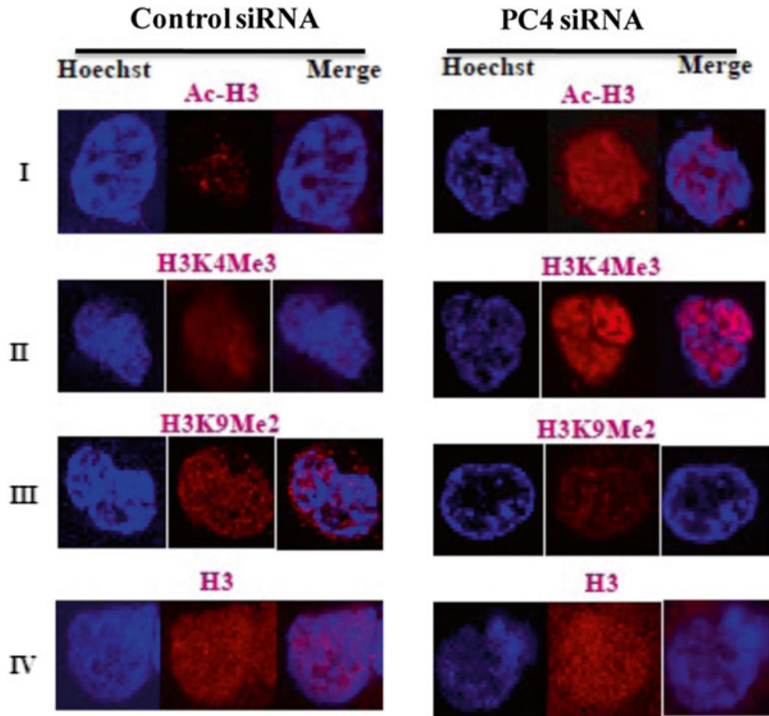

Fig. 8 Altered epigenetic marker analysis following PC4 silencing using immunofluorescence. Comparable enhancement in Ac-H3 (*panel I*) and H3K4Me3 (*panel II*) but reduction in H3K9Me2 (*panel III*) levels following PC4 silencing. PC4 expression remained unaltered in control and PC4 siRNA treatments (*panel IV*)

4. Fluorescence for Hoechst was visualized by using Carl Zeiss confocal microscope.
5. Individual plane images (Z-stacking) were captured and then merged to obtain a 3 dimensional image.

3.6.2 MNase Accessibility Assay

1. Untransfected, control siRNA (scRNA) and PC4 targeting siRNA(siRNA) transfected HeLa cells were resuspended in hypotonic buffer for 30 min in ice.
2. The cell suspension was centrifuged at 134.49 × *g* at 4 °C for 10 min.
3. The nuclei were subjected to partial MNase digestion (0.01 U/μL) in nuclei digestion buffer for 10 min at room temperature.
4. The reaction was stopped using 10 mM EDTA.
5. Individual reactions were treated with Proteinase K at 37 °C for 30 min followed by phenol- chloroform extraction.
6. Subsequently RNase treatment was carried out at 37 °C for 30 min followed by re-extraction with phenol- chloroform.
7. The DNA was precipitated by ethanol and analyzed on a 1 % agarose gel.

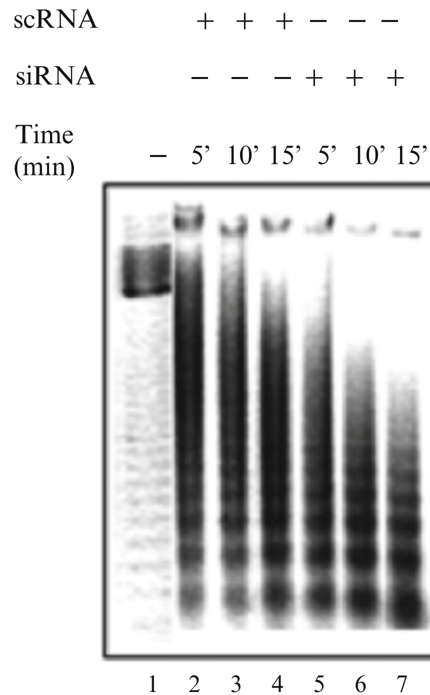

Fig. 9 MNase accessibility of the chromatin isolated from PC4 knockdown cells. HeLa cells were treated with PC4-specific or the control siRNA and the chromatin was extracted. Partial MNase digestions were carried out at three different time points using the extracted chromatin. *Lane 1*, 123 bp ladder; *lanes 2–4*, the chromatin isolated from scRNA transfected HeLa cells subjected to 5, 10, 15 min of MNase digestion and *lanes 5–7*, same time points of MNase digestions were carried out with the chromatin isolated from siRNA transfected HeLa cells

8. Similar MNase digestions were also carried out at three different time points (5, 10 and 15 min) with the chromatin isolated from siRNA and scRNA transfected HeLa cells to study the alteration in chromatin accessibility by MNase as a function of time.
9. Equal amount of DNA was loaded in 1 % agarose gel and then stained with ethidium bromide solution for 30 min in dark.
10. Image was captured using gel-doc system.

3.6.3 AFM Visualization of Chromatin

1. Approximately one million HeLa cells were cultured in a 35-mm dish.
2. Cells were washed with PBS twice followed by resuspension in buffer A for 10 min at 4 °C.
3. Cells were centrifuged at 134.49 × *g* for 5 min at 4 °C.
4. Cells were then fixed with 4 % paraformaldehyde for 20 min at room temperature.
5. For visualizing partially MNase-digested nuclei, the cell pellet was washed with buffer A followed by resuspension in buffer A without Triton X-100.

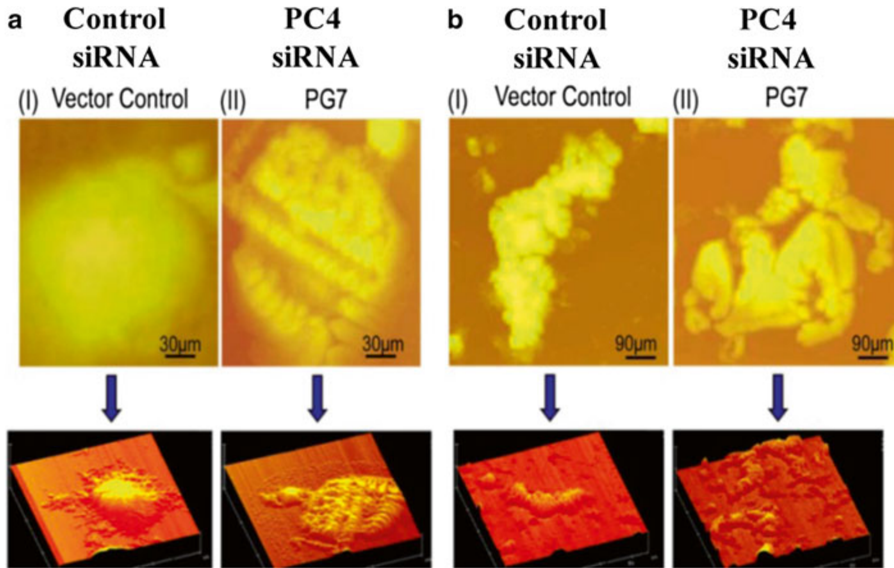

Fig. 10 AFM reveals an altered chromatin organization in PC4-silenced cells. HeLa cells were transfected with either the PC4-specific or a control siRNA. Transfection was confirmed by the coexpression of GFP encoded by the vector. Following transfection, intact (**a**) or partially MNase-digested (**b**) HeLa cell nuclei were subjected to the AFM analysis. The *top panels* in (**a**) and (**b**) show the AFM images, whereas the *bottom panels* show the three-dimensional surface topology plots of the AFM images

6. 0.6 U/μL of MNase was added to the cells in the presence of 3 mM $CaCl_2$. Cells were allowed to incubate for 20 min at 37 °C.
7. 200 mM EDTA was added to the cells and was chilled on ice to stop the reaction.
8. The cells were then centrifuged at 134.49 × *g* for 5 min at 4 °C and resuspended in 4 % paraformaldehyde.
9. Paraformaldehyde fixed nuclei or MNase pellet was layered onto a poly-L-lysine coated glass coverslip. The sample was incubated for 15 min at 25 °C and then washed with PBS supplemented with 0.1 μg/mL of Hoechst 33258.
10. The sample was completely dried under nitrogen gas.
11. AFM observation was performed with a Bioscope/Bioscope SZ NanoScope IIIa controller (Veeco Instruments, Santa Barbara, CA) using the contact mode. The cantilever (Non-Conductive Silicon Nitride, Model DNP-20, Veeco Instruments) was 0.4–0.7 mm in length with a spring constant of 0.06 N/m. The scanning frequency was 1.001 Hz, and the images were captured with the height mode in 256 × 256- and 512 × 512-pixel formats.

3.6.4 Epigenetic Marks Analysis

1. Control siRNA and PC4 siRNA treated cells were grown in poly-lysine coated glass coverslips for 24 h in desired medium.
2. Cells were fixed in 4 % paraformaldehyde solution made in PBS for 20 min at room temperature.

3. After fixation, cells were washed thrice with PBS.
4. Cells were permeabilized by permeabilizing buffer for 5 min at room temperature.
5. Permeabilized cells were subjected to blocking buffer at 37 °C for 45 min.
6. Probing was done with different antibodies at appropriate dilutions at room temperature for 1 h followed by incubation with corresponding Alexa fluorophores conjugated secondary antibodies at room temperature for 1 h.
7. Three washes with wash buffer after primary and secondary antibodies incubation was given.
8. A drop of mounting solution was put in the centre of a clean glass slide and coverslip containing cells were mounted upside down such that cells face the mounting solution.
9. Fluorescence was visualized by using confocal laser scanning microscope.

3.7 Visualization of In Vitro Chromatin Compaction Ability of PC4

3.7.1 Purification of the HeLa Core Histones

1. Actively growing HeLa cells were harvested by trypsinization followed by 1× PBS wash.
2. Cell pellet was resuspended with 10 times cell packed volume of hypotonic buffer for 10 min on ice.
3. Cell suspension was centrifuged at 86.1 × *g*, 4 °C and supernatant was discarded. The core histone octamer was then purified from HeLa nuclear pellet.
4. The nuclear pellet was resuspended in Buffer A and homogenized in a Dounce's homogenizer (Wheaton) with pestle B for 30 min on ice.
5. The suspension was cleared by centrifugation at 26361.89 × *g* for 20 min at 4 °C.
6. Supernatant was incubated with pre-soaked (10 mM Potassium phosphate buffer pH 6.7) Hydroxyapatite BioGel HTP (0.5 g beads per mL of nuclear pellet).
7. The sample was incubated for 3 h at 4 °C in an end-to-end shaker.
8. The resin was washed with buffer A with 630 mM NaCl with brief centrifuge.
9. The beads were packed into an Econoglass column and washed overnight with the same wash buffer.
10. The core histones were eluted in buffer A containing 2 M NaCl and analyzed in a 15 % SDS PAGE, dialyzed in BC100, aliquoted and stored in −80 °C.

3.7.2 Chromatin Assembly on a 100 kb Plasmid

1. 0.5 μg of the purified 100 kb plasmid DNA and 0.5 μg histone octamer (Purified from HeLa) were mixed in Hi-buffer and placed in a dialysis tube up to final volume of 50 μL.

2. The dialysis was started with 150 mL of Hi-buffer with stirring at 4 °C.
3. Lo-buffer was added to the dialysis buffer at a rate of 0.46 mL/min, and simultaneously, the dialysis buffer was pumped out at the same speed with a peristaltic pump so that the dialysis buffer contained 50 mM NaCl after 20 h.
4. The sample was collected from the dialysis tube and stored at 4 °C.

3.7.3 AFM Visualization of PC4-Mediated Chromatin Compaction

1. Recombinant PC4 and H1 proteins were mixed with the reconstituted chromatin in the molar ratio of 1:1 and incubated on ice for 60 min.
2. The samples were diluted tenfold by the fixation buffer.
3. After fixation with glutaraldehyde for 30 min at room temperature, the samples were dropped onto a freshly cleaved mica substrate, which was pretreated with 10 mM Spermidine.
4. After 15 min, the mica was washed with water and dried under nitrogen.
5. AFM observation was performed with NanoScope IIIa or IV (Digital Instruments) using the cantilever (OMCL-AC160TS-W2, Olympus) of 129 μm length with a spring constant of 33–62 N/m in air under the tapping mode. The scanning frequency was 2–3 Hz, and images were captured with the height mode in a 512 × 512 pixel format.
6. The obtained images were processed (plane-fitted and flattened) by the program accompanying the imaging module (Fig. 11).

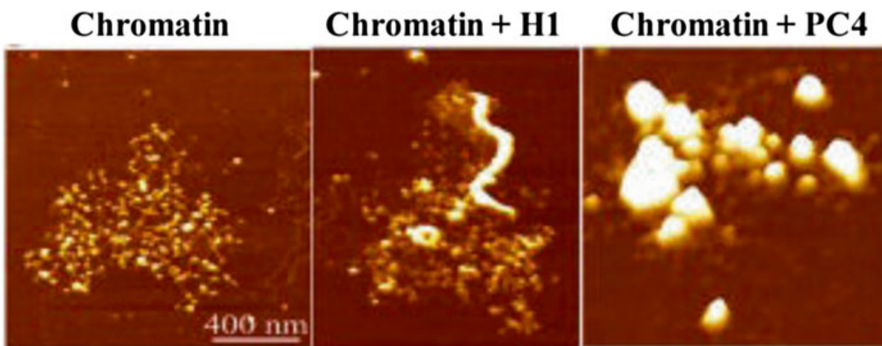

Fig. 11 PC4 condenses the chromatin fiber into a distinct globular structure. AFM images of the 100 kb reconstituted chromatin fibers incubated with or without PC4. The molar ratio of PC4/H1 to the histone octamer was 1:1. Upon 60 min incubation on ice the complexes were fixed by 0.3 % glutaraldehyde, mounted on mica, and observed under AFM

4 Notes

1. SDS running buffer, loading dye, TBE buffer, 6× gel loading dye were used as 1× and respectively diluted from otherwise highly concentrated stocks.
2. While performing western blotting, primary antibody concentration, incubation and blot washing time was standardized for each antibody used.
3. For immunofluorescence, each primary and secondary antibody dilution was standardized.
4. For anti FLAG M2 agarose affinity pull down, if the expression level of the FLAG fusion protein is relatively low, the cells should be lysed with a reduced volume of lysis buffer (Ref.: Sambrook and Russel; Volume 3 Molecular Cloning: A loboratory Manual).
5. All the reagents were procured from Sigma-Aldrich unless otherwise indicated.

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Chapter 15

Methods to Study Transcription-Coupled Repair in Chromatin

Hélène Gaillard, Ralf Erik Wellinger, and Andrés Aguilera

Abstract

The effect of endogenous and exogenous DNA damage on the cellular metabolism can be studied at the genetic and molecular level. A paradigmatic case is the repair of UV-induced pyrimidine dimers (PDs) by nucleotide excision repair (NER) in *Saccharomyces cerevisiae*. To follow the formation and repair of PDs at specific chromosome loci, cells are irradiated with UV-light and incubated in the dark to allow repair by NER. Upon DNA isolation, cyclobutane pyrimidine dimers, which account for about 90 % of PDs, can be cleaved in vitro by the DNA nicking activity of the T4 endonuclease V repair enzyme. Subsequently, strand-specific repair in a suitable restriction fragment is determined by denaturing gel electrophoresis followed by Southern blot and indirect end-labeling using a single-stranded DNA probe. Noteworthy, this protocol could potentially be adapted to other kind of DNA lesions, as long as a DNA nick is formed or a lesion-specific endonuclease is available.

Transcription-coupled repair (TC-NER) is a sub-pathway of NER that catalyzes the repair of the transcribed strand of active genes. RNA polymerase II is essential for TC-NER, and its occupancy on a damaged template can be analyzed by chromatin immunoprecipitation (ChIP). In this chapter, we provide an up-dated protocol for both the DNA repair analysis and ChIP approaches to study TC-NER in yeast chromatin.

Key words DNA damage, UV, Cyclobutane pyrimidine dimer, Nucleotide excision repair, Transcription-coupled repair, Chromatin immunoprecipitation

1 Introduction

Nucleotide excision repair (NER) is a major, evolutionarily conserved DNA repair pathway that fixes bulky DNA lesions, including UV induced photoproducts, and adducts derived from cisplatin and 4-nitroquinoline oxide treatment [1–3]. The NER reaction can be reconstituted in vitro [4], and different human disorders are characterized by defective NER including xeroderma pigmentosum (XP), trichothiodystrophy (TTD), Cockayne syndrome (CS), and UV sensitivity syndrome [5, 6]. Hypersensitivity to UV-light exposure is a characteristic of all four diseases but sun exposure is

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accompanied by an approximate 1,000-fold increase in the probability of developing skin cancer in the case of XP. Interestingly, human genes leading to these disorders include basal transcription complexes, which suggests that the cooperation between NER and transcription is important to achieve optimal genome repair. NER can be subdivided into global genome repair (GG-NER), which repairs lesions in the entire genome, and transcription coupled repair (TC-NER), which allows fast repair of the transcribed strand of active genes. GG-NER and TC-NER share the core proteins of excision repair but differ in the factors involved in damage recognition. Initial work by Phil Hanawalt and colleagues has led to a more detailed understanding of TC-NER in *E. coli*, which is mediated by a single transcription-repair coupling factor [7]. In eukaryotes the precise mechanism of TC-NER remains poorly understood [8–10], likely because of fundamental differences such as the presence of the nuclear envelope and the coupling between mRNA synthesis, processing, and export [11].

Molecular analysis of DNA damages at specific loci represents the most direct method to study TC-NER. Upon irradiation with a defined dose of UV-light, chromosomal DNA accumulates cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts, which are repaired by NER or by a photolyase in bacteria and lower eukaryotes such as *Saccharomyces cerevisiae*. As photolyase requires visible light for repair, performing experiments in the absence of photoreactivating light restricts the analysis to NER. Isolated DNA is first cleaved with suitable restriction enzymes, then nicked with the CPD recognizing T4endoV enzyme [12], and separated by electrophoresis in denaturing conditions to analyze the migration of single-stranded DNA. DNA denaturation allows the determination of strand-specific repair by Southern blot and indirect end-labeling using a single-stranded specific probe. An example is shown in which repair of the housekeeping *RPB2* gene was analyzed in the *rad7*Δ GG-NER mutant (Fig. 1a). Repair is detected by the recovery of the intact DNA fragment migrating on top of a smear-like signal. Within this smear-like signal specific bands are apparent that mark the presence of DNA damage hot-spots within the fragment analyzed. Since UV-damage is sequence specific, CPD hot-spots in the Crick strand will lead to damage cold-spots in the Watson strand and vice versa. Recovery of the intact DNA fragment at various repair time points can be measured by calculation the relative signal intensity of the intact DNA fragment versus the total signal. Repair curves are usually displayed as the percentage of repair (Fig. 1b). In wild-type cells repair of the transcribed strand occurs much faster than repair of the non-transcribed strand. In *rad7*Δ mutants repair of the transcribed strand is not affected while repair of the non-transcribed strand is severely compromised.

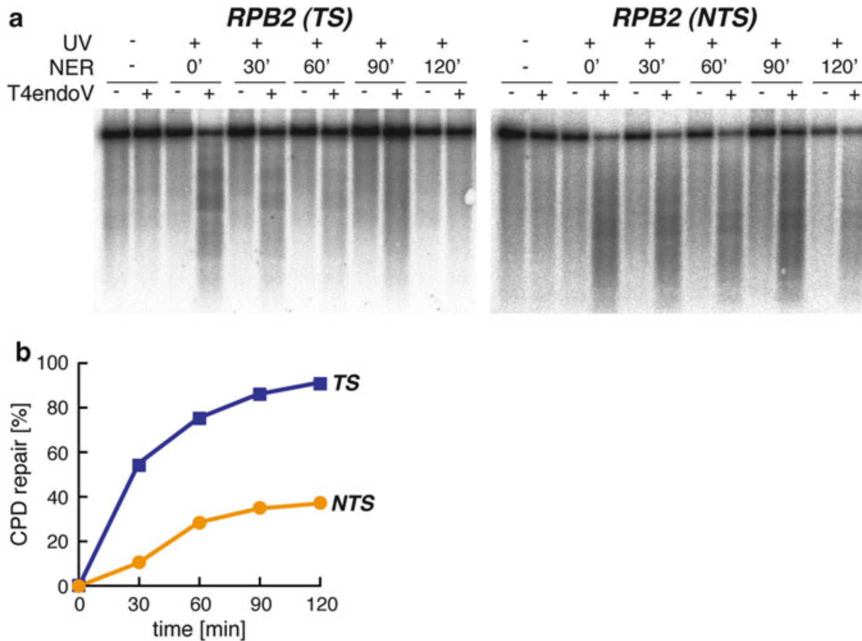

Fig. 1 Example for an experiment using the method described to analyze the CPD repair rates on the transcribed (TS) and the non-transcribed (NTS) strands of the constitutively expressed *RPB2* gene. (**a**) Yeast cells in which the Rad7 GG-NER protein is deleted were irradiated with 150 J/m^2 UV light or mock treated. Repair was analyzed by indirect end-labeling in a 4.4 kb (NsiI/PvuI) *RPB2* fragment. The remaining intact restriction fragment after treatment of damaged DNA with T4 endonuclease V (+UV, +T4endoV) corresponds to the fraction of undamaged DNA. Nonirradiated DNA (–UV) and DNA not treated with T4endoV (–T4endoV) were used as controls. (**b**) Graphical representation of the values obtained by quantification of the gels shown in (**a**)

More recent approaches have assessed the role of transcription factors in TC-NER by analyzing RNA polymerase II (RNAP II) occupancy on a damaged template by chromatin immunoprecipitation (ChIP) [13–15]. The ChIP-assay was originally developed to determine the natural site of action of the chromatin binding proteins of the POLYCOMP-complex [16] and adapted for virtually any chromatin-bound factor [17–19]. Proteins are cross-linked to DNA in vivo by formaldehyde treatment of cells, thus preventing redistribution of chromatin components during chromatin preparation, and enrichment of the protein of interest is done by subsequent immunoprecipitation. Upon reversal of the protein–DNA cross-links, the amount of co-precipitated DNA fragments can be detected by real-time quantitative PCR. In the first edition of this protocol [20], we included an in vitro photoreactivation step prior to PCR analysis because it has been shown that UV photoproducts such as CPDs block the elongation of Taq-DNA polymerases [21]. Photolyase is no longer commercially available and the omission of the in vitro photoreactivation step may lead to a

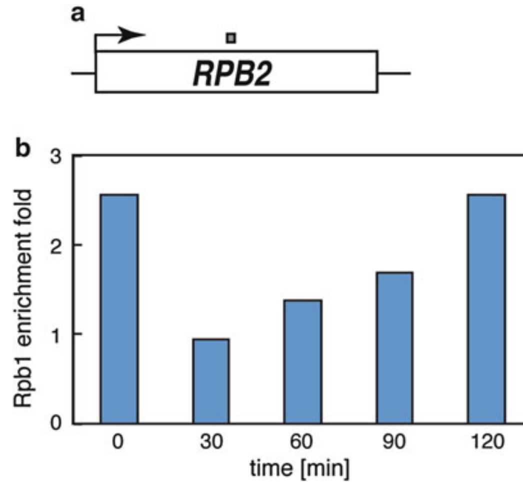

Fig. 2 Example for an experiment using the method described to analyze the association of Rpb1 (the largest subunit of RNAP II holoenzyme) with the *RPB2* gene in UV irradiated yeast cells. (**a**) Scheme of the *RPB2* gene and position of the 67-bp long amplicon used in the qPCR analysis. (**b**) Wild-type cells were irradiated with 150 J/m^2 UV light or mock treated. After formaldehyde crosslinking at the indicated time points, cell lysates were prepared and subjected to immunoprecipitation with an antibody against the Rpb1 C-terminal domain (CTD). DNA from INPUT and PRECIPITATE were isolated and analyzed by quantitative real-time qPCR analysis using primer pairs specific for the RPB2 gene and for the 9716–9863 intergenic region of chromosome V, which was used as non-transcribed control. Standard curves for all pairs of primers were performed for each PCR analysis, all PCR reactions being performed in triplicate. The fold enrichment of Rpb1 binding was calculated as the ratio between the RPB2-specific signal and the intergenic signal of the PRECIPITATE normalized with respect to the corresponding ratios of the INPUT. In response to UV irradiation, RNAPII transcription is transiently repressed and Rpb1 is degraded [24–26], resulting in the observed loss of polymerase on the analyzed gene. Recovery of transcriptional activity upon increasing repair time is an indirect measurement of DNA repair

biased amplification of short DNA fragments of highly damaged DNA. As shown in an example in wild-type cells (Fig. 2), keeping the amount of DNA lesions low to minimize interferences with the PCR elongation process leads to comparable results as with the originally published protocol. A fully updated protocol to that previously published [20] is reported here for the analysis of TC-NER in chromatin.

2 Materials

2.1 UV Irradiation and Repair

1. Yeast cultures.
2. Medium.
3. Round pyrex trays (SCHOTT, 19 cm diameter).

4. BS03 irradiation chamber equipped with UV-C lamps (Philips TUV 15 W, irradiation peak at 254 nm) and UV-Mat dosimeter (Dr. Gröbel UV-Elektronik GmbH; *see* **Note 1**).
5. Medium containing appropriate amino acids at a 3× concentration.
6. Dark rooms equipped with safety lights (Philips TLD RED).
7. Refrigerated table centrifuge (Beckman Coulter, Rotor C0650).

2.2 Purification of DNA for Repair Analysis

1. Freshly made spheroplasting buffer: 1 M sorbitol, 10 mM Tris–HCl pH 7.6, 100 mM EDTA pH 8.0, 0.1 % v/v beta-mercaptoethanol.
2. Zymolyase stock solution: 1,000 U/ml (USB).
3. Solution I: 1.6 M GuHCl, 30 mM EDTA pH 8.0, 30 mM Tris–HCl pH 7.6, 5 % Tween 20, 0.5 % Triton X-100.
4. RNase A stock solution: 10 mg/ml (Roche; *see* **Note 2**).
5. Proteinase K stock solution: 20 mg/ml (Roche).
6. NaCl stock solution: 2.5 M.
7. D/I: dichloroform–isoamyl alcohol (24:1).
8. Solution II: 1 % w/v CTAB (cetyltrimethylammonium bromide), 50 mM Tris–HCl pH 7.6, 10 mM EDTA pH 8.0.
9. Na-solution: 500 mM NaCl, 10 mM Tris–HCl pH 7.6, 1 mM EDTA pH 8.0.
10. Isopropanol (at room temperature).
11. 70 % ethanol (at room temperature).

2.3 Repair Analysis by Indirect End-Labeling

1. Appropriate DNA fragments (*see* **Note 3**).
2. Nucleotide stock solution: 10 mM dATP, 10 mM dTTP, 10 mM dGTP (Roche).
3. ^{32}PαdCTP (3,000 mCi/mmol; Amersham Biosciences).
4. Taq polymerase and 10× reaction buffer.
5. Thermocycler.
6. Restriction enzymes and appropriate 10× buffers (Roche).
7. StrataClean Resin (Stratagene).
8. 50T5E buffer: 50 mM Tris–HCl pH 7.5, 5 mM EDTA pH 8.0.
9. 50 % v/v slurry of Sephadex G-50 (SIGMA) in 50T5E buffer (keep at 4 °C).
10. Empty micro Bio-Spin chromatography columns (Biorad).
11. T4-endonuclease V (Epicentre Technologies).
12. Vacuum concentrator system (SpeedVac, Thermo Scientific).
13. Agarose.
14. Urea.

15. Water bath at 65 °C.
16. TAE electrophoresis buffer: 40 mM Tris–acetate, 1 mM EDTA pH 8.0.
17. Loading buffer: 8 M urea, 10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 1 % IGEPAL CA-630, few dusts of bromophenol blue.
18. Denaturing solution: 1.5 M NaCl, 0.5 M NaOH.
19. Neutralizing solution: 0.5 M Tris–HCl pH 7.6, 1.5 M NaCl.
20. 20× SSC: 3 M NaCl, 0.3 M sodium citrate pH 7.0.
21. 3MM Whatman paper.
22. Filter paper.
23. Hybond N blotting membrane (Amersham Biosciences).
24. Hybridization solution: 0.25 M Na_2HPO_4, 7 % SDS, 1 mM EDTA.
25. 20× SSPE: 3 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA, pH 7.4.
26. Wash solution I: 0.1× SSPE, 5 mM EDTA, 0.5 % SDS.
27. Wash solution II: 0.5× SSC, 0.1 % SDS.
28. Horizontal gel apparatus (OWL, gel size: 20 × 25 cm).

2.4 ChIP Analysis

1. Formaldehyde 37 % (Merck).
2. Glycine stock solution: 2.5 M in H_2O.
3. 10× PBS: 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.4.
4. Protein A-coated magnetic beads (Dynabeads, Novex by Life Technologies; *see* **Note 4**).
5. Magnetic rack (Life Technologies).
6. PBS/BSA buffer: 1× PBS, 5 mg/ml BSA.
7. Lysis buffer: 50 mM HEPES/KOH pH 7.5, 140 mM NaCl, 1 mM EDTA pH 8.0, 1 % Triton X-100, 0.1 % sodium deoxycholate, 1 mM PMSF, 1× Complete protease inhibitor cocktail (Roche; *see* **Note 5**).
8. Acid-washed glass beads (450–600 μm, SIGMA).
9. Bioruptor (Diagenode).
10. Affinity purified, specific antibody.
11. Lysis/NaCl Buffer: 50 mM HEPES KOH pH 7.5, 140 mM NaCl, 1 mM EDTA pH 8.0, 1 % Triton X-100, 0.1 % sodium deoxycholate, 500 mM NaCl (*see* **Note 5**).
12. Buffer III: 10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 250 mM LiCl, 0.5 % NP40, 0.5 % sodium deoxycholate (*see* **Note 5**).

13. TE buffer: 10 mM Tris–HCl pH 7.6, 1 mM EDTA pH 8.0.
14. Elution buffer: 10 mM Tris–HCl pH 7.6, 1 mM EDTA pH 8.0, 1 % SDS.
15. Pronase stock solution: 50 mg/ml (Sigma).
16. Wizard SV gel and PCR clean-up columns (Promega).
17. Power SYBR green PCR master mix (Applied Biosystems).
18. Appropriate oligonucleotides (*see* **Note 6**).
19. 7500 Fast Real-Time PCR System (Applied Biosystems).

3 Methods

3.1 UV Irradiation and Repair Kinetics of Yeast Cells

1. Grow 400 ml yeast cultures in the appropriate medium and temperature to an absorbance at 600 nm of about 0.8 (*see* **Note** 7).
2. Harvest cells by centrifugation in a JA-10 rotor (Beckman Coulter) at 6,000 × *g* and 25 °C for 5 min.
3. Resuspend cells in minimal medium without amino acids to an absorbance at 600 nm of 1.2 (*see* **Note 8**).
4. Do all steps from irradiation to lysis of spheroplasts in dark rooms equipped with safety light to avoid photoreactivation (*see* **Note 9**).
5. Pour 200 ml cell suspension into a Pyrex tray to produce a 4-mm layer. Take care to keep the remaining cell suspension away from UV light for the nonirradiated control (*see* **Note 10**).
6. Irradiate at room temperature with 150 J/m^2 of UV light (predominantly 254 nm) generated by germicidal lamps.
7. Immediately after irradiation, pour the cell suspension into an Erlenmeyer flask containing 100 ml of concentrated medium (with 3× amino acids).
8. For the nonirradiated control, pour 40 ml cell suspension into an Erlenmeyer flask containing 20 ml of concentrated medium.
9. Incubate both the irradiated and nonirradiated cells on a shaker at 30 °C (with safety light).
10. For repair analysis, remove 50 ml aliquots at 0, 30, 60, 90, and 120 min, chill on ice, and harvest cells by centrifugation at 3,000 × *g*, 4 °C for 5 min. The nonirradiated sample is taken at 0 min.
11. For ChIp analysis, remove 40 ml aliquots at 0, 15, 30, 60, and 90 min and process as described below.

3.2 Purification of DNA for Repair Analysis

1. Wash the cells with 50 ml cold H_2O.
2. Harvest cells by centrifugation at 3,000 × *g*, 4 °C for 5 min.
3. Resuspend cells in 3 ml cold H_2O and split each sample into two 2 ml eppendorf tubes.
4. Harvest cells by centrifugation at 3,000 × *g*, 4 °C for 5 min.
5. Freeze one of the tubes in liquid nitrogen and store it at −80 °C.
6. Resuspend the cell pellet in 1 ml spheroplasting buffer by vortexing (*see* **Note 11**).
7. Add 150 μl zymolyase stock solution and incubate at 30 °C for 45 min with nutation.
8. Spin down for 2 min at 5,400 × *g* in a microfuge and discard the supernatant. Be careful to remove all the supernatant.
9. Wash the pellet once with 500 μl H_2O (without resuspending the cells).
10. Resuspend the cell pellet in 200 μl H_2O. Add 250 μl solution I, vortex for 10 s, add 25 μl RNase A stock solution, then 25 μl Proteinase K stock solution and incubate in a water bath at 50 °C for 30 min (*see* **Note 12**). Invert the tube occasionally.
11. If the DNA happens to precipitate during the treatment, add 100 μl 2.5 M NaCl and incubate at 50 °C until the DNA is solved to completion.
12. Spin down for 5 min at 16,000 × *g* in a microfuge and transfer supernatant to a new tube.
13. Add 0.5 volume (env. 300 μl) D/I (prewarmed to RT), vortex for 10 s and spin down for 10 min at full speed in a microfuge. Note that a white layer is formed at the interphase. Transfer the aqueous (upper) phase into a 2 ml tube.
14. Add 2 volumes (env. 1.2 ml) solution II to the aqueous phase, invert gently approximately 20 times until the genomic DNA precipitates (note that plasmid DNA will precipitate with the genomic DNA).
15. Spin down at full speed in a microfuge at room temperature for 15 min and carefully discard the supernatant.
16. Solve the pellet in 500 μl Na-solution (*see* **Note 13**).
17. Add 0.6 volume (300 μl) isopropanol, invert several times until the DNA precipitates and spin down at full speed in a microfuge for 15 min at room temperature (*see* **Note 14**).
18. Wash the pellet with 100 μl 70 % ethanol (*see* **Note 14**). Make sure to remove all of the ethanol.
19. Solve the DNA pellet in 100 μl TE (*see* **Note 15**).
20. Check DNA concentration by agarose gel electrophoresis and ethidium bromide staining (loading 5 μl on the gel is fine, the DNA preparation usually yields enough material for five repair analyses).

3.3 Repair Analysis by Indirect End-Labeling

1. Digest the DNA (about 4 μg) to completion with the appropriate restriction enzyme in a total volume of 90 μl (*see* **Note 16**).
2. Add 1 μl StrataClean Resin, vortex, and spin down at full speed in a microfuge for 1 min.
3. Purify over Sephadex G-50 column (in 50T5E buffer; *see* **Note 17**).
4. Split the flow-through containing the DNA into two new tubes (40 μl each) (*see* **Note 18**).
5. Add 6 μl T4-endonuclease V (1 U/μl) to one tube (+T4-endoV) and 10 μl H_2O to the second one (−T4-endoV) and incubate all the tubes at 37 °C for 1 h.
6. Spin down the tubes and add 4 μl T4-endonuclease V (1 U/μl) to the (+T4-endoV) tubes and pursue incubation at 37 °C for at least 1 h (*see* **Note 18**).
7. Reduce sample volume in a SpeedVac during 20 min at 45 °C to about 5 μl.
8. To prepare the agarose–urea gel (20×25 cm, OWL; 300 ml 1.2 % agarose, 1 M urea, 1× TAE), dissolve 3.6 g agarose in 225 ml TAE and keep in the water bath at 65 °C. Prepare 700 ml 4 M urea in H_2O, filter the solution through filter paper and keep a 75 ml aliquot at 65 °C. Add the warm 75 ml urea aliquot to the agarose solution, mix well, and pour gel. Two combs should be used, one on the top and one in the middle of the gel to allow the loading of each sample in two slots (one on the top, the other one in the middle) and the subsequent hybridization of one series with a transcribed strand-specific probe and the other one with a non-transcribed strand-specific probe (*see* **Notes 19** and **20**).
9. Use the 4 M urea solution to prepare 2 L of running buffer (1 M urea, 1× TAE).
10. Prepare the loading buffer (8 M urea, 10 mM Tris–HCl pH 8, 1 mM EDTA, 1 % IGEPAL CA-630, bromophenol blue) by dissolving 2.4 g urea in 3 ml H_2O and completing to a final volume of 5 ml (*see* **Note 21**).
11. Add 70 μl loading buffer to the samples, heat to 95 °C for 5 min, spin down and load 30 μl per slot (remember we have two complete series; *see* **Notes 22** and **23**) and electrophorese samples at 150 V until the dye (bromophenol blue) migrates about 10 cm from slots (about 3 h).
12. Soak the gel for at least 10 min in H_2O on a shaker to rinse the urea off.
13. Soak the gel in denaturing solution for 20 min, then in neutralizing solution for another 20 min and finally in 20× SSC for 20 min with gentle shaking.

14. For Southern transfer, lay two sheets of 3MM Whatman paper presoaked in 20× SSC over a plastic tray bridging a tray with 20× SSC. The paper remains in contact with 20× SSC on both sides. Carefully place the gel on the paper. Cover it with a Hybond N membrane. Cover the membrane with three 3MM Whatman papers presoaked in 20× SSC, 10 cm of filter paper, a glass plate and a weight of about 250 g. Transfer DNA to the membrane overnight (or 5 h replacing the wet filter papers for fresh ones after 2 h).
15. After transfer, cross-link the DNA to the membrane by UV irradiation (700 J/m^2 on each side in a cross-linker). Rinse the membrane with 2× SSC.
16. Place the membrane in rotary cylinders with the DNA bound side facing inside and incubate them in 20 ml hybridization solution at 65 °C for 2–16 h in an hybridization oven.
17. Prepare strand-specific probes by primer extension. Primer extension reaction contains 2–5 ng appropriate and purified DNA fragment (*see* **Note 3**), 10 pmol appropriate primer, 1× Taq polymerase reaction buffer, 1 μl nucleotide stock solution, 1 U Taq polymerase, and 5 μl ^{32}PαdCTP in a total volume of 50 μl. Ten to twenty rounds of primer extension (typically 45 s denaturing step at 94 °C, 5 min annealing step at 56 °C, and 30 s elongating step at 72 °C) are used to increase the amount of radioactive DNA. Purify the probe from unbound radioactive nucleotides on a G-50 Sephadex column.
18. Replace the hybridization solution used for the prehybridization with 10 ml of hybridization solution and add the denatured probe. Hybridize at 65 °C overnight.
19. Pour off the hybridization solution, wash the membrane three times with wash solution I and once with wash solution II for 20 min each at 65 °C.
20. Air-dry the membrane on a sheet of filter paper and expose them to Phosphorimager screens (Fuji).
21. Quantify repair with a PhosphorImager (Fuji FLA5100) and the ImageGauge application software (Fuji). The background signal of the gel is determined in a background box normally set next to gel lanes or above gel slots and the average pixel value subtracted to all signal intensities. The CPD content is calculated using the Poisson distribution [22], $-\ln(RF_a/RF_b)$, where RF_a and RF_b represent the intensities of the intact restriction fragment of the T4endoV and mock-treated DNA, respectively (*see* **Note 24**). Repair curves are generally calculated as the fraction of CPDs removed versus repair time, the initial damage being set to 0 % repair. An example is shown in Fig. 1.

3.4 ChIP Analysis

1. Cross-link the cells by adding formaldehyde to a final concentration of 1 % (2.16 ml for 40 ml cell suspension) and incubate 15 min on a shaker at room temperature (*see* **Note 25**).
2. Add 2.2 ml glycine solution (125 mM final concentration) and incubate further on a shaker at room temperature for 5 min.
3. Pellet the cells in 50 ml conical tubes at 1,500 × *g*, 4 °C in a swing rotor for 5 min.
4. Wash the cells twice with 50 ml cold 1× PBS. Transfer cells to 2 ml tubes (normal tubes might be used, but screw-tops prevent leaking), spin down and discard supernatant. The cell pellets might be frozen in liquid nitrogen and kept at −80 °C at this point.
5. Prepare the antibody-coated magnetic beads as follows. Spin down the magnetic beads (30 μl will be required per ChIP sample) for 3 min at 6,000 × *g* at 4 °C and remove supernatant. Wash the beads twice with 1 ml PBS/BSA. Resuspend in the original volume PBS/BSA and add the appropriate antibody (e.g., the commercial anti-Rpb1 ab 8WG16 (Covance), in which case 7.5 μl will be required per ChIP reaction) and incubate at 4 °C overnight (min 6 h) with nutation. Wash the magnetic beads twice with 1 ml PBS/BSA and resuspend in the original volume PBS/BSA before adding to the ChIP samples (*see* **step 14** and **Note 4**).
6. Add 400 μl lysis buffer (with protease inhibitors) and 1 volume of 0.5 mm glass beads to the (frozen) pellet and keep on ice.
7. Lyse the cells by vigorous vortexing for 45 min at 4 °C.
8. Use incandescent needles to make a hole at the bottom of each tube and place them in clean 15 ml tubes. Spin down the tubes 5 s to recover the extracts, which should be transferred to clean 1.5 ml tubes and placed on ice (*see* **Note 26**).
9. Centrifuge the recovered extracts for 15 min at 16,000 × *g* and 4 °C. Discard the supernatant and resuspend the pellet in 400 μl lysis buffer (with protease inhibitors; *see* **Note 27**).
10. Sonicate each sample in a Bioruptor for 30 min, position high, 30 s "ON" followed by 1 min "OFF" at 4 °C (*see* **Note 28**).
11. Centrifuge at full speed in a table microfuge for 5 min at 4 °C to clear the extract of debris and unlysed cells. Transfer the supernatant to a new tube.
12. Centrifuge at full speed in a table microfuge for 15 min at 4 °C to clear any remaining debris and transfer the supernatant to a new tube (*see* **Note 29**).
13. Take a 20 μl aliquot (INPUT) and process immediately as described in **step 22**.

14. Add the prepared magnetic beads (*see* **step 5**) to the extracts (30 μl beads per reaction) and incubate overnight at 4 °C with nutation.
15. Place the precooled magnetic rack on ice, insert the tubes, aspirate all liquid off and resuspend the beads in 1 ml cold lysis buffer. Repeat the same procedure to achieve two washes with lysis buffer.
16. Wash the beads twice with 1 ml cold lysis/NaCl buffer on ice.
17. Wash the beads twice with 1 ml cold buffer III buffer on ice.
18. Wash the beads once with 1 ml TE.
19. Elute the precipitated material by adding 100 μl elution buffer to the washed beads. Incubate 10 min at 65 °C (*see* **Note 30**).
20. Spin down at full speed in a table microfuge for 2 min and transfer 50 μl of the supernatant to a new tube.
21. Take a 20 μl aliquot (PRECIPITATE) and process immediately as described in **step 22**. Keep the remaining at 4 °C as back up (*see* **Note 31**).
22. Add the 20 μl aliquots to PCR tubes containing 20 μl elution buffer, add 1.2 μl pronase (50 mg/ml) to each tube and incubate for 2 h at 42 °C followed by 6 h at 65 °C to reverse the crosslinks (*see* **Note 32**).
23. Purify over PCR purification columns (e.g., from Promega) according to manufacturer protocol and elute in 100 μl TE. Store samples at −20 °C (*see* **Note 33**).
24. Quantify the enriched DNA fragments in the PRECIPITATE by real-time quantitative PCR. We perform real-time quantitative PCR using FAST SYBR green dye and the absolute quantification protocol in the 7500 Real Time FAST PCR system (Applied Biosystems). Standard curves for all pairs of primers are performed for each analysis. All PCR reactions are performed in triplicate. The enrichment for each PCR of interest is calculated as the ratio between the region-specific signal and the intergenic signal of the PRECIPITATE, normalized with respect to the corresponding ratios of the INPUT. An example is shown in Fig. 2.

4 Notes

1. Alternatively, an homemade irradiation chamber containing several germicidal lamps arranged in parallel and an illumination stage at a distance of about 40 cm from the lamps might be used. UVX radiometer (UVP) with a UVX-25 photocell will be required to measure the flux and calculate the irradiation time.

2. The RNase A should be carefully boiled to eliminate residual DNAse activity.
3. To generate strand-specific probes for indirect end-labeling, DNA fragments of 200–300 bp that hybridize close to the end of the restriction fragment of interest should be used. The DNA fragment might be purified from plasmid DNA, or amplified by PCR and purified twice over commercial purification columns (e.g., Promega) to get rid of all the oligonucleotides used as primer (traces of residual primers would impede the strand-specificity of the probe generated by primer extension). Since CPD distribution is strand-specific, each strand will produce a characteristic CPD-pattern (*see* Fig. 1).
4. The use of magnetic beads speeds up the procedure, but the extract might be incubated with the antibody and the antibody-bound DNA precipitated with Protein A sepharose instead. A detailed protocol can be found in the first edition of this chapter [20].
5. Lysis buffer lacking protease inhibitors, lysis/NaCl and Buffer III might be stored at 4 °C. Once the protease inhibitors are added, the buffer should be kept on ice (or at 4 °C) and used within 2 days.
6. We use the Primer Express application software (Applied Biosystems) to design the oligonucleotides. Beside the primer pairs required to amplify the regions of interest, a primer pair amplifying a region devoided of the protein against which the immunoprecipitation is set (e.g., intergenic region in case of RNA polymerase II) is required.
7. YPED rich medium or synthetic complete (SC) medium might be used. However, it should be taken into account by using rich medium that residual medium might absorb some of the applied UV light resulting in lower initial damage. Therefore, the UV dose should be raised to 200 J/m^2 in that case. Alternatively, a washing step might be introduced before resuspending the cells in minimal medium without amino acids.
8. The cell density in the liquid culture as well as the UV-absorbance of the medium need to be considered for DNA-damage induction. Some people irradiate in medium without sugar (or even in PBS), we prefer to use sugar-containing medium to minimize the additional stresses suffered by the yeast cells. However, the presence of sugar in the media, which does absorb some of the UV light, makes it necessary to irradiate the cells at relatively high dosage to generate a reasonable amount of damage (about 0.3–2 CPD per kilobase).
9. The use of safety lights (Philips TLD RED) and brown flasks is highly recommended because photoreactivation is a fast process and could be promoted by traces of day light.

10. Rectangular plastic trays might also been used, as long as they do not absorb UV light and produce a 4-mm layer of cell suspension.
11. It is important that the cells are well resuspended. Usually, we deduce the volume of the cell pellet from the water used in the spheroplasting buffer to ensure that the final concentrations are correct.
12. The tubes might be slowly cooled down to room temperature by leaving them overnight in the turned off water bath.
13. It is important to solve the DNA pellet to completion. If it does not go into solution, add 50–100 μl 2.5 M NaCl and incubate at 50 °C for 15 min.
14. Both the ethanol and the isopropanol should be stored at room temperature to avoid co-precipitation of CTAB.
15. The best is to keep the DNA at 4 °C at least 1 day before going on with the repair analysis to ensure that it is completely solved.
16. Usually, 3 h incubation is fine to achieve complete digestion of the DNA. We use high quality restriction enzyme to avoid nicking activities, which would considerably increase background in the subsequent denaturing gel analyses.
17. To prepare Sephadex G-50 columns, place empty micro Bio-Spin chromatography columns in 2 ml eppendorf tubes, add 1 ml of Sephadex G-50 slurry and centrifuge at 2,500 × *g* for 2 min in a microfuge. The Sephadex G-50 columns are then placed in new 2 ml tubes, which serve to collect the purified DNA after centrifugation at 2,500 × *g* for 2 min in a microfuge.
18. The samples may be frozen and kept at −20 °C at this stage.
19. In this updated protocol, we use agarose–urea gels to perform denaturing gel electrophoresis [23]. A detailed protocol using alkaline gel electrophoresis can be found in the first edition of this chapter [20].
20. Once polymerized, the gel should be left at 4 °C for 30 min prior to running.
21. The loading buffer can be kept few weeks at room temperature.
22. Heating of the samples before loading is crucial, as otherwise the DNA would not denature.
23. The slots of the gel should be carefully washed out prior to loading.
24. Using this technique, region-specific damage can also be quantified (relative to total lesions). In this case, region-specific damage is calculated as the signal of that region in the T4endoV-treated DNA divided by the signal of the whole lane. The corresponding signal of the mock-treated DNA is subtracted as background.

25. Others incubate for 30 min at 30 °C and claim that for certain IPs, overnight incubation at 4 °C is helpful.
26. Two round of centrifugation may be required to recover all extracts.
27. Do not perform this step if you aim to ChIP plasmid DNA.
28. The sonification conditions should be set previously for each sonicator to generate DNA fragments between 100 and 500 bp, with a peak at 300 bp.
29. At this point 5 μl of the extracts might be run on an agarose gel to check fragment lengths. After this step, salt or detergent concentrations may be increased or decreased for more or less stringent IPs, respectively.
30. To improve the efficiency of recovery, incubation might be done for up to 30 min and repeated a second time, pooling the eluates afterwards.
31. To improve yields, 40 μl might be taken for the PRECIPITATE. In this case, simply add 2.4 μl pronase (50 mg/ml) and incubate as described in **step 22** (without adding 20 μl elution buffer).
32. It is recommended to use a thermocycler to prevent evaporation.
33. In the first edition of this protocol [20], we included an in vitro photoreactivation step prior to PCR analysis because it has been shown that UV photoproducts such as CPDs block the elongation of Taq-DNA polymerases [21]. Photolyase is no longer commercially available and the omission of the in vitro photoreactivation step may lead to a biased amplification of short DNA fragments of highly damaged DNA. However, keeping the amount of DNA damage low and using short amplicons in the real-time qPCR minimizes the interferences between damaged template and PCR elongation process (*see* Fig. 2).

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Chapter 16

Analysis of DNA Replication Associated Chromatin Decondensation: In Vivo Assay for Understanding Chromatin Remodeling Mechanisms of Selected Proteins

Sergiy Borysov, Victoria L. Bryant, and Mark G. Alexandrow

Abstract

Of critical importance to many of the events underlying transcriptional control of gene expression are modifications to core and linker histones that regulate the accessibility of *trans*-acting factors to the DNA substrate within the context of chromatin. Likewise, control over the initiation of DNA replication, as well as the ability of the replication machinery to proceed during elongation through the multiple levels of chromatin condensation that are likely to be encountered, is known to involve the creation of chromatin accessibility. In the latter case, chromatin access will likely need to be a transient event so as to prevent total genomic unraveling of the chromatin that would be deleterious to cells. While there are many molecular and biochemical approaches in use to study histone changes and their relationship to transcription and chromatin accessibility, few techniques exist that allow a molecular dissection of the events underlying DNA replication control as it pertains to chromatin changes and accessibility. Here, we outline a novel experimental strategy for addressing the ability of specific proteins to induce large-scale chromatin unfolding (decondensation) in vivo upon site-specific targeting to an engineered locus. Our laboratory has used this powerful system in novel ways to directly address the ability of DNA replication proteins to create chromatin accessibility, and have incorporated modifications to the basic approach that allow for a molecular genetic analysis of the mechanisms and associated factors involved in causing chromatin decondensation by a protein of interest. Alternative approaches involving co-expression of other proteins (competitors or stimulators), concurrent drug treatments, and analysis of co-localizing histone modifications are also addressed, all of which are illustrative of the utility of this experimental system for extending basic findings to physiologically relevant mechanisms. Although used by our group to analyze mechanisms underlying DNA replication associated chromatin accessibility, this unique and powerful experimental system has the propensity to be a valuable tool for understanding chromatin remodeling mechanisms orchestrated by other cellular processes such as DNA repair, recombination, mitotic chromosome condensation, or other chromosome dynamics involving chromatin alterations and accessibility.

Key words Acetylation, Methylation, Chromatin, Decondensation, Set8-HBD, Histone H4, Cdc45, Cdt1, HBO1, HDAC11, Remodeling, LacI-DBD, Heterochromatin, DNA replication

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1 Introduction

The analysis of chromatin decondensation by selected proteins in vivo is achieved through use of a cell-biological experimental system developed by Andrew Belmont and colleagues [1–3]. This system tests the ability of specific proteins to promote large-scale decondensation of compacted chromatin when targeted via a fused LacI DNA binding domain to a unique and identifiable chromosomal locus. To generate this system, multiple copies of a lac operator (lacO)/dihydrofolate reductase (DHFR) vector were integrated into a Chinese hamster ovary (CHO) cell line, followed by amplification with methotrexate [2]. The resulting cell line (A03_1) contains a 90-Mb homogenously staining region (HSR) of ~60 amplicons, each consisting of ~400-kb of tandemly integrated 14-kb lacO/DHFR vectors and ~1,000-kb flanking co-amplified genomic DNA. Significantly, this system allows observation of LacI-targeted proteins with conventional light microscopy, and is further capable of allowing visualization of protein-induced chromatin changes in vivo. The HSR can be directly visualized in fixed or living A03_1 cells by expressing and targeting the LacI protein, LacI-GFP derivatives, or proteins of interest fused in-frame to LacI. Under microscopic examination, the HSR normally assumes a condensed dot-like structure and is heterochromatic [2, 3]. Binding of LacI-GFP to the HSR in living cells causes no alteration in the morphology of the locus [2]. However, the expression and targeting of certain LacI-fusion proteins (e.g., LacI-VP16 and LacI-Cdc45) induces a dramatic decondensation of the HSR [4], likely by recruitment of chromatin remodeling enzymes to the LacI-fused protein [3–5]. For example, LacI-Cdc45 targeting causes dramatic chromatin unfolding [4] that is mediated by recruitment of Cdk2 and phosphorylation of histone H1, creating access to higher order condensed chromatin (e.g., in heterochromatin) during replication fork progression. Heterochromatin generally contains a high histone H1 content and is typically condensed, unless H1 is phosphorylated [4, 6–11].

This remodeling system, although artificially constructed to allow visualization of something that normally cannot be seen, is quite specific in terms of *which* proteins can remodel and in terms of *how* those proteins achieve the remodeling at the mechanistic level. Several proteins are incapable of remodeling chromatin in this system, including Cdc6 [4], geminin (unpublished results), LacI [3], Cdc45^{-CT} (unpublished results; contrasts with wt-Cdc45 below), and several mutant alleles of BRCA1 [5]. Different histone modifications contribute to the decondensation observed with proteins that are effective at unfolding chromatin: VP16-, E2F1-, human estrogen receptor (hER), and p53-decondensed chromatin correlates with core histone hyper-acetylation or hypermethylation

and recruitment of HATs [3–5, 12], BRCA1 alleles that can cause decondensation have been found associated with phospho-H2AX and the possible recruitment of COBRA1 [5]. Cdc45 decondensation involves recruitment of Cdk2 and H1-P, and is sensitive to Cdk2-inhibitory drugs or co-transfection of the Cdk inhibitor p21, but common core histone (H4, H3, H2A, H2B) acetyl-modifications do not co-localize [4]. Consistent with this, and showing the clear specificity of the system, certain Cdc45 mutants lacking the C-terminus (e.g., Cdc45^{-CT}) are unable to open the HSR (unpublished results). Importantly, it is in the C-terminus of Cdc45 where the Cdk2 enzyme mediating the remodeling is predicted to bind to RXL cyclin-binding motifs in Cdc45 (unpublished observation and ref. 13). Additionally, it has been shown that Cdc45, which is targeted by a Chk1-dependent pathway following BPDE-dependent DNA damage, is less capable of remodeling chromatin in this system when cells are co-treated with BPDE or Chk1 is co-transfected [14]. Finally, the ability of a targeted protein to promote *condensation* of the HSRs has also been demonstrated [15]. In the latter case, targeting HP1 to the HSRs causes chromatin compaction to occur, which is associated with histone lysine methylation.

A recent study by our group has further demonstrated the powerful nature and specificity of this chromatin decondensation assay for providing mechanistic information underlying how the DNA replication process is regulated via chromatin changes at origins [16, 17]. Prior studies had shown that the loading of MCM helicase subunits at origins was dependent on the HAT HBO1 interacting with Cdt1, a loader of MCMs [18]. The enzymatic activity of HBO1 and occurrence of histone H4 acetylation at analyzed origins was also required for MCM loading [19]. However, the *mechanism* whereby HBO1 and histone H4 acetylation actually promoted MCM loading remained elusive until the application of this unique chromatin remodeling system (described herein) provided an explanation [16]. Cdt1 targeting promotes chromatin decondensation and MCM recruitment to chromatin during G1 phase, which requires HBO1 and histone H4 acetylation [16]. The latter was demonstrated using this remodeling system in a novel manner in which the Set8-HBD (histone H4 binding domain) was used as a (co-expressed) competitor to block H4 acetylation in vivo, resulting in failure of Cdt1 to induce chromatin unfolding and failure to recruit MCMs [16]. Thus, this chromatin remodeling system demonstrated that the mechanism whereby H4 acetylation and Cdt1/HBO1 promote MCM recruitment to origins involves creating chromatin accessibility (at least in part). Further evidence from this study and assay system showed that recruitment of HDAC11 and Geminin by Cdt1 acted in an opposing and specific manner (versus HBO1) to promote chromatin *closure* at origins during S-phase, which prevented further unwanted

MCM loading in S-phase and suppressed reinitiation of DNA replication [16]. This yin–yang chromatin opening–closing accessibility mechanism of MCM/origin control would have been more difficult to elucidate without the utility of this unique and powerful chromatin remodeling assay system.

As evidenced by multiple reports in the literature utilizing this in vivo chromatin decondensation assay [1, 3–5, 12, 14–16, 20–22], the mechanisms that cause/regulate chromatin remodeling in this system are distinctly different for each protein tested and, importantly, derive from physiologic events related to chromatin remodeling that are relevant for each targeted protein. For these reasons, this system proves very valuable at allowing investigation of chromatin unfolding by a protein of choice in vivo, as well as investigation of how the particular protein actually brings about the unfolding that occurs. Thus, while the system is cell biological in nature, it has the propensity to allow for a molecular biological dissection of the mechanisms involved in chromatin decondensation (or compaction) events underlying important biochemical processes in cells.

2 Materials

1. Phosphate buffered saline (PBS):

   150 mM NaCl.

   5 mM Na_2HPO_4.

   1.7 mM KH_2PO_4.

   pH to 7.4 and autoclave.

   Make 1 l of 10× concentrated in water (above is for 1× solution).
2. PBS/NDS: In 1× PBS (made by diluting 10× PBS with water), add normal donkey serum (NDS; Jackson ImmunoResearch; cat#017-000-121) to 1 % final concentration (vol/vol). Normal donkey serum stock (10 ml lyophilized on receipt) is resuspended in 10 ml water, aliquoted, and stored at −20 °C.
3. Methotrexate solution (MTX): Calbiochem/EMD (cat#454125) is made in serum-free medium at 1 mM, filter-sterilized, and stored at −20 °C. Final working concentration is 0.3 μM.
4. Formaldehyde solution: 37 % formaldehyde liquid solution, Fluka (cat#47608).
5. DAPI solution: 4′-6-diamidino-2-phenylindole (DAPI) is from Sigma (cat#D-9542). Stock is made in water at 5 mg/ml and stored at −20 °C. Final working concentration is 0.2–0.5 μg/ml.
6. Coverslips/Slides: Corning number 2, 22 mm square (cat#2875-22). Precleaned, plain slides are frosted on one side, are 25 × 75 × 1 mm, and are from VWR (cat#48300-025).

7. HCl solution: 1.5 normality HCl diluted in water and stored at room temperature. As low as 1 N HCl has been used successfully in our hands to allow BrdU visualization.
8. Anti-LacI antibodies: Stratagene rabbit polyclonal anti-LacI (cat#217449-51) and Upstate mouse monoclonal anti-LacI (cat#05-503; clone 9A5) are both necessary.
9. A03_1 cells and expression vectors: Originally generated by the Belmont laboratory [2, 3], and used by other labs including the Alexandrow [4] and Li labs [5]. The pRcLac vector was designed by the Li lab, and used by Li and colleagues and the Alexandrow lab (*see also* Subheading 3.2.1). Other cell lines designed by Spector and colleagues may be used for these analyses [23, 24], but the system in these lines is different from that described here.
10. Permeabilization buffer:

    1 % NDS.

    0.2 % Triton X-100.

    Add the above to 1× PBS.

    Make fresh for each experiment as necessary; 2 ml per 35 mm plate.
11. Pre-extraction buffer I:

    0.1 % Triton X-100.

    5 mM MgCl2.

    0.1 mM EDTA (ethylenediaminetetraacetic acid) pH 8.

    Made in water as necessary for number of plates used; 2 ml per 35 mm plate.
12. Pre-extraction buffer II:

    10 mM HEPES (1-piperazineethane sulfonic acid, 4-(2-hydroxyethyl)-monosodium salt), pH 7.9 with potassium hydroxide (KOH).

    10 mM KCl.

    1.5 mM MgCl2.

    0.5 mM dithiothreitol (DTT).

    0.1 % Triton X-100.

    Made in water as necessary for number of plates used; 2 ml per 35 mm plate.
13. Mounting medium/kit: Molecular Probes ProLong Anti-Fade kit (cat#P-7481). Make fresh for each experiment. *See also* Subheadings 3.2.2 and 3.3.1.
14. Tools required: Forceps and scalpel with handle, to place coverslips into 35 mm plates and remove coverslips, respectively. Forceps are sterilized over a Bunsen burner flame prior to gripping coverslips for transfer to plates.

15. Secondary antibodies: All from Jackson ImmunoResearch. Texas Red conjugated donkey anti-rabbit (H+L) (cat#711-075-152); FITC conjugated donkey anti-mouse (H+L) (cat#715-095-150); FITC conjugated donkey anti-goat (H+L) (cat#705-095-147). All are resuspended in water as directed and then an equal amount of sterile 100 % glycerol is added such that the final glycerol is 50 %, and secondaries are stored at −20 °C. Working concentration is 2/50 μl solution on each coverslip.
16. Transfection reagent: We use FuGene 6 reagent from Roche (cat#11814443001), but Lipofectamine (or derivatives) from Invitrogen is also suitable.
17. Bromodeoxyuridine (BrdU): Sigma (cat#B-5002) dissolved in water as 10 mM stock and stored at −20 °C. Final working concentration is 10–15 μM.
18. Anti-BrdU antibodies: Roche anti-BrdU mouse monoclonal (cat#1170376) is 50 μg/500 μl, stored at −20 °C, and used at 1:20 dilution. Molecular Probes/Invitrogen anti-BrdU-Alexa-594 conjugate (cat#A-21304) is also used at 1:20 dilution and stored at −20 °C.
19. DNAse I: Amersham/Pharmacia, 10,000 U/ml (cat#27-0514-01).

3 Methods

3.1 *In Vivo* Chromatin Decondensation/Remodeling Assays

3.1.1 Cell Plating, Transfection Conditions, and Fixation

1. A03_1 cells are plated 2 days before the transfections, since often they display nonuniform adhesion to the plates the next day and many cells have not fully spread out in 24 h. A03_1 cells require culture in the presence of 0.3 μM (final) of methotrexate (MTX) to maintain the presence of the LacO repeats within the HSR. Therefore, cells are routinely cultured in minimal essential medium (MEM; from Cellgro) plus MTX, and serum from Hyclone (Fetal Clone I or II work equally well). Cells are plated in 35 mm tissue culture plates containing a sterile square glass coverslip that has been previously baked (*see* Subheading 3.2.3). Cells in a stock 10 cm tissue culture plate at high density (~90–100 %) are split at a ratio of 1:30 (10 cm plate:35 mm plates), which produces approximately 60–70 % confluency on the 35 mm plates after 2 more days of culturing, and is a suitable density for transfections and future microscopic analysis.
2. Transfections with LacI-fusion expressing plasmids are performed using a ratio of 5 μl FuGene 6 reagent to 2 μg of purified DNA. If plasmids are co-transfected, then the ratio will change (see manufacturer for details; Roche). As high as

6–7 μg of total plasmid DNA with 10 μl FuGene 6 reagent per 35 mm plate transfection has been used with success. Transient transfections last 24 h. We also include control transfections in many experiments (*see* Subheading 5, **Notes 1** and **2**). Protein expression can be verified using immunoblotting with anti-LacI antibodies (*see* Subheading 3.2.4). If one wishes to perform concurrent BrdU labeling analyses, *see* Subheading 3.3.2.

3. Cells are washed one time with 2 ml PBS at room temperature for 15 min. One may choose to pre-extract the cells prior to fixation as an alternate approach (*see* Subheading 3.3.3). Pre-extraction serves to remove loosely bound proteins from the nuclei prior to permanent fixation with formaldehyde. This is often referred to as the "chromatin-bound" fraction that remains after pre-extraction, and can alter the outcome of a given experiment or suggest new means of interpreting the data.
4. Cells are then fixed on the coverslips in the 35 mm plates for 15 min at room temperature with 2 ml per plate of PBS/2 % formaldehyde (stock is made by adding 540 μl 37 % formaldehyde to 10 ml PBS). Other fixation methods are useful as well (*see* Subheading 3.3.4).
5. Cells are finally washed two times (15 min each time) at room temperature with 2 ml PBS per plate. Plates can be stored at 4 °C for a week or two under PBS if desired.

3.1.2 Processing Cells for Immunofluorescence

1. Wash fixed cells on coverslips in 35 mm plates two times with 2 ml PBS per plate for 15 min at room temperature. Use a gentle rocking motion, preferably on an orbital shaker.
2. Permeabilize the fixed cells with 2 ml per plate of Permeabilization Buffer for 5 min at room temperature. Rock gently on an orbital shaker (*see* Subheading 5, **Note 3**). Keep 1 % NDS in all solutions as a competitor/blocking agent from this step until after staining with the secondary antibodies.
3. Wash cells in 35 mm plates two times with 2 ml PBS/1 % NDS per plate for 15 min at room temperature. Use a gentle rocking motion on an orbital shaker.
4. Prepare primary antibodies in PBS/1 % NDS at the appropriate dilution for each antibody to be used (*see* Subheading 5, **Note 4**). One will need 50 μl of diluted antibodies for each coverslip to be probed. If one is analyzing the LacI-targeted protein together with analysis of colocalization of an endogenous protein, such as a histone or enzyme, then the primary antibody to that protein must be included as well. If desired, 0.1 % TX100 can be included in the dilutions to achieve a higher level of stringency. To analyze two primary monoclonal antibody probings simultaneously, then refer to Subheading 3.3.5.

5. Label frosted slides for each coverslip to be probed. The antibody dilution (50 μl) is placed as a drop onto the appropriate slide. Using the scalpel, carefully pry up the coverslip from the 35 mm plate and use the other hand to grasp the coverslip on its sides. Carefully transfer the coverslip to the frosted slide, turn over, and place face down (cell side down) onto the drop of diluted antibodies. If the coverslip is accidentally dropped, then use the scalpel to gently "slice" a line through one side of the coverslip. If a line appears, the "cell-side" of the coverslip has been "sliced" and should be placed face down.
6. Place slides/coverslips into the humidified chamber (*see* Subheading 3.2.5) in a flat manner. Cover the chamber and incubate for 1–2 h at room temperature.
7. Remove coverslips from slides and carefully transfer coverslips back to the same 35 mm plates from where they originated. Add 2 ml per plate of PBS/1 % NDS and wash two times at room temperature for 15 min on an orbital shaker.
8. Prepare secondary-conjugated antibodies as described above for the primary antibodies (Subheading 3.1.2, **step 4**). Secondary antibodies are directed against the species of the primary antibody used above in Subheading 3.1.2, **step 4** (e.g., anti-rabbit, anti-mouse, or anti-goat). Combine Texas Red- and FITC-conjugated antibodies into the same mixture in PBS/1 % NDS. A 1:50 dilution of secondaries from Jackson ImmunoResearch has been used. Use the same approaches as described above to place 50 μl antibody drops onto slides, remove and turn over coverslips, and incubate for 1–2 h at room temperature in the humidified chamber (Subheading 3.1.2, **steps 5** and **6**). Keep the slides in the dark from this step forward to reduce background fluorescence.
9. Remove slides/coverslips, and carefully transfer coverslip back to their proper 35 mm plate from where they originally came. Add 2 ml per plate of PBS (no need for NDS from this step forward) and wash two times at room temperature for 15 min on an orbital shaker.
10. Stain with DAPI solution for 3–5 min at room temperature in PBS. Use a 1:10,000 dilution of the 5 mg/ml stock of DAPI (final is 0.5 μg/ml, but can go as low as 0.2 μg/ml). Rock on an orbital shaker.
11. Wash two times at room temperature with PBS for 10 min each wash.
12. Mount coverslips on slides using 50 μl of Prolong Antifade reagent (*see* Subheading 3.2.2) that is dropped onto each slide, and onto which the coverslips are placed cell-side down as a described above (Subheading 3.1.2, **step 5**). An alternate mounting medium can be used if desired (*see* Subheading 3.3.1).

13. Place slides/coverslips on a flat surface in the dark overnight at room temperature to dry.
14. Seal the edges of the coverslips on the slides the next morning, or after up to 3 days, with clear nail polish. Slides can be stored at 4 °C for a short term (up to a week), or at −20 °C for longer periods prior to viewing by microscopy (*see* Subheading 4, **Interpretations 1–3**).

3.2 Support Protocols

3.2.1 Subcloning into pRcLac

The pRcLac vector was engineered by Li and colleagues [5], and has within it one AscI restriction enzyme site useful for subcloning cDNAs into the vector for in-line expression with an amino-terminal LacI DNA binding domain tag/targeting domain. To subclone, one of two approaches should be used. First, polymerase chain reaction (PCR) can be used to add AscI sites to both ends of the cDNA, followed by direct ligation into pRcLac. Alternatively, a vector we have generated (pcAscIx2; vector and map available from us upon request) can be used as a shuttle vector to add AscI sites to both ends of the cDNA. For transferring cDNAs into the vector, BamHI and EcoRI restriction sites have been incorporated into pcAscIx2 such that cDNAs will be in the proper reading frame with LacI in the final pRcLac construct. This necessitates that the user already have, or add (e.g., using PCR methods), such restriction sites in-frame with their cDNAs prior to this subcloning (*see also* Subheading **5**, **Note 5**).

3.2.2 Preparation of Mounting Medium

Mounting medium from Molecular Probes/Invitrogen consists of two components, A and B. Component A is a chemical pellet at the bottom of a small brown tube; Component B is a viscous solution that is largely solid when stored frozen. Component B must be thawed at least an hour prior to use, but we have found that 5–10 s in a microwave oven, with the lid off, is suitable for quick thawing. Dropwise, add 1 ml of Component B to a clear microcentrifuge tube. Then transfer the 1 ml solution to the Component B tube. Wait for 15 min at room temperature. Use a micropipette tip to stir and dislodge the pellet. Vortex briefly. Spin out bubbles at full speed in a microcentrifuge. 50 μl are used to mount each coverslip, but we have found that one tube will only be suitable for 17–18 slides (rather than 20).

3.2.3 Coverslip Preparation and Transfer

Coverslips are placed into a glass petri dish, covered with aluminum foil, and baked at 80 °C overnight for ~16 h to sterilize. To transfer into 35 mm tissue culture plates, forceps that have been sterilized just prior with a Bunsen burner flame are used to carefully pick up and transfer only one coverslip. Accidental transfer of two coverslips will yield mounted slides in the end that are unreadable with the microscope. Coverslips from Corning prepared in this way do not need to be acid treated.

3.2.4 Simplified Immunoblotting Analysis

To detect LacI-fusion protein expression, transfected cells are washed on 35 mm plates with excess PBS two times, then collected into microcentrifuge tubes with a cell scraper. After a quick spin in a microcentrifuge to pellet the cells (~100 × *g* for 20 s), PBS is aspirated and 100 μl of 1× Laemmli protein loading dye [25] is added to each pellet and boiled 10 min. 5 μl of each sample is loaded onto gels, transferred, and analyzed by immunoblotting with ~1:20,000 dilution of the anti-LacI antibodies.

3.2.5 Humidified Chamber Preparation

A plastic dark room developer tray (8 × 10 in., 1.5–2 in. deep), or similar, is used to prepare a humidified chamber. Paper towels are wetted with water, then laid in the tray as smooth as possible. Plastic wrap (e.g., Saran Wrap) is used to cover the chamber, and cardboard plates, such are used to sandwich autoradiography cassettes, are laid on top. The chamber is allowed to evaporate water for about 2 h prior to use to create vapor pressure that prevents slides from drying out.

3.3 Alternate Protocols

3.3.1 Alternate Mounting Medium Preparation

The ProLong Antifade mounting reagent from Molecular Probes/Invitrogen is preferred for durability of mounting and prevention of ultraviolet bleaching of samples. However, an alternative mount can be made as follows: Mix 8.5 ml glycerol (Polysciences cat#00084) with 750 μl PBS. Add 10 mg 1,4-phenylenediamine (Aldrich Chemical cat#27,515-8), wrap in aluminum foil and stir for half a day to overnight. Check that the pH is ~pH 6 (using pH paper). Add 20–30 drops of carbonate–bicarbonate buffer to give a final of pH 8. Store in aliquots at −20 °C wrapped in foil. The carbonate–bicarbonate buffer is itself made by making 0.2 M solution of anhydrous sodium carbonate and 0.2 M solution of sodium bicarbonate, then combining 4 ml of 0.2 M solution of anhydrous sodium carbonate with 46 ml of 0.2 M solution of sodium bicarbonate, and bringing to a final volume of 200 ml with water. The pH of the mixed solutions will be 9.2.

3.3.2 Concurrent Bromodeoxyuridine (BrdU) Analysis

To analyze DNA replication concurrently with chromatin unfolding, cells are pulse labeled with 10–20 μM BrdU for 30 min prior to fixation. The BrdU solution is added directly to the tissue culture medium. To process cells for BrdU incorporation, proceed through Subheading 3.1.2, **step 3**, and keep non-BrdU-labeled cells rocking in PBS/1 % NDS. BrdU-labeled cells are treated with 1.5 N HCl (in water) for 30 min at room temperature on an orbital shaker. Alternatively, use DNAse I to create access for anti-BrdU antibodies (*see* Subheading 3.3.6). Cells are washed three times with PBS/1 % NDS at room temperature for 10 min each time. The protocol then resumes at Subheading 3.1.2, **step 4**. When diluting anti-BrdU antibodies, we use a 1:20 dilution of the Roche or Molecular Probes/Invitrogen antibodies onto the slides/coverslips in PBS/1 % NDS (*see also* Subheading 5, **Note 4**). Samples

treated with 1.5 N HCl still retain the ability to bind DAPI effectively (at Subheading 3.1.2, **step 10**).

3.3.3 Pre-extracting with Detergents Prior to Fixation

Treat non-fixed, PBS-washed cells (from Subheading 3.1.1, **step 3**) for 10 min at 4 °C with (cold) pre-extraction buffer I or II prior to fixation. Then resume with formaldehyde fixation in Subheading 3.1.1, **step 4**.

3.3.4 Alternate Fixation Methods

MeOH (100 %, cold) or a 1:1 mixture of MeOH:Acetone (cold) can be used to fix cells instead of formaldehyde. A 10 min incubation at −20 °C in either solution will fix cells. Following fixation, dry for 15–30 min at room temperature. Formaldehyde fixation can block certain epitopes for antibody recognition, and these other approaches may alleviate this problem.

3.3.5 Using Two Primary Monoclonal Antibodies Simultaneously

It is not possible to use two primary monoclonal antibodies simultaneously followed by secondary antibody probings that can differentiate between the two primary antibodies. However, if using two monoclonals as primaries is the only way to proceed, then at least one of the primary monoclonal antibodies must be pre-conjugated to a fluorophore. For example, *see* Subheading 5, **Note 4**. To perform the analysis in this manner, proceed through the immunofluorescence assays from Subheading 3.1.2, **steps 1–9**, using the first non-conjugated primary antibody and secondary antibody probings. Then, fix the coverslips/cells again with formaldehyde as in Subheading 3.1.1, **step 4**. Continue through the procedure from Subheading 3.1.1, **step 4**, until Subheading 3.1.2, **step 4**, at which time add diluted primary antibody of the second monoclonal that is pre-conjugated to a fluorophore. Follow the procedure from Subheading 3.1.2, **step 4** through Subheading 3.1.2, **step 6**. There is no need to probe with another secondary, since the second primary antibody is pre-conjugated. Therefore, finish the assays by resuming at Subheading 3.1.2, **step 9**. In the end, the procedure will have been performed essentially 1.5 times to achieve analysis of two monoclonals simultaneously.

3.3.6 DNAse I Treatment for BrdU Analysis

DNAse I treatment can be used rather than HCl to denature DNA for anti-BrdU access and recognition. For this, use 50–100 μl of 10 U/ml DNAse I in PBS/1 % NDS on each coverslip turned over onto a slide face down at 37 °C for 1–2 h in a humidified tissue culture incubator (to prevent drying out of the slides). Then proceed with anti-BrdU staining as described above (*see* Subheading 3.3.2). We must caution, however, that DNAse I treatment/accessibility has proven unsuccessful in our hands. For more information on this approach, the reader is referred to a Kennedy and Harlow paper [26].

4 Interpretations

1. *Data acquisition*
   Microscopic examination is used to detect the chromatin structures (via fluorescence of the anti-LacI antibody + secondary conjugate that marks the region where the HSR is located and the targeted protein is concentrated). This may entail using a traditional wide-field ultraviolet microscope, or a more sensitive confocal laser scanning microscope system. The A03_1 cells may often display two HSRs, with some examples displaying one closed HSR and one open HSR in the same cell. The importance of this is that this chromatin unfolding system derives from a complex cell biological environment. There are many variables influencing the ability of a targeted protein to unfold chromatin (or not). Simple targeting of any protein will not open chromatin, and even proteins that do usually open chromatin may not necessarily lead to a decondensation event at all targeted HSRs for reasons that are unknown.

2. *Criteria for chromatin decondensation/non-decondensation*
   This experimental approach is admittedly a subjective cell biological assay for chromatin unfolding capability of a protein of interest. However, we use an analysis method that is aimed at mitigating the subjectivity of the assay and incorporates a degree of objectivity into the results. Rather than assign an "open" definition as being larger than 2 % of the area of the nucleus [2, 3], which is a measurement that even some arguably closed chromatin structures would fall into based on our studies, we have assigned "open" to structures that are unambiguously open and rather large in shape. Such open structures are easily seen by almost any observer and are generally greater than 10 % of the nuclear area. The "closed" definition we use to describe chromatin structures that are clearly still dots and condensed to almost any observer. The "closed" structures tend to comprise 2–5 % of the nuclear area in our analyses [4]. In many experiments, approximately 4–12 % of the total samples show chromatin structures that are not clearly open or closed, and are referred to as "indeterminate" structures. The size of such "indeterminates" often comprises 5–10 % of the nuclear area, usually toward the smaller side. Although these structures are counted, they are generally excluded from our interpretations, leaving us with strictly open or closed structures that are clear to most observers. In this manner, our approach at analysis lends a more objective interpretation to the ability (or lack thereof) of a tested protein to induce chromatin unfolding in vivo.

3. *Important considerations, and co-expression of regulatory proteins*
   While this chromatin unfolding analysis system seems capable of further investigating the control of DNA replication or tran-

scription at the HSRs after targeting proteins of interest, it is important to keep in mind that this system was never designed for this purpose. It is strictly a targeting system that allows one to test whether a protein of interest *can* remodel chromatin or not. However, using co-transfections (co-expression of potential regulatory proteins), drug treatments, or other combinatorial approaches, the initial inroads into deciphering how a protein may be remodeling, and whether this remodeling is physiologically relevant due to supporting evidence gained from the combinatorial approaches, can be made. A nice example of this derives from a recent study where we used the Set8-HBD to block histone H4 acetylation in vivo, which resulted in failure of Cdt1 (via LacI-Cdt1 expression) to promote chromatin decondensation and MCM helicase recruitment [16]. Such a co-expression approach demonstrated that H4 acetylation was mechanistically involved in chromatin unfolding and the resultant MCM recruitment in the steps leading up to the initiation of DNA replication [16, 17]. Similarly, co-expression of HBO1, HDAC11, or Geminin produced changes in the ability of LacI-Cdt1 to open chromatin in this system, which helped to elucidate their roles in the Cdt1-regulated process of MCM recruitment at origins [16].

Common sense dictates that fusion of a large DNA binding domain (LacI; ~40 kDa) will likely interfere in some manner with normal function of the proteins fused to LacI, since this is not the normal structure of the proteins. Nonetheless, this system clearly has tremendous advantages that allow one to make initial discoveries into the nature and possible mechanisms whereby a specific protein decondenses chromatin. It is up to the investigator to find means of relaying these findings to the physiological significance and relevance of the tested protein's role in normal cell processes. In the end, much of the novelty of this system is dependent on the creativity of the person using the system.

5 Notes

1. Controls may include a LacI-VP16 vector (pNYE4-GFP-VP16; *see* ref. 3) and/or an "empty" vector that only expresses the LacI DNA binding domain alone (pRcLac alone). We have used the LacI-VP16 vector as a comparison when performing colocalization analyses with antibodies against modified histones (e.g., acetylated or methylated histones). This aids in verifying that the antibodies against endogenous modified histones are working as expected, and that the microscopic settings are correct for visualizing colocalization.
2. Co-transfection of other plasmids encoding proteins that modulate the activity of the LacI-targeted protein, or the activity of

proposed mediators recruited by the LacI-tagged protein, can be performed. In addition, the transfected cells can be treated with various drugs that modulate the enzymatic activities of proteins thought to be mediators of the chromatin unfolding. In either case, the timing of co-transfection or drug treatment can also be varied to suit the experimental requirements. Both of these approaches offer a novel means to elaborate on mechanisms that may be involved in the chromatin unfolding mediated by a particular LacI-fused protein being analyzed.

3. It is preferable to use the same species of blocking normal serum as the species of secondary antibody conjugate used in the immunofluorescence assays. This reduces background fluorescence [25]. We use secondaries raised in donkey and normal donkey serum throughout.
4. The anti-LacI antibodies used may need titration. The rabbit and mouse anti-LacI antibodies that we use require 1:20,000 dilutions into PBS/1 % NDS. In addition, an alternative to using primary antibodies followed by secondary antibody staining is to use pre-conjugated primary antibodies that have a fluorophore already chemically attached. For example, anti-BrdU conjugated to Alexa-594 (Molecular Probes/Invitrogen) can be used.
5. An internal in-frame green fluorescent protein (GFP) in the constructs can be used as a control, or to aid in visualizing the targeted proteins without antibody staining procedures. Likewise, to analyze fragments of proteins for domains that can remodel, it may be necessary to include a nuclear localization sequence in the final construct to guarantee nuclear import during the transfection.

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Chapter 17

Non-radioactive Assay Methods for the Assessment of Telomerase Activity and Telomere Length

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Abstract

The Telomeric repeat amplification protocol (TRAP) is a highly sensitive PCR-based assay and prove to be an important tool for understanding the role of telomerase in cancer and various tissues that harbors telomerase positive stem cells. This assay measures telomerase activity where the amount of target is dependent upon the activity of the enzyme. This protocol consists of two steps: first, telomeric repeats are added to the substrate by telomerase present in the cell and second, the extended products are amplified by Taq-DNA polymerase. The amplified TRAP products are separated on 10 % native PAGE and detected by SYBR Green I dye.

Key words Telomerase, TRAP assay, Telomere, SYBR Green I, Southern blot

1 Introduction

Telomerase, a ribonucleoprotein enzyme with specialized reverse transcriptase activity, catalyzes the synthesis and extension of telomeric DNA [1]. Telomerase is present in germ line cells, cancer-derived cell lines, and spontaneously immortalized cells in culture. It is activated in 85–90 % of malignant tumors but usually absent in normal somatic cells which results in the progressive loss of telomeres with each cell division [2]. Activation of telomerase allows cells to overcome replicative senescence, therefore, be a rate-limiting step in cellular immortality and oncogenesis [3]. The telomerase complex is composed of telomerase reverse transcriptase (TERT) [4], telomerase RNA component (TERC) [5], telomerase associated proteins (TEP1) [6], and chaperone proteins (p23, Hsp90) [7]. TERC, TEP1, p23, and Hsp90 are expressed in a wide variety of cells, irrespective of the presence or absence of telomerase activity. On the other hand, a strong correlation is observed between hTERT mRNA expression and telomerase activity in a variety of epithelial cancers [8–11], indicating that measuring

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telomerase activity could be an important tool for the detection of tumorigenesis.

Similarly, telomeres play a critical role in chromosomes structure and function. They prevent aberrant recombination [12] and attachment of chromosomes ends to the nuclear envelope by capping the ends of chromosomes [12]. In the somatic cells, telomere loss occurs with each replication cycle due to incomplete DNA replication in the absence of telomerase [13]. Critically shortened length leads to chromosomal abnormalities, which ultimately resulting in replicative senescence of cells. In contrast, in immortal cell lines telomere length will be stabilized by the presence of telomerase [14]. Thus, stabilization of telomere length appears to be one of the earliest and most prevalent genetic alterations acquired in the multistep process of malignant transformation. Therefore, telomere length assessment could be a useful biomarker for monitoring early diagnosis of cancer. Since the telomerase activity is also present in normal (non-tumorigenic) stem cells, this assay can be used to quantify telomerase activity in normal tissues containing stem cells [15].

Here, we describe telomerase activity (TRAP) assay first and then telomere length assay.

1.1 The TRAP Assay

This assay has two steps (as shown in Scheme 1). In the first step, telomerase in cell or tissue extracts extend the TS oligonucleotide with three or more TTAGGG repeats (lower case). These telomerase products are specifically amplified by PCR with the upstream TS [2] and the downstream primer ACX [16]. The ACX primer has 6 bp "anchor" at the 5′-end that is neither telomeric nor complementary to telomeric sequences, followed by sequences that hybridize to telomeric repeats. The presence of the 6 bp anchor and non-complementary nucleotides in the primer reduces primer dimer PCR artifact formation. The anchored primer also prevents 3′ elongation of telomerase products by capping the 3′-end of telomerase product after the first PCR cycle. Thus the length of PCR products produced in a TRAP assay utilizing the ACX primer accurately reflects the activity of the telomerase being tested. The TRAP internal control utilized in the assay, TSNT, is amplified by TS primer and its own dedicated return primer, NT, which is not a substrate for telomerase (Fig. 1). Thus, the internal control is semi-competitive in that it shares only one of the primers, TS, used to amplify the telomerase products. False negative results can be easily identified by the disappearance of the internal control band with the incorporation of internal control (Fig. 2). This non-radioactive TRAP assay can be used to quantify the level of telomerase activity in normal and cancer cells as well as in tissues.

1.2 The Telomere Length Assay

This assay consists of multiple steps. The first step is the isolation of genomic DNA. The genomic DNA is then digested and the DNA fragments are separated by gel electrophoresis.

Step one:

TS primer

5' – AATCCGTCGAGCAGAGTT - 3'

Telomerase

ttaggg

Telomere repeats

5' – AATCCGTCGAGCAGAGTT**agggttagggttagggttag**

TS Telomeric Product

Step two:

5' – AATCCGTCGAGCAGAGTT**agg gt t a ggg t t agggt t aggg t t ag**– 3'

3' – C CAA T CCCA TTCCCA TTCCCA T TCGGCGCG - 5'

ACX primer

5' – AATCCGTCGAGCAGAGTT - 3

TS primer

Scheme 1 Flow chart showing steps of TRAP assay

Telomerase inhibitor treated CaP cells

NC

TRAP products

Internal control

Fig. 1 Determination of telomerase activity in prostate cancer cells (LNCaP) with or without treatments of a telomerase inhibitor for 3 days in culture. Telomerase was extracted from cell pellets and subjected to TRAP assay. *NC* negative control using lysis buffer only

Following Southern transfer of the DNA fragments to a nylon membrane. The blotter DNA fragment will be hybridized with digoxigenin (DIG-labeled probe), a chemiluminescent probe specific for telomeric repeats and incubated with a DIG-specific antibody coupled with alkaline phosphatase. The immobilized telomere probe is visualized by adding alkaline phosphatase

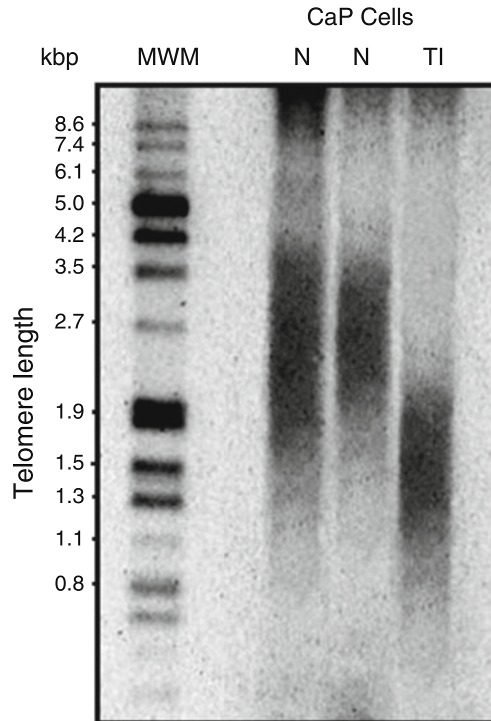

Fig. 2 Determination of telomere length in prostate cancer cells (LNCaP) with or without the treatment of a telomerase inhibitor for 3 days. *N* normal (untreated) LNCaP cells; mean telomere length is 2.5 kb; *TI* LNCaP cells treated with telomerase inhibitor; mean telomere length is 1.5 kb. *MWM* molecular weight marker

metabolizing substrate, CDP-Star. Lastly, the mean telomere repeat fragment is determined by measuring the chemiluminescence signal using an imaging system or by visually comparing the mean size of the smear to the molecular weight marker.

2 Materials

2.1 TRAP Assay

2.1.1 Buffers and Solutions (See Notes 1 and 2)

1. Phosphate buffered saline (PBS): 10 g sodium chloride (NaCl), 0.25 g potassium chloride (KCl), 1.43 g sodium phosphate dibasic (Na_2HPO_4), and 0.25 g potassium phosphate monobasic (KH_2PO_4). Make up to 1,000 ml w/dH_2O.
2. Wash buffer: 10 mM HEPES–KOH (pH 7.5), 1.5 mM magnesium chloride ($MgCl_2$), 10 mM KCl, and 1 mM dithiothreitol (DTT).
3. Cell/tissue lysis buffer: 10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.1 mM phenylmethylsulfonyl-fluoride (PMSF), 5 mM 2-mercaptoethanol, 0.5 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate hydrate (CHAPS), and 10 % Glycerol.

4. 5× TRAP buffer: 100 mM Tris–HCl pH 8.3, 7.5 mM $MgCl_2$, 315 mM KCl, 0.025 % Tween 20, 5 mM EGTA, and 0.5 mg/ml bovine serum albumin (BSA).
5. 5× TBE buffer: 54 g of Tris base, 27.5 g of boric acid, and 20 ml of 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0). Make up to 1,000 ml w/dH_2O.

2.1.2 Oligonucleotides

1. TS primer: 5′-AATCCGTCGAGCAGAGTT-3′.
2. ACX primer: 5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-3′.
3. NT primer: 5′-ATCGCTTCTCGGCCTTTT-3′.
4. TSNT primer: 5′-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3′ (Primers were synthesized from Integrated DNA Technologies, Iowa. Use primers 1–3 at 10 pM concentration and primer TSNT at 10 amol).

2.1.3 10 % PAGE for Telomere Analysis

1. Running gel: 5× TBE buffer, 5.0 ml; 30 % acrylamide, 16.6 ml; tetramethylethylenediamine (TEMED), 0.035 ml; 10 % ammonium persulfate (APS), 0.1875 ml; H_2O to 50 ml.
2. Stacking gel: Tris–HCl (pH 6.8), 0.698 ml; 30 % acrylamide, 0.752 ml; TEMED, 0.0067 ml; 10 % APS, 0.0336 ml; H_2O to 6 ml.

2.1.4 Additional Reagents

1. SYBR Green I dye (Molecular Probes, Inc.)
2. Taq-DNA polymerase (New England Biolabs).
3. dNTP mix (Invitrogen).

2.2 The Telomere Length Assay

2.2.1 Buffers and Solutions

1. Phosphate buffered saline (PBS): 10 g NaCl, 0.25 g KCl, 1.43 g Na_2HPO_4, and 0.25 g KH_2PO_4. Make up to 1,000 ml w/dH_2O.
2. DNA buffer: 1 M Tris–HCl, pH 8.0, 5 ml; 0.5 M EDTA, 5 ml; Distilled water 15 ml.
3. TAE buffer: 0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0.
4. Sodium dodecyl sulfate (SDS), 10 %.
5. Phenol–chloroform–isoamyl alcohol 25:24:1 saturated with 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
6. Sodium acetate, 3 M, pH 5.2.
7. Isopropanol (Molecular Biology grade, Fluka BioChemica).
8. Ethanol, 70 %.
9. Denaturation solution: 0.5 M NaOH, 1.5 M NaCl.
10. Neutralization buffer: 0.5 M Tris–HCl, pH 7.5.
11. 20× SSC 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
12. Stringent wash buffer I: 2× SSC, 0.1 % SDS.

13. Stringent wash buffer II: 0.2× SSC, 0.1 % SDS.
14. Blocking buffer: Dilute an appropriate volume of 10× blocking buffer (Roche) 1:10 with 1× maleic acid buffer (Roche).

2.2.2 Additional Reagents

1. Proteinase K (10 mg/ml) (Sigma Aldrich).
2. Trypsin-EDTA (Mediatech, Cellgro).
3. Agarose (Gibco BRL).
4. 0.25 HCl solution.
5. DNA digestion kit (Roche).
6. Nylon membrane (Schleicher & Schuell).

2.3 Equipment

1. Refrigerated microcentrifuge.
2. Spectrophotometer.
3. Thermocycler (with heated lid).
4. Vertical and horizontal electrophoresis system.
5. UV/Chemiluminescent image analyzer.
6. DNase- and RNase-free microfuge tubes (1.5 ml).
7. DNase- and RNase-free PCR tubes (0.2 and 0.5 ml).
8. Aerosol-barrier tips.
9. Two separate sets of pipets for pre-PCR and post-PCR use.

3 Methods

3.1 Harvesting Cells and Tissue

3.1.1 Dispersion of Cells from Solid Tissue

1. Cut the tissue into small pieces (1 mm^3), wash three times with Hank's balanced salt solution (HBSS).
2. Treat small tissue fragments with 200 U/ml collagenase (Sigma) in HBSS at 37 °C with shaking for 60 min.
3. Collect dispersed cells by centrifugation (500 × *g*) and wash with HBSS.

3.1.2 Preparation of Cell Lysate for Telomerase Activity

1. Wash cells or tissue in ice-cold PBS and pellet at 1,500 rpm for 5 min at 4 °C.
2. Resuspend cells or tissue in ice-cold wash buffer and centrifuge again at 1,500 rpm for 5 min at 4 °C.
3. Resuspend cells in ice-cold lysis buffer. For tissue, chop it into small pieces and then homogenize gently on ice. Incubate on ice for 30 min.
4. Centrifuge at 20,000 × *g* for 20 min at 4 °C.
5. Carefully separate supernatant and measure the protein concentration. At this step, samples can be stored at −80 °C in small aliquots to avoid repeated freezing and thawing.

3.1.3 TRAP Assay (See Notes 3 and 4)

1. Prepare the following TRAP reaction in a sterile 0.1 ml-PCR tube:

5× TRAP buffer	5.0 µl
dNTP (50 mM)	1.0 µl
TS primer	1.0 µl
Taq-DNA polymerase	0.5 µl
Protein lysate (1–5 µg)	
H_2O	To 25.0 µl

2. Set up the negative control reaction at the same time as the above test reaction, include all of the components listed above, but omit the protein lysate.
3. Mix the component gently, and incubate the reaction for 30 min at 23 °C followed by 90 °C for 3 min in a thermocycler.
4. To each tube add the following:

ACX primer	1.0 µl
NT primer	1.0 µl
TSNT primer	1.0 µl

5. Amplify the telomere repeats using the denaturation, annealing, and polymerization times and temperature listed below:

Cycle number	Denaturation	Annealing	Polymerization
27 cycles	30 s at 94 °C	30 s at 50 °C	1.5 min at 72 °C

3.1.4 PAGE Analysis

1. Use a vertical electrophoresis apparatus constructed to hold 22 cm glass plates for casting the gel. Spacers vary in thickness from 0.5 to 2.00 mm. However, 1 mm spacers are recommended to run TRAP products, as they produce the sharpest and flattest DNA bands.
2. After polymerization is complete, place the gel in the electrophoresis tank. Fill the reservoirs of the electrophoresis tank with 0.5× TBE buffer.
3. Mix an appropriate amount of DNA gel loading buffer with the TRAP samples and load the mixture into the wells using a gel loading micropipette. Run the gel until the marker dye migrates to the bottom of the plate.
4. Dilute SYBR Green I in TBE buffer (pH 8.0) 1: 10,000 just before use [17].
5. Transfer the gel to a shallow tray containing diluted SYBR Green I. 50 ml of staining solution is required to completely

submerge the 20/20 cm gel. Cover the tray with aluminum foil or place it in the dark and incubate the gel at room temperature with gentle agitation for 5 min. No destaining is required.

6. View the bands under UV in an imaging system. SYBR Green I is maximally excited at 497 nm and the fluorescence emission of SYBR Green I stain bound to DNA is at 520 nm.

3.2 The Telomere Length Assay

3.2.1 Isolation of Genomic DNA

1. Use a cell scraper or trypsin to detach cells from the tissue culture dish. Centrifuge the cells at 300×*g* for 10 min at 10 °C. Remove the supernatant and resuspend the cell pellet in 1× PBS. Wash the cell pellet twice with 10 ml 1× PBS, centrifuging between washes.
2. Resuspend the cell pellet in 1 ml DNA buffer. Centrifuge the cells at 300×*g* for 10 min at 10 °C. Remove the supernatant.
3. Add 300 μl of DNA buffer to resuspend the cell pellet. Then add 5 μl of Proteinase K (10 mg/ml) and 15.25 μl of 10 % SDS. Shake gently and incubate overnight at 45 °C.
4. Add 360 μl of phenol–chloroform–isoamyl alcohol or a total amount equal to the volume of the supernatant. Shake by hand for 10 min at room temperature. Centrifuge at 955×*g* for 10 min at 10 °C.
5. Transfer the supernatant to a new tube and measure the volume. Add 1/10 the volume of 3 M sodium acetate (pH 5.2) and 3× the volume of 100 % isopropanol. Shake gently until the DNA is precipitated.
6. Use a sterile glass pipette to transfer the precipitated DNA into a tube with 200 μl of 70 % ethanol. Place the tube inverted on a rack for 2 h. Transfer the DNA into a sterile eppendorf tube.
7. Centrifuge at 10,600×*g* for 10 min at 10 °C. Dry the DNA pellet in a SpeedVac for 5 min. Dissolve the DNA in 100–200 μl of sterile water. Place the tube in an eppendorf thermomixer shaker overnight at 37 °C.
8. Measure the DNA concentration and run 1–5 μl of the DNA on a 1 % agarose gel.

3.2.2 Digestion of Genomic DNA (See **Note 5**)

1. Prepare DNA digestion enzyme mixture by mixing 20 U/μl each of Hinfl and Rsa1 (Roche). 20 U/μl enzyme mix needed for each sample.
2. Dilute 1–2 μg of purified genomic DNA with nuclease free water to a final volume of 17 μl.
3. For each sample, add 2 μl of 10× digestion buffer (Roche), and 1 ml of enzyme mixture and mix gently.
4. Incubate the reaction mixture at 37 °C for 2 h.
5. After the incubation add appropriate amount of DNA gel loading buffer to stop the reaction and quick spin vials.

3.2.3 Gel Electrophoresis

1. Prepare a 0.8 % agarose gel in 1× TAE buffer.
2. Load digested genomic DNA samples.
3. Mix 4 μl DIG molecular weight marker (Roche), 12 μl nuclease free water and appropriate volume of DNA gel loading buffer. Load 10 μl on either side of the samples to measure length accurately.
4. Run gel at 5 V/cm in 1× TAE buffer until the bromophenol blue dye is separated about 10 cm from the starting wells.
5. Submerge the gel in HCl solution for 10 min until the bromophenol blue stain changes to yellow, with agitation, at room temperature.
6. Rinse the gel two times with H_2O.
7. Submerge the gel in the denaturation solution for 2 × 15 min at room temperature.
8. Rinse the gel two times with H_2O.
9. Submerge the gel in the neutralization solution for 2 × 15 min at room temperature.

3.2.4 Southern Transfer to Nylon Membrane

It is recommended that powder-free rubber gloves be worn and handle membrane with forceps only at the edges.

1. Set up the transfer in a large electrophoresis tray. Fill the tray with transfer buffer (10× SSC). Assemble the following items (gel-sized and saturated with transfer buffer) in the middle support. Carefully remove all air bubbles.
   - Wick (Whatman 3MM paper): This should be the same width as your gel and long enough to drape into the transfer buffer.
   - Three pieces of Whatman 3MM paper.
   - Gel (upside down).
   - Nylon membrane (Handle with clean gloves and blunt-ended forceps. Do not adjust the membrane once it is placed on the gel.). Cut off the lower left hand corner of the nylon membrane for orientation.
   - Three pieces of Whatman 3MM paper.
   - Dry 5 cm stack of paper towels cut to the size of the gel.
   - Glass plate and then a weight on top.
2. Surround the transfer tray with plastic wrap or Parafilm to prevent evaporation of the transfer buffer. Let transfer overnight.
3. After the Southern transfer, fix the transferred DNA on the wet blotting membrane by UV-cross-linking (120 mJ).
4. Wash the blotting membrane with 2× SSC.
5. If not used immediately for the hybridization, the membrane can be air-dried and stored at 4 °C.

3.2.5 Hybridization and Chemiluminescence Detection (See **Notes 6–8***)*

1. Prewarm 25 ml of DIG hybridization solution to 42 °C.
2. Prehybridize the blot by submersing the blot in 18 ml of prewarmed DIG hybridization buffer and incubate for 30–60 min at 42 °C with gentle agitation.
3. Prepare hybridization buffer solution by adding 1 ml telomere probe (Roche) in 5 ml fresh prewarmed DIG hybridization buffer and mix.
4. Discard prehybridization buffer and immediately add hybridization solution to the membrane.
5. Incubate for 3 h at 42 °C with gently agitation.
6. Wash the membrane three times with sufficient stringent wash buffer I for 5 min at room temperature with gentle agitation.
7. Wash the membrane three times with sufficient prewarmed stringent wash buffer II for 20 min at room temperature at 50 °C with gently agitation.
8. Rinse the membrane in 25 ml 1× washing buffer for 5 min at room temperature in gentle agitation.
9. Incubate the membrane in 25 ml freshly prepared blocking solution for 30 min at room temperature with gentle agitation.
10. Dilute appropriate volume of Anti-DIG-AP (Roche) with blocking solution to a final concentration of 75 mU/ml.
11. Remove blocking solution and incubate the membrane in 25 ml Anti-DIG-AP solution for 30 min at room temperature with gentle agitation.
12. Wash the membrane two times for 15 min with 25 ml of 1× washing buffer (Roche) at room temperature with gentle agitation.
13. Incubate the membrane in 20 ml of detection buffer (Roche) at room temperature with gentle agitation.
14. Discard detection buffer and remove excess liquid by placing on absorbent paper. Do not dry the membrane.
15. Immediately place the wet membrane, the DNA side facing up, on a sheet of polyethylene wrap and quickly apply approximately 40 drops substrate solution (Roche) onto the membrane.
16. Immediately cover the membrane with the second sheet of polyethylene wrap to spread the substrate solution homogeneously and without air bubbles over the membrane.
17. Expose the membrane to the imaging device or to X-ray film for 5–20 min.

3.2.6 Telomere Repeat Fragment Analysis

After exposure of the blot to an X-ray film, an estimation of the mean TRF length can be obtained by visually comparing the mean size of the telomeric DNA to the molecular weight marker.

4 Notes

1. Add PMSF to cell/tissue lysis buffer just before use (Subheading 2.1.1).
2. Filter 5× TRAP buffer and store at −20 °C in aliquots (Subheading 2.1.1).
3. Taking into account the size of the electrophoresis glass plates and the thickness of the spacers, calculate the volume of gel required. Samples should be run in 10 % PAGE with 22 cm long plates in order to see well-separated telomere products as shown in representative picture (Subheading 3.1.3).
4. SYBR Green I is a light-sensitive dye. Protect the solution from light all the time (Subheading 3.1.3).
5. Prepare and pipette the genomic DNA digestion mixture on ice. Prepare master-mix for all samples to be analyzed (Subheading 3.2.2).
6. The volume of DIG hybridization solution, washing buffer, telomere probe, and blocking solution are recommended for 200 cm^2 membrane size (Subheading 3.2.5).
7. Prepare Anti-DIG-AP solution before use and do not store (Subheading 3.2.5).
8. Luminescence intensity of the membrane, after treated with detection buffer, will be high during the first hour. However, it continues for at least 24 h (Subheading 3.2.5).

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Chapter 18

Detecting ATM-Dependent Chromatin Modification in DNA Damage Response

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Abstract

Loss of function or mutation of the ataxia–telangiectasia mutated gene product (ATM) results in inherited genetic disorders characterized by neurodegeneration, immunodeficiency, and cancer. Ataxia-telangiectasia mutated (ATM) gene product belongs to the PI3K-like protein kinase (PIKKs) family and is functionally implicated in mitogenic signal transduction, chromosome condensation, meiotic recombination, cell-cycle control, and telomere maintenance. The ATM protein kinase is primarily activated in response to DNA double strand breaks (DSBs), the most deleterious form of DNA damage produced by ionizing radiation (IR) or radiomimetic drugs. It is detected at DNA damage sites, where ATM autophosphorylation causes dissociation of the inactive homodimeric form to the activated monomeric form. Interestingly, heat shock can activate ATM independent of the presence of DNA strand breaks. ATM is an integral part of the sensory machinery that detects DSBs during meiosis, mitosis, or DNA breaks mediated by free radicals. These DNA lesions can trigger higher order chromatin reorganization fuelled by posttranslational modifications of histones and histone binding proteins. Our group, and others, have shown that ATM activation is tightly regulated by chromatin modifications. This review summarizes the multiple approaches used to discern the role of ATM and other associated proteins in chromatin modification in response to DNA damage.

Key words Ataxia–telangiectasia, Telomerase, Double-stranded DNA breaks, Chromatin modification

1 Introduction

Maintenance of genomic stability depends on appropriate cellular responses to DNA damage [1, 2]. The response is based on a complex network of signaling pathways that activate numerous processes that ultimately lead to damage repair and cell survival or death. The precise sequence of events that follow IR-induced DNA damage in cells is still not well understood but it is increasingly evident that a complex series of proteins are recruited in a time dependent manner at different stages of the cell cycle to maintain genomic stability after DNA damage. The ATM protein

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plays a key role in this mechanism by sensing, processing, and signaling of IR-induced DNA damage through its interactions with various proteins [3]. Notably, cells deficient in ATM display a high frequency of spontaneous chromosomal aberrations, high rates of intrachromosomal recombination, and error-prone recombination [4–7]. The cells also have a higher level of initial and residual chromosomal aberrations in G_1 and G_2 phase cells after IR exposure, as determined by analysis of prematurely condensed chromosomes [8–10] and by karyotypic examination of metaphase chromosome spreads [10, 11].

1.1 ATM Activation and DNA Repair

Mammalian cells contain many highly specialized chromatin structures such as telomeres, replication forks, and compact heterochromatin. These structures have specific patterns of histone modifications, differences in nucleosome packing density and varied interactions with binding proteins (reviewed in [12]) On the other hand, proper recognition and recruitment of DNA repair proteins requires efficient access to the damaged site. Hence, the complex and diverse organizational pattern of chromatin poses a series of challenges to the DSB repair machinery which necessitates the ability to rapidly reorganize chromatin and rapidly complete DNA repair with a minimal number of steps [13]. Chromatin structural alterations initiated by posttranslational modification such as phosphorylation, ubiquitination, acetylation, and deacetylation have all been linked to the repair of damaged DNA. Under normal conditions, cellular ATM is found in a homodimeric inactive state but upon IR exposure ATM undergoes rapid autophosphorylation at serine 1981 and dissociates into active monomers, with kinase activity (*see* **Note 1**). It is proposed that the ATM conversion from dimer to monomer is regulated by the state of the chromatin structure itself [14]. Activated ATM phosphorylates histone H2AX at the carboxy-terminal serine 139 immediately following ionizing radiation exposure [15], or Spo11-induced DSB formation during meiotic prophase [16], and together with dephosphorylation of Tyr 142, binds MDC1 (mediator of DNA damage checkpoint 1 protein). Phosphorylation of H2AX spreads along the chromatin for hundreds of kilobases away from the DSB site in mammalian cells [17, 18]. Analysis of H2AX-deficient mice has demonstrated a role for H2AX in a variety of responses to DSBs, including DNA repair, checkpoint signaling, and immunoglobulin gene class switching [19] (*see* **Notes 2** and **3**). Mice deficient in H2AX (*H2ax*$^{-/-}$) exhibit male-specific sterility, likely due to defects in chromatin remodeling during meiosis. In addition to H2AX, activated ATM has been shown to phosphorylate several other substrates [3] including proteins involved in checkpoint activation (p53, and Chk2), and DNA repair such as BRCA1 CtIP, and 53BP1 [20, 21]. ATM also has a critical role in maintaining chromosome condensation in the vicinity of recombination intermediates [4, 22] and ATM phosphorylates the structural maintenance of chromosomes protein

(Smc1) necessary for sister chromatid cohesion [4, 23, 24]. Several studies have indicated that the MRN (Mre11/Rad50/NBS1) complex as well as TRF2 either influence activation of ATM or serve to modulate/amplify ATM activity [1, 4, 25–27]. Thus, ATM is a "hierarchical kinase," capable of initiating many pathways simultaneously [4, 5, 28] (Fig. 1). Studies on DNA and chromosome damage after IR exposure suggested that ATM defective A-T cells more efficiently convert DNA damage into chromosome damage [8, 9] presumably due to an inherent chromatin alteration [29]. The chromatin-modifying factor human males absence on the first (hMOF) hMOF influences IR-induced initiation of ATM kinase activity [30]. Furthermore, detailed studies have revealed that heat shock, which induces chromatin alterations independent of DNA damage, also activates ATM [11] (*see* **Note 4**).

1.2 Chromatin Modifiers and DNA Damage Response

The DNA repair involves alterations in chromatin structure, which are required for direct access to DNA damage sites. This process is facilitated by chromatin modifying factors, which temporarily allow DNA loss or detachment from histones and other bound proteins. Posttranslational histone acetylation has emerged as key regulatory events and a widespread modification in DNA damage responses. Acetylation is regulated by the balance between histone acetyl-transferases (HATs), which transfer an acetyl moiety, and histone deacety-

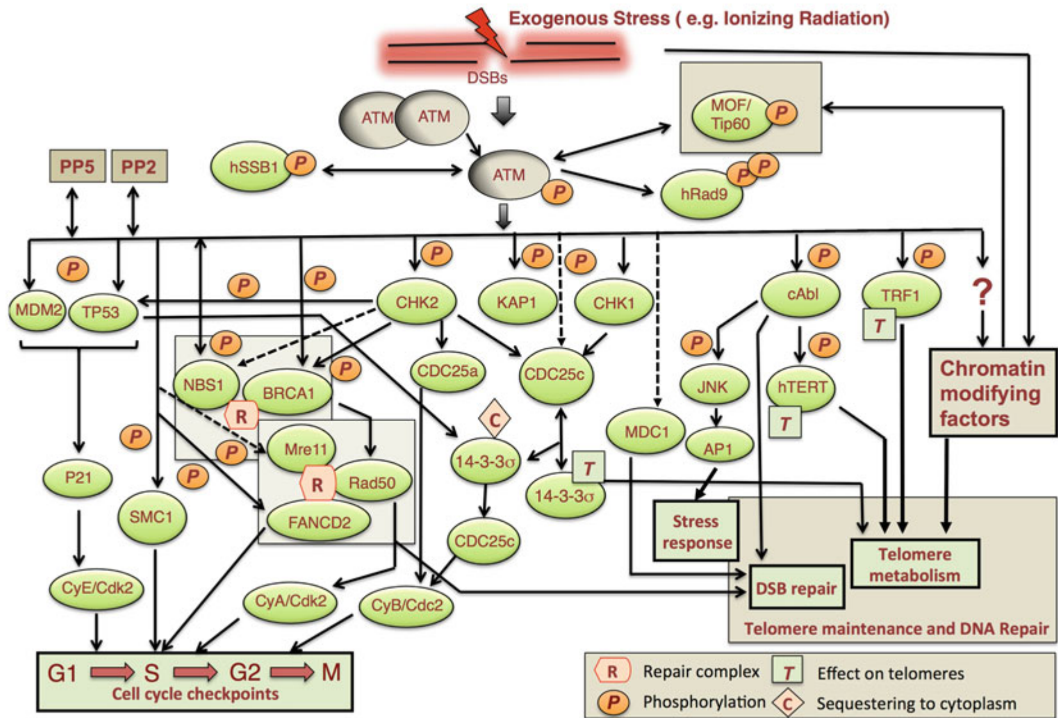

Fig. 1 A schematic showing the various signaling pathways modulated by ATM kinase in response to DNA damage by ionizing radiation. Several phosphorylation as well as acetylation events are regulated by ATM, enabling the appropriate response to DNA damage

lases (HDACs), which reverse the reaction. Reversible acetylation of four lysines (K), at positions 5, 8, 12, and 16 in the amino-terminal tail of histone H4 occurs in vivo in all eukaryotes [31, 32]. Hyperacetylation of histone H4 can lead to unfolding of the nucleosomal fiber [33]. Acetylation of histone H4 at K16 occurs on the transcriptionally hyperactive male X chromosome of *Drosophila* polytene chromosomes [34], an indirect indication of greater access to the DNA by RNA polymerase. Studies have shown that histone H4K16 controls chromatin structure and protein interactions [35]. Tip60 (Tat interacting protein) interacts with ATM, acetylates histones (H2A, H3, H4), and also plays a role in DNA repair [36, 37]. In *Drosophila* Tip60 acetylates nucleosomal phospho-H2Av and exchanges it with an unmodified H2Av [38]. Bird et al. [39] reported that the acetylation of histone H4 by Esa1 (essential SAS2-related acetyltransferase) is required for DNA repair in yeast and suggested that a similar modification may function in mammalian cells. Acetylation of H3K56 has been shown to play a crucial role in DNA replication and genomic stability [40, 41]. ATM has been reported to regulate the function of several histone-modifying factors. We previously identified hMOF as an ATM interacting protein and have provided evidence that hMOF participates in the activation of ATM in response to DNA damage [30]. More recently, we have further shown that ATM-mediated MOF phosphorylation modulates the function of 53BP1, an adapter/mediator critical for processing damaged DNA ends, thus establishing a regulatory role for MOF in DSB repair pathway choice in S/G2 phase cells [42, 43].

In spite of accumulating literature concerning histone modifications that participate in specific repair processes, pathway choice, or checkpoint control, mechanistic studies are still limited. Identification of chromatin modifying factors that play a role in the IR response will help to understand the mechanisms by which IR-induced chromatin modifications trigger ATM activation. The protocols presented in this chapter describe how to analyze DNA damage using different methods (*see* **Note 5**).

1.3 Determination of ATM Interacting Proteins

Various approaches are used to determine the interactions between chromatin and chromatin binding proteins. For in vivo analysis, the most commonly used techniques are described below.

2 Materials

2.1 Co-immunoprecipitation and Immunoprecipitation

1. Total cell extracts or nuclear extracts from cell lines under study. The cells should be growing exponentially at the time of protein extraction using the extraction buffers described below.
2. Protein A/G beads and antibodies targeting the protein of interest.
3. Immunoprecipitation (IP) buffer: (Mild) NP40 buffer; 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 % NP-40, (More Stringent) RIPA buffer; 10 mM Tris–HCl (pH 8.0), 1 mM

EDTA, 0.5 mM EGTA, 1 % Triton X-100, 0.1 % sodium deoxycholate, 0.1 % SDS, 140 mM NaCl.

4. Protease inhibitor cocktail (100×): 18 mg/ml PMSF, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 70 μg/ml pepstatin in 100 % methanol; store at −20 °C.
5. Sample loading buffer: 50 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol (DTT), 2 % SDS, 0.02 % bromophenol blue, 10 % glycerol.

2.2 Resolution of Proteins with SDS-PAGE and Western Blotting Analysis

2.2.1 SDS-PAGE Separation

1. SDS-PAGE 4–20 % with varying acrylamide concentration depending on the molecular weight of proteins to be resolved. The protocol for casting 8 % and 12 % gels is described below.
   (a) Stacking gel (10-ml total volume): 6.3 ml H_2O, 2.5 ml 0.5 M Tris–HCl, pH 6.8, 1.0 ml 40 % acrylamide solution (37.5:1 = acrylamide–bis), 100 μl 10 % SDS, 100 μl 10 % APS (ammonium persulfate), 20 μl TEMED.
   (b) 8 % SDS-PAGE separating gel (10-ml total volume): 5.3 ml H_2O, 2.5 ml 1.5 M Tris–HCl, pH 8.8, 2.0 ml 40 % acrylamide solution, 100 μl 10 % SDS, 100 μl 10 % APS, 10 μl TEMED.
   (c) 12 % SDS-PAGE separating gel (based on 10-ml total volume): 4.3 ml H_2O, 2.5 ml 1.5 M Tris–HCl, pH 8.8, 3.0 ml 40 % acrylamide solution, 100 μl 10 % SDS, 100 μl 10 % APS, 10 μl TEMED.
2. SDS-PAGE running buffer (10×): 30.3 g Tris–HCl, 144 g glycine, 10 g SDS, pH to 8.3 to 1 l with ddH_2O.

2.2.2 Western Blotting

1. Blocking solution (5 % dry milk powder in TBST [pH 7.4]).
2. ECL (enhanced chemiluminescent) detection kit.
3. SDS-PAGE transfer buffer (1×): 700 ml H_2O, 100 ml of 10× SDS-PAGE running buffer without SDS (30.3 g Tris–HCl, 144 g glycine, pH 8.3 to 1 l dH_2O), and 200 ml methanol.
4. 10× Phosphate-buffered saline (10× PBS) (pH 7.4): To prepare 1 l of 10× PBS, dissolve the reagents (80 g NaCl, 2.0 g KCl, 14.4 g Na_2HPO4, 2.4g KH_2PO_4) in 800 ml of H_2O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H_2O to 1 l.
5. Primary and secondary (Horseradish peroxidase (HRP)-conjugated) antibodies.
6. TBS-T (pH 7.4) 20 mM Tris–HCl, 150 mM NaCl, and 0.2 % Tween 20.
7. Microcentrifugation tube (1.5-ml).
8. Electrotransfer apparatus.
9. Enhanced chemiluminescent detection equipment.

10. Water bath (for boiling samples).
11. PVDF membrane or nitrocellulose membrane.
12. SDS-polyacrylamide gel electrophoresis equipment.

2.3 Immunofluorescence Staining

1. Specific primary antibodies to proteins of interest, and fluorescence labeled secondary antibodies.
2. Blocking solution (5 % normal goat serum in PBS).
3. Paraformaldehyde (PFA) (4 %) in 1× PBS: 4 % paraformaldehyde solution: Add 4 g of EM grade paraformaldehyde to 100 ml 1× PBS and dissolve with heat carefully.
4. 1× PBS and Hardset mounting medium with DAPI.
5. 2-wells or 4-wells glass chamber slides.
6. Fluorescence microscope or confocal microscope.

2.4 ATM Kinase Assay

1. Lysis buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % Tween 20, 0.2 % NP-40, 1 mM NaF, 1 mM $NaVO_4$, 50 mM glycerophosphate, 10 % glycerol, 1 mM PMSF, 2 μg/ml pepstatin A, 5 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM dithiothreitol (DTT).
2. Anti-ATM antibodies and Protein A/G Sepharose beads.
3. 0.5 M LiCl.
4. Kinase buffer: 50 mM Hepes pH 7.5, 150 mM NaCl, 1 % Tween 20, 0.2 % NP-40, 1 mM NaF, 1 mM $NaVO_4$, 1 mM DTT, 10 mM $MnCl_2$.

2.5 Chromatin Immunoprecipitation (ChIP)

1. Swelling buffer: 25 mM Hepes, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 % NP-40, 1 mM DTT, 0.5 mM PMSF, and Protease inhibitor cocktail.
2. Sonication buffer: 50 mM Hepes pH 7.9, 140 mM NaCl, 1 mM EDTA, and 1 % Triton X-100, 0.1 % Na-deoxycholate, 0.1 % SDS, 0.5 mM PMSF, and Protease inhibitor cocktail.
3. Wash buffer A: 50 mM Hepes pH 7.9, 500 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % Na-deoxycholate, 0.1 % SDS, 0.5 mM PMSF, and Protease inhibitor cocktail, Wash buffer B: 50 mM Hepes pH 7.9, 250 mM LiCl, 1 % NP-40, 1 % Na-deoxycholate, 1 mM EDTA, and Protease inhibitor cocktail (Roche).
4. Elution buffer: 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 % SDS.
5. Sonicator.
6. Antibodies to target proteins.
7. PCR reagents.

2.6 Histone Acetyl Transferase Assay

1. Histone acetyl transferase kit with reagents and controls.
2. Coenzyme A.
3. DMSO.
4. Recombinant proteins, and acetyl transferases of interest.

2.7 Replication Restart Assay

1. 5-iododeoxyuridine (IdU).
2. Hydroxyurea (HU).
3. 5-chlorodeoxyuridine (CldU).
4. Hypotonic lysis solution: 10 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$, 1 mM PMSF, and 0.5 % NP-40.
5. 5 % bovine serum albumin (BSA).
6. Primary antibodies against IdU and CldU.
7. Secondary antibodies: anti-rabbit Alexa Fluor 488-conjugated, and anti-mouse Alexa Fluor 568-conjugated.
8. 0.1 % Triton X-100.
9. VECTASHIELD mounting medium without 4′,6-diamidino-2-phenylindole (DAPI).
10. Phosphate buffered saline (PBS).
11. ImageJ software for analysis.

2.8 Chromosomal DNA Strand Break Analysis

2.8.1 Pulsed Field Gel Electrophoresis (PFGE)

1. Tris-EDTA buffer [10 mM Tris–HCl, 1 mL EDTA (pH 8)].
2. 0.8 % agarose gels, ethidium bromide, NaCl, and Triton X-100.
3. CHEF DRIII system (Bio-Rad, Hercules, CA).
4. Charge-coupled device camera system under UV transillumination.

2.8.2 Neutral Filter Elution

1. Cells of interest and appropriate growth medium for cells.
2. [Methyl ^{3}H]thymidine (20 Ci/mmol) or [2-^{14}C]thymidine (50 mCi/mmol) for radioactive determination.
3. Unlabeled thymidine and test compound.
4. PBS, ice-cold.
5. Cell lysis solution, pH 9.6: 2 % SDS, 25 mM EDTA, and 0.1 M glycine (optional). Adjust pH to 9.6 with 1 N NaOH.
6. Cell lysis solution (above) containing 0.5 mg/ml proteinase K.
7. Alkaline elution solution, pH 12.3: 0.1 % SDS, 20 mM EDTA, 0.8 % tetrapropylammonium hydroxide. Adjust pH to 12.3 with 20 % (w/v) tetrapropylammonium hydroxide in water.

8. Scintillation fluid (e.g., Ecolume, ICN Biomedicals) containing 0.7 % (v/v) acetic acid 0.2 M tetrasodium EDTA, pH 10 (pH adjusted with 1 N NaOH).
9. 1 N HCl.
10. 0.4 N NaOH.
11. 1 M potassium dihydrogen phosphate.
12. 17 mM KH_2PO_4.
13. 150 μM Hoechst 33258 stock solution.
14. Tabletop centrifuge and 2.0-μm polycarbonate filter (25-mm diameter).
15. 1-l vacuum flask, 60 °C oven or water bath.
16. Alkaline elution funnel apparatus (e.g., Millipore).
17. Aluminum foil or black paper cylinder.
18. Multichannel peristaltic pump and suitable Tygon tubing.
19. Fraction collector.
20. Glass scintillation vials and liquid scintillation counter or fluorometer.

3 Methods

3.1 Immunoprecipitation and Co-immunoprecipitation

Several approaches are used to determine ATM autophosphorylation and ATM-dependent phosphorylation. These include co-immunoprecipitation, western blotting, and cytological analysis. First phosphorylation sites of protein substrate are identified by MALDI-TOF mass spectroscopy. Once the residue(s) are identified, phosphospecific antibodies are generated. Co-immunoprecipitation is carried out followed by western blotting to resolve and quantitate the phosphorylation status of the interacting proteins, or immunostaining of fixed cells to detect the phosphorylated form of proteins. The beads selected to bind the antibodies depend on the species and subclass of the antibody used (e.g., Protein A beads for rabbit, mouse IgG2a, IgG2b, and IgG3; and Protein G beads for mouse IgG1 and most subclasses of rat IgGs).

1. Wash 50 μl of Protein A/G bead suspension by adding 500 μl cold IP buffer in a 1.5 ml eppendorf tube and spinning for 5 s, 15,300 × *g* in a microcentrifuge. Remove supernatant and repeat the washing step twice. Remove supernatant.
2. To the washed beads, add 5–10 μg of specific antibody or control antibody.
3. Incubate for 1 h at 4 °C rotating gently.

4. Wash beads three times with IP buffer as in **step 1** to remove unbound antibodies. Add cold protein extract in IP buffer with protease inhibitors (500–1,000 μg). Adjust the volume to 500 μl with IP buffer. Incubate at gentle rotation at 4 °C for 1–4 h.
5. Carefully remove the supernatant completely after spinning down the beads and wash/pellet the beads three to five times using 500 μl of IP buffer.
6. After the last wash, aspirate supernatant and add 50 μl of sample loading buffer to bead pellet. Vortex and heat to 100 °C for 5 min. Spin at 10,000 × *g* for 5 min collect the supernatant and load onto an SDS-polyacrylamide gel.

3.2 Resolution of Proteins with SDS-PAGE and Western Blotting Analysis

3.2.1 SDS PAGE Analysis

1. Remove medium from the cells, approximately 1×10^6 in 60 mm dish. Rinse the cells once with 1,000 μl of PBS and add 200 μl of trypsin-EDTA. Incubate for 1 min at 37 °C; suspend the cells and add 1,000 μl of DMEM medium to quench the trypsin.
2. Transfer the suspended cells to a chilled 1.5-ml centrifugation tube. Collect the cells by centrifugation at 300 × *g* for 2 min at 4 °C. Resuspend the cell pellet in 500 μl ice-cold PBS and centrifuge again.
3. Resuspend the cells in 500 μl ice cold buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 % NP40, 1 mM DTT, 1 mM EDTA, 50 mM glycerophosphate, 10 % glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 5 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM dithiothreitol (DTT) at 4 °C for 10 min. then sonicate three times for 10 s each on ice. Centrifuge for 10 min at 15,000 × *g* to pellet insoluble cell debris.
4. Recover the supernatant and determine the protein concentration by Bradford protocol. For gel analysis, use 20 μg of supernatant protein in 1× Laemmli buffer and incubate the sample for 3 min in a boiling water bath and vortex.
5. Separate the proteins by loading onto an SDS-polyacrylamide gel using an acrylamide concentration appropriate to resolve the molecular weight of the targeted protein.
6. Transfer proteins from the gel to a PVDF or nitrocellulose membrane using an electrotransfer apparatus.

3.2.2 Western Blotting Analysis

1. Incubate the membrane in blocking solution (PBS solution containing 0.05 % Tween 20, and 5 % nonfat dried milk, to block nonspecific binding) for 1 h at 37 °C.
2. Replenish the blocking solution with fresh blocking solution and add the primary antibody at the appropriate dilution.

Incubate for 1–2 h at room temperature or over night at 4 °C. Wash the blot four times with TBST for 10 min.

3. For ECL detection, incubate the blot with either HRP-conjugated secondary antibody at a suitable dilution in blocking solution for 1–2 h at room temperature. After washing with TBST four times 10 min each, perform ECL detection.

3.3 Immunostaining

1. Grow cells on coverslips and wash with PBS.
2. Incubate the samples in 4 % PFA 10–20 min at room temperature. Rinse the specimens three times in 1× PBS for 5 min each (total time of 15 min).
3. Permeabilize the cells for 20 min in 0.5 % NP-40. Circle the area of interest on each slide with a hydrophobic PAP pen. Gently pipette ~200–300 μl of blocking solution onto each slide. Incubate the slides for 1–2 h at room temperature.
4. Remove the blocking solution by draining the excess. Add ~100–200 μl of primary antibody diluted in blocking solution to each slide. Incubate the slides overnight at 4 °C.
5. Remove the primary antibody by rinsing the slides three times in PBS for 10 min each at room temperature. Drain the excess PBS, and add 100–200 μl of secondary antibody diluted in blocking solution· Incubate the slides for 1–2 h at room temperature.
6. Remove the secondary antibody by rinsing the slides three times in PBS for 10 min each at room temperature. Drain as much PBS from the slides as possible. Add one drop of mounting medium directly onto each section.
7. Carefully place a coverslip onto each slide. Allow mounting medium to harden for at least 3 h at room temperature before imaging. Image the slides on a confocal or fluorescent microscope.

3.4 ATM Kinase Assay

ATM is a serine/threonine kinase and specifically phosphorylates SQ/TQ sites. To determine the substrates of ATM phosphorylation, in vitro kinase assays are performed.

1. Cells are broken open in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % Tween 20, 0.2 % NP-40, 1 mM NaF, 1 mM $NaVO_4$, 50 mM glycerophosphate, 10 % glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 5 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM DTT).
2. Lysates are pre-cleared for 1 h at 4 °C with 1 mg rabbit purified IgG and 40 μl protein A/G beads.

3. Endogenous ATM proteins are immunoprecipitated with anti-ATM antibodies and protein A/G Sepharose. Immunoprecipitants are washed three times with lysis buffer, once with lysis buffer and 0.5 M LiCl, and three times with kinase buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1 % Tween 20, 0.2 % NP-40, 1 mM NaF, 1 mM $NaVO_4$, 1 mM DTT, 10 mM $MnCl_2$).
4. Immunoprecipitant beads are mixed with kinase buffer containing 10 μCi [γ-^{32}P]ATP and 0.1 mg GST-$p53_{1-101}$, incubated for 20 min at 30 °C. The kinase reactions are stopped with SDS-PAGE loading buffer and reaction mixtures are separated by SDS-PAGE.
5. Western transferred ATM and substrates are visualized and quantitated on a PhosphorImager. ATM proteins are detected using anti-ATM antibodies.

3.5 Chromatin Immunoprecipitation (ChIP) Assay

The Chromatin Immunoprecipitation (ChIP) assay is used to analyze the association of proteins with specific DNA regions. This technique involves cross-linking of the proteins to the bound DNA then preparing a soluble form of the chromatin followed by immunoprecipitation with an antibody for the protein of interest. The region of DNA associated with the protein is then identified by PCR amplification of the co-immunoprecipitated genomic DNA [43, 44]. The general methodology is described below.

1. Culture cells by growing in 150 mm dishes, containing $2–5 \times 10^7$ cells per plate. Replace media with fresh serum-free medium (27 ml). Cross-link by adding 3 ml formaldehyde (10 %) and mix immediately. Incubate at room temperature for 10 min.
2. Add 3 ml glycine (1.25 M) and mix immediately to stop the reaction and incubate for 5 min at room temperature.
3. Place plates on ice and wash three times with 20 ml ice-cold PBS/0.5 mM PMSF. Scrape cells into 20 ml ice cold PBS/PMSF and centrifuge for 5 min at 1,000 rpm.
4. Resuspend pellet in 10 volumes of swelling buffer and dounce-homogenize to isolate nuclei by centrifugation at 5,000 rpm for 5 min.
5. Resuspend the pellets in 5–10 ml sonication buffer and sonicate ten times for 10–20 s at 80 % setting on ice. Centrifuge the samples twice at 14,000 rpm for 15 min and centrifuge the supernatant again for 5 min.
6. Pre-clear the lysate by incubating by constant rotation with Protein-A or G Sepharose. Redistribute the samples to 1 ml aliquots for IP reaction.
7. Add about 5 μg of antibody and incubate at 4 °C for 2 h; add 40 μl of Protein-A or G Sepharose per IP (pre-equilibrated

with appropriate IP buffer) and incubate overnight by constant rotation at 4 °C.

8. Centrifuge the beads at 6,000 rpm for 3 min and wash with 1 ml sonication buffer, and the wash step should be repeated three times with 10 min each time.
9. Wash again two times with buffer A, and two times with buffer B, and then with TE buffer.
10. Add 200 μl of elution buffer to the beads before incubating at 65 °C for 10 min and centrifuging at 14,000 rpm for 1 min.
11. Carry out the de-cross-linking step by adding required amount of NaCl and incubating at 65 °C overnight or for at least 5 h.
12. Add RNase A and incubate at 37 °C for 1 h.
13. Add EDTA and proteinase K and incubate at 42 °C for 2 h.
14. Extract the samples two times with phenol–chloroform–isoamyl alcohol and once with chloroform–isoamyl alcohol. Further, make final concentration of 0.05 μg/μl glycogen, 0.3 M Na-acetate, and 70 % ethanol and incubate at −20 °C overnight.
15. Centrifuge at 14,000 rpm 30 min and wash with 70 % ethanol. Then resuspend the IP and INPUT samples in 100 μl of 10 mM Tris (pH 7.5) and carry out PCR analysis, or submit them for sequencing.

3.6 Histone Acetyl-Transferase Assay

Histone acetylation alters nucleosomal interactions of the chromatin fiber to facilitate decondensation and enhance access to nucleosomal DNA. We have shown that the absence of an acetyl transferase, MOF, influences ATM activation and results in delayed appearance of gamma-H2AX foci. We have further shown that ATM-mediated MOF-T392 phosphorylation is involved in repair pathway choice [43]. A simple, robust in vitro assay to measure histone acetyl transferase activity is described below [45]. The assay can be adapted for other substrates by generating other fusion proteins and acetyl transferases of interest by modifying conditions.

1. Recombinant proteins are immunoprecipitated from 100 μg of nuclear extract using either anti-FLAG or anti-HA antibodies plus Protein A-agarose (Sigma).
2. Immunoprecipitates are washed twice in phosphate buffered saline containing 0.1 % Tween 20 (PBS-T), and once in acetyltransferase assay buffer (50 mM Tris-HCl pH 8.0, 10 % glycerol, 10 mM butyric acid, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF).
3. Each reaction contains immunoprecipitated proteins, mixed with 10 μM acetyl CoA, and different quantities of purified substrates as per the kit protocol.

4. Acetylation reactions are incubated for 45 min at 30 °C on a rotating platform, followed by SDS-PAGE sample buffer elution, followed by electrophoresis and western blotting.

3.7 Replication Restart Assay

DNA replication occurs during the synthesis (S) phase of the cell cycle and starts at predefined replication origins or origin firing [46, 47]. Once the origins of replication have fired, the DNA replication proteins organize into a structure called the replication fork. These forks are highly susceptible to stalling or collapse when they encounter obstacles such as unrepaired DNA damage, chromatin bound proteins, or secondary structures of the DNA. Chemicals such as hydroxyurea (HU) also lead to fork stalling or collapse. While the stalled forks are capable of reversing when the stress is removed, the collapsed forks are essentially irreversible due to generation of double strand breaks or dissociation of replication machinery [48]. In recent years, the single-molecule analysis of DNA replication has become the standard assay for replication fork restart efficiency as a response to inhibition of DSB repair pathways. While several lines of clear evidence point to the role of ATR and its substrates [49–52], and nuclear structural proteins [53] in modulating the replication fork restart, only limited evidence exists that shows ATM involvement in replication restart in cooperation with ATR [54, 55]. Nevertheless, this assay system accurately analyzes the involvement of DNA helicases, nucleases, and homologous recombination factors during replicative stress. The general methodology is described below.

1. The exponentially growing cells are pulsed with 50 mM 5-iododeoxyuridine (IdU) for 20 min, and then washed three times with phosphate-buffered saline (PBS).
2. Then the cells are treated with 2 mM HU for the appropriate intervals, washed three times with PBS.
3. Then the cells are incubated in fresh medium containing 50 mM 5-chlorodeoxyuridine (CldU) for 20 min, and then washed three times in PBS.
4. The cells labeled with IdU and CldU are mixed with unlabeled cells in a ratio of 1:10, and 2-μl cell suspensions are dropped onto a glass slide and then mixed with a 20-μl hypotonic lysis solution (10 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 0.5 % Nonidet P-40) for 8 min.
5. The slides are air-dried and fixed with methanol, followed by 70 % ethanol, and washed with 1× PBS.
6. The slides are then blocked with 5 % bovine serum albumin (BSA) for 15 min, and incubated with primary antibodies against IdU and CldU and secondary antibodies, anti-rabbit

Alexa Fluor 488-conjugated, and anti-mouse Alexa Fluor 568-conjugated for 1 h.

7. The slides are washed with 1× PBS with 0.1 % Triton X-100 and mounted with VECTASHIELD mounting medium without 4′,6-diamidino-2-phenylindole (DAPI).
8. ImageJ software is used to analyze and quantitate the DNA fibers.

3.8 Chromosomal DNA Strand Break Analysis

Two different approaches are used to measure DNA strand breaks after treatment with heat shock or IR exposure or heat shock + IR exposure. Biochemical methods include (a) pulsed field gel electrophoresis (PFGE) [11] and (b) Neutral filter elution [9, 30] while cytological approaches involve either the analysis of chromosomes at metaphase or interphase using the premature chromosome condensation technique as described previously [8, 56]. The biochemical approaches are described here mainly.

3.8.1 Pulse Field Gel Electrophoresis

1. In PFGE, cells are harvested, embedded in agarose plugs, and lysed in situ.
2. Plugs are washed in Tris-EDTA buffer [10 mM Tris–HCl, 1 mM EDTA (pH 8)] and PFGE is carried out with a CHEF DRIII system in 0.8 % agarose gels.
3. The gels are run at 14 °C with linearly increasing pulse times from 50 to 5,000 s for 192 h at field strength of 1.5 V/cm.
4. Subsequently, gels are ethidium bromide stained and photographed with a charge-coupled device camera system under UV transillumination.
5. Quantitative analysis to determine the fraction of DNA entering the gel provides a measure for the relative number of double-strand breaks. Control cell DNA is normalized to zero and DNA of cells treated with 10 Gy is given a value of 1.

3.8.2 Neutral Filter Elution

Filter elution is a commonly used technique for measuring DNA strand breakage (*see* **Note 6**). The assay uses a filter to mechanically impede passage of long (unbroken) DNA strands during elution. Double-strand DNA breaks are detected with “neutral” filter elution (performed at pH 9.6). This pH is nondenaturing, so no DNA strand unwinding occurs. The eluting solution is composed of 0.05 M Tris-Cl/0.05 M glycine/0.025 M EDTA/2 % (w/v) sodium dodecyl sulfate. The pH of the eluting solution is adjusted to pH 9.6 with 10 N NaOH. DNA quantitation is conducted by prelabeling cells with radiolabeled thymidine or by using fluorometry.

1. *Label cells with [^{14}C] or [^{3}H]thymidine*—Expose 10^5 cells/ml in suspension or a monolayer culture of 10^6 cells to 0.1 μCi/ml [methyl ^{3}H]-thymidine plus 10^{-5} M unlabeled thymidine or 0.01–0.02 μCi/ml of [2-^{14}C]-thymidine for 24 h.

2. Recover cells in suspension (by centrifuging 5 min at $200 \times g$, 4 °C) or in monolayer (by rinsing twice with PBS before trypsinization, and centrifuging 5 min at $200 \times g$, 4 °C) and remove the supernatant.
3. Rinse pellets with ice-cold PBS, centrifuge again, remove the supernatant, and transfer an appropriate number of cells into the appropriate medium for treatment with test agent.
4. *Assemble and prepare filter apparatus*—Secure the filter within the funnel apparatus and attach to a suction flask that is in turn connected to a vacuum line. Add 20 ml of ice-cold PBS to the funnel. Apply a full vacuum for a few seconds to expel air from the filter. Adjust vacuum to a slow flow rate.
5. *Apply cells to filter*—Add $0.5–2 \times 10^6$ cells, diluted in 20 ml ice-cold PBS, to the funnel. Continue to apply a gentle vacuum to pull the cells onto the filter.
6. Just before the solution level reaches the filter, add 5–10 ml of additional ice-cold PBS to wash the cells. Repeat twice. During the last wash, turn off the vacuum when the fluid is ~1 cm above the filter and allow the last of the solution to flow through the filter by gravity. Cover the funnel apparatus, with foil or a black paper cylinder.
7. *Lyse the cells*—Disconnect the funnel/filter apparatus from the suction flask. Fill the upper chamber of the filter holder with cell lysis solution, pH 9.6, by using a pipettor with a plastic tip that will fit through the filter orifice. Withdraw the tip slightly to allow for air bubbles to escape from the upper chamber. Pipet 1 ml at a time, slowly. Add a total of 5 ml of cell lysis solution. Allow the lysis solution to drip by gravity into a scintillation vial.
8. *Digest protein*—Connect the outflow tubes to tubing leading to a peristaltic pump (with the pump turned off at this point). Fill the upper chamber of the filter holder with 2 ml of cell lysis solution containing 0.5 mg/ml of proteinase K, using the procedure described above for adding the cell lysis solution. Pump through the filter at 0.035 ml/min. Turn off the pump.
9. *Elute damaged DNA*—Aspirate excess proteinase K solution from the filter funnel and add 5 ml of alkaline elution solution, pH 12.3, by slowly running the solution down the wall of the funnel. Pour an additional 30–40 ml of the solution into the funnel. Turn on the pump at a speed of 0.035 ml per min and collect fractions into scintillation vials at 90- or 180-min intervals for 15 h. Turn pump off.
10. *Quantitate DNA using scintillation counting of radiolabeled DNA*—Pour off any remaining elution solution.

11. Turn pump on to maximum speed to empty solution remaining in the line and filter holder. Collect this fraction into a scintillation vial.
12. Turn off the pump and remove the filter. Place it in a scintillation vial containing 0.4 ml of 1 N HCl. Cap the vial and heat at 60 °C in oven or water bath for 1 h to depurinate the DNA.
13. Add 2.5 ml of 0.4 N NaOH, shake vigorously. Allow to stand for 1 h at room temperature. Shake again.
14. Flush the filter holder and line tubing with 10 ml of 0.4 N NaOH by pumping at high speed. Collect into another scintillation vial. Wash again with 2.5 ml 0.4 N NaOH and count to check if all radioactivities have been removed.
15. Add 10 ml of scintillation fluid (e.g., Ecolume, ICN Biomedicals) containing 0.7 % (v/v) acetic acid to each scintillation vial.
16. Count the radioactivity in each of the vials containing the filters, rinse solution, and eluted fractions.
17. Calculate results by adding the radioactivity (cpm) from the filter to the radioactivity measured in the wash of the filter holder. Add this amount to the cumulative amount of radioactivity in the fractions.
18. Plot data on a semilogarithmic plot of DNA retention [i.e., the fraction of total DNA retained on the filter (log scale)] versus elution time.

3.9 Cytogenetic Approach

This method is cell-based analysis of individual chromosome damage, and provides the most sensitive assay for determining the induction of DNA strand breaks after stress. Essentially, cells are treated with colcemid after IR exposure. Chromosome aberrations are assessed by counting chromatid breaks and gaps per cell. The details of cytogenetical approaches for assessing chromosomal DNA damage have been well described [6, 8, 9, 30, 56–62].

4 Notes

1. Chromatin structural perturbations induced by DNA double-strand breaks have been proposed to serve as a trigger for ATM activation as determined by ATM autophosphorylation and enhanced kinase activity.
2. γ-H2AX foci are usually the consequence of chromosomal DNA double strand breaks, however, this does not stand true for heat-induced γ-H2AX foci, because hyperthermia does not induce DNA double strand breaks.

3. Ionizing radiation induced chromosomal DNA breaks correlate with the number of γ-H2AX foci; however, γ-H2AX foci are seen in S-phase cells without exposure to DNA damaging agents.
4. Two areas of research that are being actively pursued are: (1) determination of which types of chromatin alterations are responsible for the ATM activation and (2) the mechanism by which such alterations activate ATM function.
5. To determine cellular ATM activation, cells must be physiologically viable as determined at least by trypan blue dye exclusion assay.
6. Both neutral filter elution and pulse field gel electrophoresis allow measurement of gross chromosomal DNA double strand breaks, whereas cytogenetic approaches allow measurement of DNA double strand breaks of individual chromosomes of each cell. Thus the cytogenetic approach to quantitate chromosomal DNA damage is more sensitive.

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Chapter 19

Imaging Local Deposition of Newly Synthesized Histones in UVC-Damaged Chromatin

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Abstract

DNA damage not only jeopardizes genome integrity but also challenges the well-organized association of DNA with histone proteins into chromatin, which is key for regulating gene expression and cell functions. The extent to which the original chromatin structure is altered after repair of DNA lesions is thus a critical issue. Dissecting histone dynamics at sites of DNA damage has provided mechanistic insights into chromatin plasticity in response to genotoxic stress. Here, we present an experimental protocol for visualizing the deposition of newly synthesized histone H3 variants at sites of UVC damage in human cells that couples SNAP-tag based labeling of new histones with local UVC irradiation of cells through micropore filters.

Key words Local UVC damage, Histone H3 variants, Chromatin assembly, SNAP technology, Fluorescence microscopy

1 Introduction

In eukaryotic cells, the DNA damage response takes place on a chromatin substrate, where DNA is associated with histone proteins [1]. These proteins, through their posttranslational modifications [2] and the existence of sequence variants [3], contribute to encoding epigenetic information, which modulates gene expression without changes to the DNA sequence. The inheritance of epigenetic marks through cell generations is key for maintaining cell function and identity. Understanding how the epigenetic information is preserved after a genotoxic assault is thus a matter of intense investigation. The current model, known as "Access/Prime-Repair-Restore" [4, 5], postulates that chromatin organization is first destabilized after DNA damage induction to allow repair machineries to have access to the damage and repair the DNA. Chromatin structure is subsequently restored, and the latter step involves histone deposition onto DNA by specific proteins called histone chaperones [6, 7].

*equal contribution

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The first evidence of chromatin reorganization upon genotoxic stress came from monitoring the accessibility to nuclease digestion of purified chromatin from UVC-irradiated human fibroblasts [8, 9]. These pioneering experiments revealed a transient disassembly of chromatin regions undergoing repair. Chromatin reassembly coupled to DNA repair was further studied in vitro on DNA templates damaged by UVC irradiation or nuclease digestion, by analyzing the supercoiling of damaged plasmids or the association of histone proteins with damaged DNA immobilized onto magnetic beads (methods described in ref. [10, 11]). More recently, the in vivo analysis of histone dynamics in response to DNA damage has been facilitated by the development of multiple methods for inflicting localized DNA damage in living cells [12, 13]. Among them, localized UVC irradiation through micropore filters [14, 15] is one of the easiest to implement. Furthermore, this method generates predominantly pyrimidine dimers, which are repaired by the well-characterized and evolutionarily conserved NER pathway (Nucleotide Excision Repair) [16]. NER is an attractive model system that already provided significant insights into chromatin alterations and histone dynamics in response to DNA damage (reviewed in ref. [11]).

One important advance in our understanding of chromatin reassembly after DNA damage has been the realization that restoration of damaged chromatin structure involves new histone incorporation [17, 18]. Given that posttranslational modifications on new soluble histones are distinct from parental histone marks [19], the replacement of parental histones by new histones is likely to dilute the original information in damaged chromatin regions, thus challenging the maintenance of epigenetic information. The recent development of in vivo methods that discriminate between parental and newly synthesized histones was instrumental for investigating such plasticity of chromatin in response to DNA damage. The first method to be developed is based on analyzing cells short term after transient transfection of Flag-HA-tagged H3.1 histone variant [17]. H3.1 belongs to the family of replicative histone variants, which are expressed at highest levels during S-phase, as opposed to replacement variants. H3.1 was chosen for this assay because its deposition into chromatin is coupled to DNA synthesis and restricted to S-phase cells in the absence of DNA damage, thus facilitating the visualization of de novo incorporation events at sites of UVC irradiation outside S-phase. However, quantitative analysis of new histone deposition is hampered in this case by the heterogeneity of tagged-H3.1 expression inherent to transient transfection. In addition, this method is poorly suited for dissecting the dynamics of replacement histone variants whose global incorporation into chromatin in a replication-independent manner obscures their local mobilization at sites of DNA damage. For example, it is the case of the H3.3 replacement variant, which is deposited into chromatin all along the cell cycle and in non-cycling cells [20].

Here, we describe a recently implemented method that circumvents these caveats and allows specific tracking of newly synthesized histone H3 variants at damage sites. This method combines local UVC irradiation through micropore filters with SNAP-tag based imaging of newly synthesized H3 variants in human cells stably expressing SNAP-tagged histones [18]. SNAP-tag based labeling of histone proteins was pioneered by studies on the centromeric H3 variant CENPA in human cells [21, 22] and proved to be a powerful technique for visualizing the de novo deposition of several H3 variants in vivo [18, 23–25].

2 Materials

2.1 Cell Culture

1. Human U2OS cells stably expressing H3.1- or H3.3-SNAP [23] (*see* **Note 1**).
2. Culture medium: Dulbecco's Modified Eagle Medium with high glucose, GlutaMAX, sodium pyruvate and phenol red (DMEM, Gibco) supplemented with 10 % fetal bovine serum (Eurobio), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), and 100 μg/ml G418 disulfate salt solution (Sigma). Store at 4 °C.
3. Dulbecco's Phosphate Buffered Saline without calcium, magnesium, phenol red (DPBS 1×, Gibco).
4. Sterile round glass coverslips 12 mm diameter, thickness No. 1 (Marienfeld).
5. 4-well cell culture plates (1.9 cm^2/well, NUNC).

2.2 SNAP Reagents

1. Quenching SNAP substrate: Prepare 4 mM stock solution by dissolving 100 nmol SNAP-Cell® Block (New England Biolabs) into 25 μl sterile dimethyl sulfoxide solution (DMSO, Sigma). Store at −20 °C.
2. Fluorescent SNAP substrate: Prepare 200 μM stock solution by dissolving 30 nmol SNAP-Cell® TMR-STAR (New England Biolabs) into 150 μl sterile dimethyl sulfoxide solution (DMSO, Sigma). Store 30 μl aliquots at −20 °C (*see* **Note 2**).

2.3 Local UVC Irradiation

1. UVC lamp 254 nm, 6 W on lamp stand (Vilber Lourmat).
2. VLX-3W dosimeter equipped with a 254 nm sensor (Vilber Lourmat).
3. Isopore membrane filters 5 μm pore size, 13 mm diameter (Millipore).
4. 6-cm diameter cell culture dish (TPP).
5. Face shield or protective glasses (Vilber Lourmat).
6. Acrylic benchtop radiation shield (Nalgene).

2.4 Immunofluorescence

1. 1× Phosphate Buffer Saline (PBS, Gibco).
2. CytoSKeleton (CSK) buffer: 10 mM PIPES pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$. Store at −20 °C.
3. CSK-Tx: 0.5 % Triton X-100 (Euromedex) in CSK buffer, freshly made.
4. 16 % Paraformaldehyde solution EM grade (PAF, Electron Microscopy Sciences). Freshly prepare 2 % PAF by diluting in 1× PBS.
5. PBS-T: 0.1 % Tween 20 (Sigma) diluted in 1× PBS. Keep at room temperature.
6. Blocking buffer: 5 % Bovine Serum Albumin (BSA, Sigma) dissolved in PBS-T, freshly made.
7. Sodium azide solution: Sodium azide (Sigma-Aldrich) 2 % w/v in H_2O. Store at 4 °C.
8. Primary antibody mouse monoclonal anti-human XPA (clone 12F5, BD Biosciences), 0.5 mg/ml stock solution.
9. Secondary antibody Alexa Fluor 488 goat anti-mouse IgG highly cross-adsorbed (Molecular Probes), 2 mg/ml stock solution.
10. VECTASHIELD mounting medium with DAPI (Vector Laboratories).
11. Superfrost microscope slides (Menzel-Gläser).
12. Leica epifluorescence microscope with 63× Apochromat N.A. 1.4 oil-immersion objective.

3 Methods

Here, we describe a protocol for monitoring the dynamics of newly synthesized histone H3 variants at sites of DNA damage in human cells. This method combines SNAP-tag-based imaging [22] with local UVC irradiation of cells through micropore filters [10, 14, 15]. The SNAP-tag is a modified version of the human suicide-enzyme AGT that catalyzes its own irreversible binding to benzylguanine derivatives [26, 27]. Such SNAP substrates exist in a cell-permeable form, suitable for labeling intracellular proteins including histones. Here, newly synthesized SNAP-tagged histones are specifically labeled with a red-fluorescent SNAP substrate (step 1) by first quenching all preexisting SNAP-tagged histones with a nonfluorescent SNAP substrate [18, 21, 22, 24]. Local UVC irradiation (step 2) induces damage in discrete regions that are randomly distributed in the cells. Immunodetection of the repair factor XPA (steps 3 and 4) is thus used to visualize the sites of DNA damage.

The whole protocol can be completed in 1 day (*see* Fig. 1, **Note 3**).

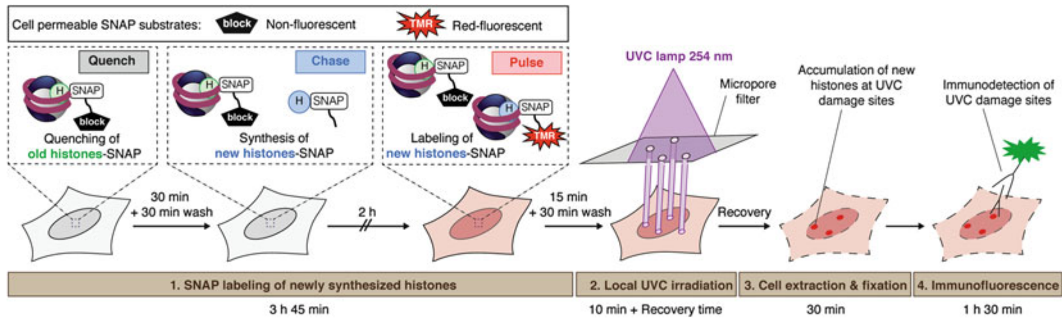

Fig. 1 Scheme of the assay for monitoring new histone dynamics upon local UVC irradiation in human cell lines stably expressing SNAP-tagged histones. By quenching preexisting SNAP-tagged histones with a nonfluorescent substrate (block), only histones neo-synthesized during the chase period are labeled with the red fluorescent substrate TMR-star (TMR) during the pulse step. Local UVC damage is induced by irradiating cells with a UVC lamp through micropore filters and UVC damaged regions are marked by immunofluorescence

3.1 SNAP Labeling of Newly Synthesized Histones (See Note 4*)*

1. Grow cells on glass coverslips in 4-well plates with 0.5 ml culture medium per well (*see* **Note 5**). Incubate in a humidified 37 °C incubator with 5 % CO_2 until cells reach 70 % confluency.
2. Dilute the quenching SNAP substrate in culture medium to a final concentration of 10 μM. Quench preexisting SNAP-tagged histones by incubating cells with 200 μl of this solution per well for 30 min at 37 °C, 5 % CO_2.
3. Wash out the excess of quenching substrate by rinsing cells twice with 1 ml PBS and incubate cells in 1 ml culture medium per well for 30 min at 37 °C, 5 % CO_2.
4. Chase time: Rinse cells twice with 1 ml PBS and incubate cells in 1 ml culture medium per well for 2 h at 37 °C, 5 % CO_2 (*see* **Note 6**).
5. Dilute the fluorescent SNAP substrate in culture medium to a final concentration of 2 μM (*see* **Notes 7** and **8**). Pulse-label newly synthesized SNAP-tagged histones by incubating cells with 200 μl of this solution per well for 15 min at 37 °C, 5 % CO_2.
6. Wash out the excess of fluorescent substrate by rinsing cells twice with 1 ml PBS and incubate cells in 1 ml culture medium per well for 30 min at 37 °C, 5 % CO_2 (*see* **Note 9**).
7. Rinse cells twice with 1 ml PBS and re-incubate in 1 ml culture medium per well.

3.2 Local UVC Irradiation (See Note 10*)*

1. Set up the UVC lamp behind an acrylic benchtop radiation shield (*see* **Note 11**).
2. Warm up the UVC lamp for 1–2 min until the power stabilizes. Determine two positions on the lamp stand receiving identical fluence rates as monitored with the dosimeter, so that the cells are irradiated at one position and the UV fluence rate can be monitored simultaneously at the other position (*see* **Note 12**).
3. Drain and transfer one coverslip (cells up) to the center of a 6-cm diameter culture dish. Cover the coverslip with a micropore filter (*see* **Note 13**). Close the lid of the culture dish.
4. To irradiate the cells, place the closed dish at the determined position on the lamp stand and remove the lid of the culture dish.
5. When a UV dose of 150 J/m^2 is reached (0.0150 J/cm^2 read on the dosimeter), stop irradiation by placing the lid back on the culture dish (*see* **Note 14**).
6. Remove the micropore filter from the coverslip by pouring PBS into the culture dish (*see* **Note 15**). Transfer the irradiated coverslip (cells up) back into the 4-well plate containing culture medium.
7. Incubate cells for at least 30 min at 37 °C, 5 % CO_2 (*see* **Note 16**).

3.3 Cell Extraction and Fixation

All steps are performed at room temperature and volumes are given per well of a 4-well plate.

1. 45 min after irradiation (*see* **Note 17**), rinse cells on coverslips once with 500 μl PBS and once with 500 μl CSK.
2. To remove soluble cell components, incubate for 5 min with 500 μl CSK-Tx (*see* **Note 18**).
3. Stop extraction by rinsing cells once with 500 μl CSK and once with 500 μl PBS (*see* **Note 19**, also **Note 15**).
4. Fix cells with 500 μl PAF solution for 20 min.
5. Rinse three times with 500 μl PBS (*see* **Note 20**).

3.4 Immunofluorescence (See Note 21*)*

All steps are performed at room temperature and volumes are given per well of a 4-well plate unless stated otherwise.

1. Block aspecific sites by incubating cells on coverslips with 500 μl BSA blocking buffer for 10 min.
2. Transfer coverslips (cells up) to Parafilm and cover each coverslip with 50 μl anti-XPA primary antibody (*see* **Note 22**) diluted 1:500 in blocking buffer containing 2 % sodium azide (*see* also **Note 23**). Incubate for 45 min.
3. Transfer coverslips (cells up) back into the 4-well plate and rinse three times with 500 μl PBS-T.

4. Retrieve the primary antibody solution and keep it at 4 °C for reuse (*see* **Note 23**).
5. Incubate cells for 30 min at room temperature with 250 μl of Alexa Fluor 488 anti-mouse secondary antibody diluted 1:1,000 in blocking buffer.
6. Rinse cells twice with 500 μl PBS-T and once with 500 μl PBS.
7. Mount coverslips (cells down) on Superfrost slides with 10 μl mounting medium with DAPI per coverslip. Seal coverslips with clear nail polish.
8. Store at 4 °C for at least 15 min before microscope observation (*see* **Note 24**).
9. Images are acquired using a Leica epifluorescence microscope with 63× Apochromat N.A. 1.4 oil-immersion objective. The Y3 filter is used for visualizing TMR star fluorescence (*see* Fig. 2).

4 Notes

1. This protocol is optimized for U2OS cells stably expressing SNAP-tagged H3 variants, but it can be adapted to other cell types, stably or transiently expressing SNAP-tagged histones. Stable monoclonal cell lines are preferred to ensure minimal cell-to-cell variability in transgene expression.
2. The fluorescent SNAP substrate must be protected from light and is sensitive to repeated freeze–thaw cycles. It is thus advisable to prepare aliquots of the stock solution.
3. For a deeper understanding of the mechanisms underlying histone dynamics at UV sites, this protocol can be combined with siRNA-mediated depletion of histone chaperones and NER factors or with inhibition of enzymatic activities involved in the UV damage response [18].
4. The SNAP labeling of newly synthesized histones is usually done under sterile conditions, but it is not absolutely required as cells are fixed a few hours later.
5. We generally work in 4-well plates with 12-mm diameter coverslips in order to minimize the amount of reagents used, but the method can be scaled-up to process larger coverslips in 6-well plates.
6. The chase time can be adapted depending on the expression level of SNAP-tagged histones and on the turnover of histone proteins. You can also verify quenching efficiency by performing the pulse immediately after the quench step, omitting both wash and chase procedures.

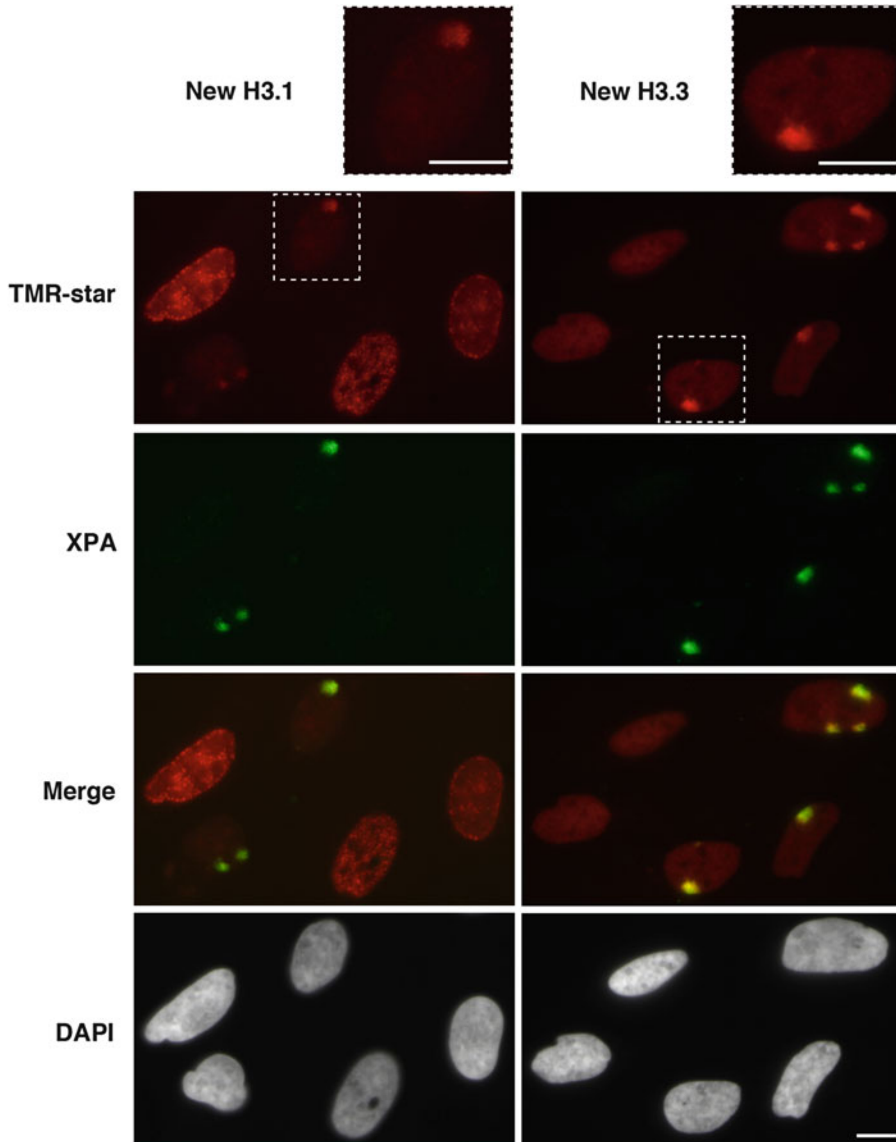

Fig. 2 New H3.1 and H3.3 deposition at UVC damage sites. Newly synthesized SNAP-tagged H3.1 and H3.3 histone variants labeled with TMR-star accumulate at UVC damage sites marked by the repair factor XPA. Cells are fixed 45 min after local UVC irradiation. The focal patterns observed with new H3.1 in undamaged nuclei correspond to replication-coupled deposition. Cell nuclei are co-stained with DAPI. Scale bars, 10 μm

7. Centrifuge the fluorescent SNAP substrate before pipetting to avoid aggregates.
8. The red-fluorescent substrate SNAP-Cell® TMR-star can be replaced by the green-fluorescent SNAP-Cell® Oregon-Green® at a final concentration of 5 μM. In this case, the secondary antibody used for immunofluorescence should be labeled with Alexa Fluor 594 or 568.

9. The washing procedures are critical to minimize background signal. The specificity of SNAP labeling can be controlled on the parent U2OS cell line, which does not express SNAP-tagged proteins and thus should not be stained.
10. Local UVC irradiation can be performed either after completing the SNAP labeling procedure as described in this protocol or immediately before the pulse step with similar results. We did not observe bleaching of TMR star fluorescence by UVC light.
11. Wear protective gloves, glasses and lab coat when performing UVC irradiation.
12. In our irradiation settings, the average exposure time to UVC is 30 s with a fluence rate stabilizing around 0.5 mW/cm^2 for a lamp-to-specimen distance of 13 cm. However, the fluence rate varies with time and between UVC lamps, so it is necessary to monitor systematically the UV fluence with a dosimeter.
13. Around 98 % of UVC light is stopped by the micropore filter.
14. The UVC dose can be varied from 50 to 500 J/m^2 to assess the dose-dependency of the observed response.
15. Cells normally do not detach from the coverslip but if this happens it can be prevented by coating the coverslips with poly-L-lysine or collagen-fibronectin before seeding the cells.
16. The local accumulation of fluorescently labeled SNAP-tagged H3 variants at damage sites can also be visualized at this stage in live cells.
17. You can vary the recovery time between UVC irradiation and cell fixation to analyze the kinetics of new histone deposition at UVC damage sites.
18. Triton extraction before fixation removes soluble proteins and thus allows specific visualization of chromatin-bound histones. If one wants to assess the production of SNAP-tagged histones rather than their incorporation into chromatin, the cells can be fixed directly after the SNAP labeling procedure without Triton pre-extraction. This control is particularly important if you observe a defect in chromatin-bound histones at damage sites, to determine if it results from defective histone protein synthesis or deposition.
19. Wash gently as cells can detach from coverslips during the extraction procedure. The extraction conditions can be adjusted (duration, temperature) depending on cell type.
20. Fixed cells can be kept at 4 °C in PBS for up to 1 week before performing immunofluorescence.

21. As the fluorescent SNAP substrate is light sensitive, it is advisable to protect cells from light all along the immunofluorescence protocol.
22. Instead of detecting XPA, we have also successfully visualized UVC-damaged regions by immunofluorescence against the NER factor XPB using anti-XPB primary antibody (rabbit polyclonal anti-TFIIH p89, Santa Cruz Biotechnology, 1:400 dilution) and Alexa Fluor 488 anti-rabbit secondary antibody (Molecular Probes). Note that at late time points after UVC irradiation (over 2 h), NER factors become barely detectable at UV sites. In this case, UVC damage should be labeled directly using for example an antibody against the most abundant and slowly repaired UVC lesions Cyclobutane Pyrimidine Dimers (mouse monoclonal anti-CPD, clone KTM53, Kamiya Biomedical Company, 1:1,000 dilution). Importantly, CPD immunodetection requires 5 min denaturation with 0.5 M NaOH and neutralization by three washes in PBS before blocking.
23. Sodium azide allows proper conservation of primary antibody solutions by preventing microbial contamination. The antibody dilutions can thus be kept for several months at 4 °C and can be reused multiple times.
24. The slides can be kept at 4 °C in the dark for up to 1 week before image acquisition or at −20 °C for longer storage.

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Chapter 20

In Vitro Replication Assay with Mammalian Cell Extracts

Wasia Rizwani and Srikumar P. Chellappan

Abstract

Regulatory mechanisms are crucial to control DNA replication during cell cycle in eukaryotic cells. Cell-free in vitro replication assay (IVRA) is one of the widely used assays to understand the complex mammalian replication system. IVRA can provide a snapshot of the regulatory mechanisms controlling replication in higher eukaryotes by using a single plasmid, pEPI-1. This chapter outlines the general strategies and protocols used to perform IVRA to study the differential recruitment of replication factors either independently or in combination, based on the experience in studying the role of prohibitin in replication as well as other published protocols. This method can be employed to identify not only proteins that assist replication but also proteins that inhibit replication of mammalian genome.

Key words Replication assay, pEPI-1 plasmid, Nuclear and cytosolic extracts

1 Introduction

Genome replication in prokaryotes and some eukaryotic viruses such as simian virus 40 (SV40) initiates with the binding of specific initiator proteins to genomic sites, termed "origins" and results in the localized unwinding of the DNA duplex to form replication forks [1]. SV40 expresses a single initiator protein, T antigen, which binds with high specificity to the viral origin, a specific 60 bp long DNA segment that functions as a helicase separating the complementary DNA strands [2]. Contrary to this, the replication of eukaryotic cellular genomes is strictly coordinated within the cell cycles and efficiently controlled to complete the replication only once per cell cycle [3]. This is accomplished by several levels of regulation starting with the stepwise assembly of pre-replication complexes (pre-RC) in the early G1 phase of the cell cycle, consisting of the DNA-bound multi-subunit origin recognition complexes (ORC with its components Orc1–Orc6) that serves as a platform for the association of additional proteins such as Cdc6, Cdt1, and the minichromosome maintenance proteins (Mcm2–Mcm7) [4, 5]. This is followed by the activation of specific protein kinases to

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begin the transition into S phase, which is marked by the conversion of pre-RC to initiation complexes. Partial degradation of the pre-RC, including the loss of Mcm proteins, prevents re-replication during the S phase [6–10]. Dissociation of the Orc1p subunit of ORC could also protect mammalian cells from undergoing re-replication [11].

This process so far was best understood in yeast, *Saccharomyces cerevisiae*. Presence of autonomously replicating sequences (ARSs) in yeast that direct the extrachromosomal replication of ARS-bearing plasmids made it possible to understand how specific eukaryotic replication is and the significance of defined origin elements [12, 13]. Also in yeast, the replication origins are genetically determined. In higher eukaryotes, it is believed that replication start sites are totally determined not only by the underlying DNA sequences but also by epigenetic factors such as the presence of specifically bound transcription factors or the conformation of the chromatin [14]. Developing an in vitro assay system comparable to the ARS system in yeast to investigate the importance of origin specificity and replication proteins in mammalian cells was indispensable. For this purpose, there was a need for completely soluble proteins from mammalian cells and a biochemically amenable plasmid that not only replicated episomally in cultured mammalian cells but also contained an authentic origin sequence to prevent rapid incorporation into the host genome or complete loss [15, 16]. Two regions appeared to be important for extrachromosomal replication, an active CMV promoter with the adjacent downstream sequences and atleast partial S/MAR coding region [17, 18]. Hence, plasmid pEPI-1 possessing these two features was constructed by Piechaczek et al. [19] to study in vitro replication (Fig. 1). The plasmid pEPI-1 of size 6.7 kb replicates autonomously

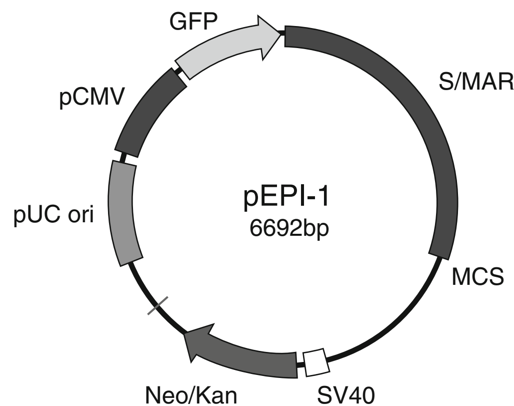

Fig. 1 A map of pEPI-1 plasmid. S/MAR represents the human scaffold/matrix attached region cloned into the multiple cloning site (MCS) of the vector pGFP-C1. The plasmid has SV40 origin, which is a T antigen target site to initiate in vitro replication. Adapted from Piechaczek et al. [19]

in a once-per-cell-cycle manner and is stably transmitted over many generations (~100) without selection, as observed in CHO cells [19] and HeLa cells [18]. pEPI-1 has a unique combination of genetic elements including a strong eukaryotic promoter (CMV) and a scaffold/matrix attachment region (S/MAR). It probably associates with mitotic chromosomes via an interaction of its S/MAR element with the scaffold attachment factor, SAF-A in cellular chromosomes [20, 21], and is therefore correctly transmitted from parental to progeny cells. To mimic in vivo replication system, a mixture of proteins with chain-elongating functions and replication initiation properties was necessary [5]. For a long time, Xenopus egg extracts were used to study replication and was successful in investigations into the events leading to pre-RC formation [22–25] and to characterize important replication factors such as Cdt1 and Mcm8 [26, 27]. However, the *Xenopus* egg extract contains high amounts of initiator proteins such as ORC and Mcm proteins, stored for subsequent rounds of cell divisions in early embryogenesis that differs from the situation in proliferating adult mammalian cells with their limited amounts of ORC. For this reason, the Xenopus extracts have been replaced with cytosolic mammalian cell extracts rich in replicative chain elongation factors and either T antigen or high salt nuclear extract for replication initiation [28, 29], thereby providing an in vitro replication system that is sufficiently robust to investigate questions related to replication initiation in mammalian cells. This chapter will describe in detail the protocols to be followed for conducting an in vitro replication assay using pEPI-1 plasmid. A novel improvised vector pEPito, derived from the pEPI-1 plasmid replicon, has been developed recently to study biotechnological assays in vitro (*see* **Note 1**) [30]. The assay involves the preparation of cytosolic and nuclear extracts from mammalian cells, purification of the pEPI-1 plasmid, and the replication assay itself. For the replication assay, as a positive control, T antigen may be used in place of nuclear extract to initiate replication. This assay was employed by our lab to study the effect of prohibitin, a tumor suppressor protein, on mammalian DNA replication [31]. Figure 2a demonstrates the optimal concentrations of cytosolic and nuclear MCF7 cell extracts to achieve maximum detectable pEPI-1 replication. T antigen was added in all the assays and prohibitin shows a significant repression of pEPI-1 replication in vitro (Fig. 2b) [31]. Dpn I digestion is an optional step to confirm the amount of replication quantitatively. Replication products are resistant to Dpn I digestion only when hemimethylated or unmethylated, thereby indicating that DNA has undergone atleast one round of replication in vitro [29]. Hence, for the Dpn I digestion assay to work, the plasmid has to be purified either from the pEPI-1 transfected mammalian cells or from pEPI-1 transfected methylation-deprived bacterial strains. This is because bacterial plasmids undergo methylation on both

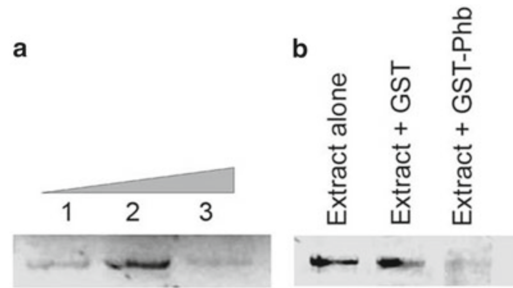

Fig. 2 Prohibitin suppresses replication of pEPI-1 plasmid under in vitro conditions. Cytosolic (S100) and nuclear fractions (S300) were prepared from asynchronously growing MCF7 cells [34]. In vitro replication assay was performed using pEPI-1 plasmid according to the protocol described by Baltin et al. [29]. (**a**) Different concentrations of protein fractions were used to optimize maximal efficiency of replication of the plasmid: (1) 45 μg S100 and 16 μg S300 extracts, (2) 45 μg S100 and 32 μg S300 extracts, (3) 90 μg S100 and 64 μg S300 extracts. Combination of 45 μg S100 and 32 μg S300 extracts gave a better signal; therefore it was used for all future assays. (**b**) In vitro replication assay was done in the presence of GST or GST-prohibitin. Replication of pEPI-1 plasmid was significantly reduced in the presence of GST-prohibitin when compared to S300/S100 extract alone or extract with GST. Adapted from Rizwani et al. [31]

the strands and are susceptible to Dpn I digestion. This assay is extensively employed to examine the formation of functional pre-replication complexes under biochemical conditions.

2 Materials

1. 30 % Acrylamide Solution (0.8 % bis): 60 g acrylamide and 1.6 g bis. Heat to dissolve in approximately 100 ml of water. QS to 200 ml with water and filter with Whatman #1 filter paper into a foil wrapped bottle.
2. 40 % Acrylamide Solution (19:1 acrylamide–bis): 80 g acrylamide and 4.21 g bis. Heat to dissolve in approximately 100 ml of water. QS to 200 ml with water and filter with Whatman #1 filter paper into a foil wrapped bottle.
3. 50 % Acrylamide Solution (19:1 acrylamide–bis): 237.5 g (97 %) acrylamide and 12.5 g bis. Dissolve in 250 ml hot water. Filter to remove debris and wrap in foil.
4. Buffer A: 20 mM HEPES, pH 7.4, 5 mM KCl, 1.5 mM $MgCl_2$, and 0.1 mM dithiothreitol. Store at room temperature.
5. Buffer A containing 250 mM sucrose. Store at room temperature.
6. Buffer A containing 450 mM potassium acetate. Store at room temperature.

7. Buffer NE1: 10 mM HEPES, pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, and 1× complete, EDTA-free protease inhibitor.
8. Buffer NE2: 20 mM HEPES, pH 8.0, 1.5 mM $MgCl_2$, 25 % glycerol, 420 mM NaCl, 1 mM DTT, 0.5 mM PMSF, and 1× complete, EDTA-free protease inhibitor.
9. Creatine Kinase BB fraction, human (Sigma, cat # C9983-100UG). Dissolve in 1 ml water. Store at −20 °C in aliquots.
10. Creatine phosphate disodium salt tetrahydrate. (Sigma, cat # 27920). Dissolve 163 mg in 1 ml water to get a stock of 500 mM concentration. Store in aliquots at −20 °C.
11. Deoxynucleotide phosphates (100 mM stock solutions from Promega)—dATP (cat # U120-B), dCTP (cat # U121-B), dGTP (cat # U122-B), and dTTP (cat # U123-B).
12. Dpn I restriction enzyme. (Promega, cat # R0176S).
13. 50 % Glycerol—25 ml 100 % glycerol and 25 ml water. Autoclave.
14. NE buffer 4 (Promega, cat # B7004S, 10× concentrate).
15. Nucleotide phosphates (100 mM stock solutions from Promega)—ATP (cat # E601B), UTP (cat # E602B), GTP (cat # E603B), and CTP (cat # E604B).
16. Phosphate-buffered saline, pH 6.5 (20×): 160 g NaCl, 4 g KCl, 28.8 g Na_2HPO_4, 4.8 g KH_2PO_4 in 1 l of water. It can be stored at room temperature. For 1× PBS, 50 ml of 20× PBS is made up to 1 l, pH adjusts to 7.2 upon dilution with water. The buffer has to be ice-cold when used.
17. Protease inhibitor (Complete, EDTA-free from Roche Applied Sciences, cat # 04693132001). Dissolve one tablet in 2 ml of water and aliquot into 200 μl and store at −20 °C.
18. Proteinase K (Promega, cat # V302A): Dissolve 10 mg powder in 1 ml of TE buffer and store at −20 °C in aliquots. Thaw on ice before use.
19. RNase A (Sigma, cat # R-5503): Dissolve 10 mg in 1 ml of 1× TE, pH 7.5. Boil for 30 min.
20. 3 M sodium acetate, pH 5.2.
21. Stop mix: 60 mM EDTA, 2 % SDS.
22. 50× TAE (1 M Tris–HCl, 57.1 ml acetic acid, 50 mM EDTA, pH 8.3): 242.2 g Tris, 57.1 ml acetic acid, 18.6 g EDTA. Adjust pH to 8.3. QS to 1 l. Autoclave (optional, refer to **Note 2**).
23. 10× TBE (1 M Tris–HCl, 1 M boric acid, 20 mM EDTA, pH 8.3): 242.2 g Tris, 123.66 g boric acid, 14.89 g EDTA. Adjust pH to 8.3. QS to 2 l. Autoclave (refer to **Note 2**). (*Biotechniques* 10:182, 1991 suggests that filtering up to a 20× TBE solution through 0.2–0.45 μm cellulose acetate or

cellulose nitrate filters prevents formation of precipitants during long-term storage. The solution may be re-autoclaved to dissolve precipitates that form).

24. 1× TE buffer, pH 7.5: 10 mM Tris–HCl and 1 mM EDTA.
25. S.O.C. media (Invitrogen, cat# 15544-034). Store at room temperature.
26. Kanamycin—30 mg/ml in water. Store at −20 °C.
27. [α-^{32}P] dATP-10 μCi/μl.
28. Water—molecular biology grade from Sigma (refer to **Note 3**).
29. QIAprep Spin Miniprep Kit (Qiagen, cat# 2714).
30. Recipe for electrophoresis of DNA in polyacrylamide gels.

Gel Sizes

Small: 75 × 100 mm

Medium: 100 × 150 mm

Large: 160 × 180 mm

5 % Analytical Gels

Reagent	1 mm Small	1 mm Medium	1 mm Large
10× TBE buffer (ml)	1.0	1.75	2.5
40 % bis–acrylamide (ml)	1.25	2.2	3.1
Water (ml)	5.75	10.05	14.4
50 % glycerol (ml)	2.0	3.5	5.0
APS (mg)	11.0	20.0	28.5
TEMED (μl)	4.27	7.5	10.7
Total volume (ml)	10	17.5	25

With other concentrations of a bis–acrylamide stock substitute:

Reagent	1 mm Small	1 mm Medium	1 mm Large
25 % bis–acrylamide (ml)	2.0	3.50	5.0
Water (ml)	5.0	9.25	12.5
30 % bis–acrylamide (ml)	1.67	2.90	4.15
Water (ml)	5.33	9.25	13.35
50 % bis–acrylamide (ml)	1.0	1.75	2.5
Water (ml)	6.0	10.50	15.0

3 Methods

3.1 Cell Culture and Preparation of Cytosolic and Nuclear Extracts

The extracts can be isolated by two different methods as described below:

3.1.1 Protocol 1

1. 293T cells (contain endogenously expressing T antigen) or breast cancer MCF7 cells are cultured in DMEM containing 1 % antibiotic-antimycotic solution and 10 % fetal bovine serum until 80–90 % confluency in a 100 mm culture dish. One plate should be sufficient to make cell extracts for few assays (refer to **Note 4**).
2. Wash the plates with cells twice with 10 ml of ice-cold 1× PBS, collect them in eppendorf with 1 ml of buffer A containing sucrose and wash twice with buffer A (*see* **Note 5**).
3. Keep the cells on ice for 10 min in 100–200 μl of buffer A (depending on the cell pellet size, preferable to keep the buffer volume small to obtain concentrated protein) and disrupt in a type S Dounce homogenizer.
4. Pellet the nuclei by centrifugation at 16,000 × *g* for 10 min, which are processed further as described later in this section.
5. The supernatant is then cleared by centrifugation at 100,000 × *g* for 1 h to prepare the S100 extract. Approximately 7–10 μg protein/μl is obtained. The protein has to be stored at −70 °C in aliquots.
6. Wash the nuclear pellet once in buffer A and incubate in approximately 100 μl of buffer A containing potassium acetate for 90 min on ice.
7. After preclearing at 12,000 × *g* for 5 min, the supernatant is centrifuged at 300,000 × *g* for 1 h and collected as S300 extracts (2–3 μg protein/μl). Store at −70 °C in aliquots until further use.

3.1.2 Protocol 2

1. Cells should be 80 % confluent for extraction. 10 × 10 cm dishes of cells will yield around 50 μl of nuclear extract (*see* **Note 6**).
2. Wash the plates with ice-cold PBS twice, scrape and collect cells in 1 ml PBS per plate. Place in eppendorf and spin at 800 × *g* for 2 min at 4 °C. Resuspend all pellets into 1 ml of PBS and spin again under same conditions (*see* **Note 5**).
3. Aspirate PBS completely and estimate packed cell volume (PCV). Resuspend in 1PCV of NE1 buffer. Leave on ice for 15 min.

4. Take up the suspension into a 1 ml syringe that has been washed with buffer NE1. In one stroke, squirt the cell suspension through a 23-gauge needle into an eppendorf. Repeat this step four more times. (Place syringes in freezer before use).
5. Microfuge at high speed (20,000 × *g*), 4 °C for 20 s to collect the supernatant. This is the cytosolic fraction.
6. Resuspend the nuclear pellet in 2/3rd PCV of NE2 buffer. Leave on ice with regular stirring for 30 min.
7. Pellet the nuclear debris at high speed for 5 min in cold microfuge.
8. Collect the supernatant, which is the nuclear fraction.

3.2 Preparation of pEPI-1 Plasmid from Transfected Mammalian Cells

3.2.1 By Hirt Extraction Protocol [32]

1. Culture pEPI-1 transfected mammalian cells in 100 mm dishes (~10^6–10^7 cells/plate). Wash the plate with ice-cold PBS twice, scrape the cells in 1 ml PBS and collect the pellet in an eppendorf tube by centrifuging for 2 min at 800 × *g* at 4 °C.
2. Resuspend the pellet in 0.5 ml of 1× TE buffer and then add another 0.5 ml of TE containing 2 % SDS. Gently invert the tube several times to mix. Pipetting is to be avoided to prevent shearing of the DNA.
3. The volume will now be approximately 1.1 ml. Hold at room temperature for 10 min.
4. Add 5 M NaCl such that the final NaCl concentration will come to 1 M (i.e., around 220 μl of 5 M NaCl). Slowly invert the tube several times to mix and keep at 4 °C for 8 h to overnight.
5. Microfuge at 17,000 × *g* for 1 h at 4 °C and transfer the supernatant to a separate 2 ml eppendorf tube carefully avoiding any remnants of the pellet to appear in the supernatant. Squirt 1 ml of 10 mM EDTA, pH 8.0 containing 0.6 % SDS.
6. Carefully transfer it to a new eppendorf which now contains the plasmid along with RNA and subject to proteinase K and RNase A treatment. The pellet contains genomic DNA and can be processed similarly in case it needs to be assessed.
7. For this, add 10 μl of 10 mg/ml proteinase K (final concentration will be 100 μg/ml) and 1 μl of 10 mg/ml RNase A. Leave at 37 °C for 1.5 h to digest the pellet.
8. Add equal volume of phenol–chloroform and extract the plasmid. Repeat this step once followed by extraction with chloroform or isopropanol alone.
9. Transfer the aqueous phase to a fresh eppendorf tube(s) and add 2 volumes of 100 % ethanol and leave at −20 °C for 1 h to overnight or at −80 °C for 30 min.

10. Spin at high speed for 30 min–1 h at 4 °C in a microfuge. Decant the supernatant and air-dry the pellet (mark the region where pellet is visible before drying because it becomes invisible after drying).
11. Redissolve the pellet in 50 μl sterile water (*see* **Note 3**).
12. Plasmid can be checked by running on 0.8 % agarose gel or analyzed by Dpn I digestion (*see* Subheading "Dpn I Digestion").

3.2.2 By Using QIAprep Spin Miniprep Kit

This is a modified protocol [33] basically designed for the purification of high-copy plasmid DNA from bacterial cultures (http://www.qiagen.com/literature/plklit.asphashplasmidmini). It is now being used to purify low molecular weight DNA such as SV40 containing plasmids from transfected mammalian cells (*see* **Notes 7–9**).

1. Culture pEPI-1 transfected mammalian cells in 60 mm dish. Rinse the plate once with ice-cold PBS.
2. Add 250 μl each of buffers P1 and P2 directly to the plate and incubate for 5 min at room temperature.
3. Gently transfer the cell lysate to a microfuge tube using a rubber policeman and digest with proteinase K at a final concentration of 800 μg/ml at 55 °C for 1 h.
4. Add 350 μl of buffer N3 to precipitate cellular DNA and cell debris by mixing gently, incubate on ice for 5 min and spin at 16,000 × *g* for 10 min.
5. Load the supernatant onto the spin column and centrifuge for a minute at 16,000 × *g*. Wash the spin column once each with buffers PB and PE. Give an additional 1 min spin to remove residual wash buffer and dry the column for 5 min to evaporate residual ethanol. Presence of ethanol might inhibit subsequent enzymatic reactions.
6. Elute the plasmid with 50 μl of water (*see* **Note 3**) by adding it to the center of the column, incubate at 37 °C for 5 min and centrifuge at 16,000 × *g* for 1 min. Repeat this step, if desirable.

3.2.3 By Transformation into DAM or DCM Minus Bacterial Strains Followed by Purification Using QIAprep Spin Miniprep Kit

1. Thaw 50 μl DAM^- or DCM^- competent cells on ice in an eppendorf, add 1 μl plasmid and mix gently with a pipet.
2. Incubate on ice for 30 min.
3. Give heat shock by holding the tube in 37 °C water bath for 20 s.
4. Keep on ice for 2 min, and then add 950 μl S.O.C. media inside a sterile biosafety hood.
5. Allow the cells to grow for an hour at 37 °C with shaking conditions of 225 rpm.

6. Spread 50 μl culture on LB plate containing 30 μg/ml Kanamycin and incubate at 37 °C overnight.
7. The plate with bacterial colonies can be stored at 4 °C. Glycerol stocks can be made for long-term storage.
8. Inoculate a single colony into 3 ml LB broth containing 30 μg/ml Kanamycin and incubate at 37 °C overnight rotating at 250 rpm in a bacterial shaker. The culture is pelleted at 800 × *g* for 5 min and subjected to plasmid purification using QIAprep spin miniprep kit.

3.3 In Vitro Replication Assay

3.3.1 By Using pEPI-1 Plasmid with Mammalian Nuclear and Cytosolic Extracts

1. In a total volume of 35 μl with S300 nuclear extract (16–32 μg, preferably more nuclear extract to increase the probability of pre-RC formation), add pEPI-1 DNA (160–320 ng) at 2 mM ATP and 80 mM potassium acetate in buffer A plus 1 μl of Complete, EDTA-free protease inhibitor (*see* **Note 3**). Pre-incubate this mix at 37 °C for 60 min.
2. Initiate the replication by the addition of the S100 extract (45 μg), 40 mM creatine phosphate, 0.6 μg/μl of creatine kinase, 30 mM potassium acetate, 80 μM CTP, GTP, and UTP, 100 μM dGTP, dCTP, and dTTP, 30 μM dATP, 0.5 μg ± T antigen, and 10 μCi of [α-^{32}P] dATP in a total volume of 50 μl (*see* **Note 4**).
3. Incubate at 37 °C for 60 min and then stop the reaction by 30 μl of stop mix.
4. Extract the DNA by proteinase K digestion and phenol–chloroform treatment, followed by ethanol precipitation as described below (Subheading "DNA Extraction Following Replication Assay").

DNA Extraction Following Replication Assay

1. After the replication assay, adjust the reaction volume to 200 μl with water (molecular biology grade).
2. To this, add 10 % of 3 M sodium acetate, pH 5.2 and 1 ml ethanol and vortex for a few seconds to mix. Allow the DNA to precipitate by incubating at −80 °C for 15 min, centrifuge at high speed (20,000 × *g*) for 15 min at 4 °C.
3. Decant the solvent and air-dry the pellet. An optional 70 % ethanol wash can also be done before air-drying the DNA pellet.
4. Redissolve the pellet in 200 μl of water and perform proteinase K digestion (50 μg) at 37 °C for 30 min. Phenol–chloroform-extract the DNA by adding 200 μl of buffered phenol, mix by vortexing for a minute and centrifuge at high speed for 10 min at room temperature. Collect the aqueous phase and repeat **steps 2** and **3**.
5. Resuspend the pellet in 20 μl of sterile water.

Dpn I Digestion

1. Reaction mix (total volume 20 μl) contains plasmid (10 μl), NEB 4 (2 μl), Dpn I (2 μl), 10× BSA (2 μl) and water (4 μl). Incubate the mix for 4 h to overnight at 37 °C and follow with phenol–chloroform extraction and ethanol precipitation.
2. Redissolve the DNA pellet in 20 μl of water. Run it either on 0.8 % agarose at 80–100 V or 5 % polyacrylamide gel at 200 V. Ethidium bromide staining will help in visualizing the bands. If testing for replication, dry the gel for 1 h and expose to autoradiography anywhere from 2 days to a week.
3. If running an agarose gel, before drying, it is preferable to wash the gel in 7 % TCA in water for 30 min with gentle rocking (*see* **Note 2**).

3.3.2 By Using SV40 DNA Replication Assay Kit

Alternatively, this kit from CHIMERx, WI, USA (cat # 8050) can be utilized to study mammalian chromosomal DNA replication in vitro. The kit contains HeLa cell extract, plasmids pUC-HSO and pUC 8–4, reaction buffers, creatine phosphokinase, 20× dNTPs/NTPs, phosphocreatine, yeast RNA coprecipitant. Addition of SV40 Large T antigen initiates replication in the presence of host proteins.

4 Notes

1. pEPito vector is derived from parent pEPI-1 plasmid but has considerably higher efficacy in vitro and in vivo. The pEPito vector is significantly smaller in size, contains only one transcription unit and has 60 % less CpG motives as compared to pEPI-1 plasmid. It shows enhanced performance due to the presence of the human CMV enhancer/human elongation factor 1 alpha promoter that is known to be minimally affected by epigenetic silencing events [30]. The pEPito contains MAR sequence hence is applicable to study in vitro DNA replication; however, it has not been tested for mammalian replication assays yet.
2. It is advisable to autoclave buffers used in making the plasmid, carrying out IVRA or running gel to prevent plasmid degradation by nucleases.
3. A very important point to remember is to use EDTA-free solutions and water in order to prevent unwanted termination of IVRA because EDTA is used to stop the replication assay.
4. Under similar biochemical conditions, it was found that replication of plasmid was only 2–3 % when cytosolic and nuclear extracts from MCF7 cells were used. Replication was significantly higher with 293T cell extracts and best achieved by addition of commercially available large T antigen. This is

quite plausible because the initiator T antigen is added in high amounts to the incubation mixtures and does not need to interact with other proteins for the recognition and the unwinding of the viral origin. The plasmid contains a separate SV40 origin. In contrast, the dozen or so cellular initiator functions are inevitably diluted when extracted from the nuclei of cultured cells and assemble on one of many possible sites on the plasmid template where they must interact in a highly complex manner before they are ready to initiate a replication round. Hence, as a positive control large T antigen could be used since pEPI-1 plasmid has SV40 origin to assess replication in in vitro conditions. T antigen can be purchased from the company, CHIMERx, (cat # 5800-01). Another reason why replication efficiency is low when nuclear extracts from cells are used, instead of T antigen, could be the presence of limiting factors such as proteins that negatively control replication in the nuclear extracts. Immunodepletion or silencing of potential replication inhibiting proteins could help in accelerating the replication efficiency. Reversibly, the effect of these proteins can be seen by addition of excessive amounts of inhibiting proteins.

5. It is essential to perform all steps of cell lysis on ice or at 4 °C to prevent unwanted degradation of proteins. Likewise, addition of protease inhibitors wherever mentioned will preserve the integrity of proteins.
6. Precautions during nuclear and cytosolic extract preparation—Make sure the homogenization is proper in order to eliminate cytosolic protein contamination in the nuclear fraction. Usually the nuclear extract obtained is dialyzed before use, but not in this assay because it needs a high salt nuclear extract that contains many proteins known or believed to be involved in mammalian genome replication such as Mcm's, Orc's, Cdt1, or Cdc6 among others.
7. It is preferable to extract pEPI-1 plasmid directly from transfected mammalian cells by Hirt extraction protocol or to amplify the plasmid in bacterial strains—DAM^{-} or DCM^{-} strains, which prevents methylation of plasmids. The reason for observing these preventative measures is parental pEPI-1 plasmid obtained from bacteria is potentially methylated on both strands and hence susceptible to Dpn I digestion.
8. Hirt extraction of plasmid from cells could be a time-consuming and labor-intensive procedure. Qiagen has a kit available to isolate these plasmids called as QIAprep Spin Miniprep Kit with some modifications as described in the methods, Subheading 3.2.2. The advantage of this kit over Hirt extraction are phenol–chloroform step can be omitted and experimental inconsistencies such as pipetting errors can be avoided.

9. The spin kit uses silica gel membrane for DNA binding, and hence, care should be taken to prevent overloading of the sample on the columns to obtain high DNA yield of good quality.

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Chapter 21

Fluorescent In Situ Hybridization on Comets: FISH Comet

Sergey Shaposhnikov, Naouale El Yamani, and Andrew R. Collins

Abstract

The DNA in eukaryotic cells is organized into loop domains that represent basic structural and functional units of chromatin packaging. The comet assay, a sensitive method for monitoring DNA damage and repair, involves electrophoresis of nucleoids comprising supercoiled DNA attached to the nuclear matrix. Breaks in the DNA relax the supercoiling and allow DNA loops to expand, and on electrophoresis to move towards the anode, giving the appearance of a comet tail. We use fluorescent in situ hybridization (FISH) to investigate the structure of the chromatin within comet preparations and to study specific DNA sequences within comets. In this chapter we describe our FISH comets protocols, deal with some technical questions and outline the theory. FISH with comets should be useful to researchers interested in the structural organization of DNA and chromatin, the localization of DNA damage, and the kinetics of repair of damage.

Key words FISH-comet, Comet assay, Fluorescent in situ hybridization, DNA damage, DNA probes

1 Introduction

The comet assay involves electrophoresis of nucleoids of supercoiled DNA attached to the nuclear matrix. Breaks in the DNA relax the supercoiling and allow DNA loops to expand, and on electrophoresis to move towards the anode, giving the appearance of a comet tail [1]. The percentage of DNA in the tail reflects the level of DNA damage in individual cells. By applying FISH on comet preparations a resolution at the level of individual DNA sequences can be achieved [2].

A variety of DNA probes can be applied on comets. Most popular non-gene-specific DNA probes are repetitive sequences (centromere, telomere, ribosomal DNA repeats, and interspersed repetitive elements—SINEs and LINEs), DNA from microdissected chromosomes (chromosome arm- or band-specific painting probes), as well as DNA from flow-sorted chromosomes (chromosome painting probes). These probes are often produced commercially. Specific genes and other DNA regions are studied using

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unique probes such as oligonucleotides and PCR products, cDNAs, and genomic DNA fragments cloned in cosmids, P1-artificial chromosomes (PACs), bacterial artificial chromosomes (BACs), or yeast artificial chromosomes (YACs). Peptide nucleic acid (PNA) probes [3], as well as padlock DNA probes [4] can be also used on comets.

The first FISH comet results were published by Santos et al. who studied structural chromatin organization in comets using probes from centromeric and telomeric DNA regions, as well as segments of gene *MGMT* [5].

We used PAC clones from human chromosome 16q22.1 as probes to investigate the behavior of DNA loops under neutral and alkaline conditions of the comet assay: in neutral comets the probed sequence appeared as a linear array, consistent with extension from a fixed point on the nuclear matrix; in alkaline comets linear DNA has coalesced into a granular form [6].

FISH comets can be also used to study gene-specific DNA repair by inducing damage and following the relocation of gene-specific signals from tail to head during a repair incubation period. Using this approach we reported preferential repair of gene *TP53* after H_2O_2 treatment [7].

Here we describe our FISH comets protocols and deal with some technical questions.

2 Materials

2.1 Equipment and Supplies

1. Staining jars (vertical or horizontal).
2. Glass slides with frosted ends.
3. Glass coverslips (9 mm round, 20 × 20 mm and 22 × 22 mm square).
4. Parafilm.
5. Metal plate.
6. Incubator (37 °C/55 °C).
7. Moist chamber (improvised from a glass or plastic box with a layer of water below a platform holding slides).
8. Electrophoresis tank (horizontal) and power supply (approx. 500 mA, at least 25 V).
9. Fluorescence microscope.

2.2 Reagents and Solutions

1. Normal melting point agarose (NMP agarose): 1 % in H_2O. A few hundred mL is enough to pre-coat several hundred slides.
2. Low melting point agarose (LMP agarose): 1 % in PBS. Store at 4 °C in 10 mL aliquots.
3. Phosphate buffered saline (PBS).

4. Lysis solution: 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10 (set with 10 M NaOH), stored at 4 °C. 1 mL of Triton X-100 per 100 mL is added immediately before use.
5. Alkaline electrophoresis solution: 0.1 M NaOH, 1 mM EDTA.
6. Neutral electrophoresis solution (1× TBE): 0.09 M Tris-borate, 0.002 M EDTA. Set to pH 7.5 by adding concentrated HCl.
7. Enzyme reaction buffer: 40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0 with KOH (made as 10× stock, adjusted to pH 8.0 and frozen at −20 °C).
8. Enzymes (*see* **Note 1**): Endonuclease III (endo III), formamidopyrimidine DNA glycosylase (FPG) and T4 endonuclease V are commercially available in purified form, or may be obtained as s crude extract from bacteria containing overproducing plasmids. Nonspecific nuclease activity in the crude extract is not significant at the concentrations used.
9. DNase I 10× Nick translation buffer: 0.5 M Tris–HCl pH 7.5, 0.1 M $MgSO_4$, 1 mM DTT, 0.5 mg/mL bovine serum albumin: aliquots stored at −20 °C.
10. Stock solutions of deoxyribonucleoside triphosphates (dNTPs).
11. Biotin-14-dCTP (0.4 mM).
12. Digoxigenin-11-dUTP (1 mM).
13. Cot-I DNA (*see* **Note 2**).
14. DNA polymerase I, *E.coli*: stored at −20 °C.
15. Salmon sperm DNA: stored at −20 °C.
16. Formamide (ultrapure).
17. “Fixo gum” rubber cement.
18. 20× SSC.
19. Tween 20.
20. Nonfat milk powder.
21. Antibody dilution solution: 4× SSC, 0.05 % Tween 20, 5 % nonfat milk powder (*see* **Note 3**).
22. Cy3-conjugated streptavidin and Biotinylated anti-avidin D (*see* **Note 3**).
23. Fluorescent antibody enhancer set for digoxigenin detection (*see* **Note 3**):
    - Solution 1: anti-DIG monoclonal antibody against digoxigenin, mouse IgG1.
    - Solution 2: anti-mouse-Ig-DIG, F(ab')2, fragment from sheep.
    - Solution 3: anti-DIG-Fluorescein, Fab fragments from sheep.

3 Methods

3.1 Slide Preparation for the Comet Assay (Precoating)

1. Slides should be grease-free; clean if necessary by soaking in alcohol and then wiping dry with a clean tissue.
2. Dip slides in a vertical staining jar of melted 1 % standard agarose in H_2O.
3. Drain off excess agarose, wipe the back clean and leave on a clean bench overnight to dry.

3.2 Embedding Cells in Agarose

(Work quickly as the agarose sets quickly at room temperature!)

1. Melt the LMP agarose in a microwave oven (loosen the cap!). Then place in water bath at 37 °C and allow equilibrating to this temperature.
2. Suspend cells at 10^6/mL in PBS. (Cells can be unattached such as white blood cells, or trypsinized cells from culture, or disaggregated tissue cells.)
3. Place a 40 μL aliquot in a microcentrifuge tube.
4. Quickly add 140 μL of 1 % LMP agarose at 37 °C: aspirate with pipettor, up and down once, to mix with cells.
5. Place two 70 μL aliquots on one slide (using a single pipettor tip, for speed).
6. Cover each drop with a 20 × 20 mm coverslip. Leave slides at 4 °C for 5 min.

3.3 Lysis

1. Add 1 mL Triton X-100 to 100 mL of lysis solution (4 °C).
2. Remove coverslips from gels and place slides in lysis solution in a horizontal staining jar.
3. Leave at 4 °C for 1 h.

3.4 Enzyme Treatment (Optional) (See Note 1)

1. Prepare 300 mL of enzyme reaction buffer. Keep 1 mL separate, for diluting enzymes.
2. Place slides in this buffer (4 °C) in staining jar, leave for 5 min, decant buffer and replace with fresh, leave for 5 min and then repeat process (three washes in total).
3. Meanwhile, prepare enzyme at appropriate dilution (this will vary from batch to batch; refer to supplier).
4. Remove slides from last wash and carefully remove excess liquid with a tissue.
5. Place 40–50 μL of enzyme solution, or buffer (control) onto each gel, and cover with a coverslip.
6. Put slides into moist box and incubate at 37 °C for 30 min.

3.5 Alkaline Electrophoresis (See Notes 4 and 5)

1. Place slides on platform in electrophoresis tank, forming complete rows (gaps filled with blank slides). Check that the tank is level. Add alkaline electrophoresis solution so that gels are just covered. Leave for 40 min.
2. With tanks of standard size, run at 25 V (constant voltage setting) for 30 min.
3. The current should not exceed the limit for the power supply; if it does, it will be impossible to run at 25 V. Removing some solution to reduce the depth above the slides will reduce the current. Normally the current is around 300 mA (not crucial).
4. Neutralize by washing with PBS in staining jar at 4 °C for 10 min, and then for 10 min in water.
5. Prepare for FISH as described below, or
6. Dry and store at room temperature for later staining.

3.6 Neutral Electrophoresis (See Notes 4 and 5)

1. Incubate the slides in 1× TBE buffer at 4 °C (two changes of buffer).
2. Place on platform in electrophoresis tank, forming complete rows (gaps filled with blank slides). Check that the tank is level.
3. Add 1× TBE buffer to just cover the slides and electrophorese at 25 V for 30 min at 4 °C.
4. Prepare for FISH as described below.
5. Dry and store at room temperature for later staining.

3.7 Preparing Slides for FISH (See Notes 6 and 7)

After electrophoresis, incubate slides in 96 % ethanol for 30 min at 4 °C, then in 0.5 M NaOH for 25 min, and finally dehydrate through 70, 84, and 96 % ice-cold ethanol.

3.8 Probes for FISH

If commercial FISH probes are used, follow protocols recommended by the supplier, with some modifications as described in the technical notes. However, if there are no commercial probes available for your applications, you must prepare them yourself (*see* **Note 8**).

3.9 Labeling of DNA Probes by Nick Translation

Label human Cot-I DNA or cosmids, and PAC, BAC, or YAC clones containing human genomic DNA inserts with botin-14-dCTP or digoxigenin-11-dUTP by conventional nick translation. Labeling of 1–4 μg DNA in a final volume of 100 μL (adjusted with sterile distilled water) is carried out as follows (scaling up or down is possible):

1. Prepare 10× nick translation buffer: 0.5 M Tris–HCl pH 7.5, 0.1 M $MgSO_4$, 1 mM DTT, 0.5 mg/mL bovine serum albumin; aliquot and store at −20 °C.
2. From the stock solutions of deoxyribonucleoside triphosphates (dNTPs), 100 mM each, prepare mixture of dNTPs (0.5 mM

each) containing either no dCTP (dNTP-C), or no dATP (dNTP-A), or no dTTP (dNTP-T) (aliquot and store at –20 °C).

3. Prepare the reaction mix by adding the reagents in order. Work on ice.
   (a) Biotin-14-dCTP reaction mix:
      - Distilled water (to give a final volume of 100 μL).
      - 10× nick translation buffer: 10 μL.
      - DNA probe: 1–4 μg.
      - dNTP-C: 7.5 μL (0.5 mM each).
      - Biotin-14-dCTP: 6.25 μL.
      - DNA polymerase I: 40 units.
   (b) Digoxigenin-11-dUTP reaction mix:
      - Distilled water (to give a final volume of 100 μL).
      - 10× Nick translation buffer: 10 μL.
      - DNA probe: 1–4 μg.
      - (dNTP-T): 6 μL (0.5 mM each).
      - (dNTP-A): 1.5 μL (0.5 mM each).
      - Digoxigenin-11-dUTP: 2 μL.
      - DNA polymerase I: 40 units.
4. Mix briefly by tapping the tube with a finger and spin down using bench microcentrifuge.
5. Add 4 μL of DNase I at concentration established by titration (*see* Subheading 3.10 below). Mix by tapping the tube with a finger and spin down using bench microcentrifuge.
6. Place the tube in water bath at 13–14 °C for 30 min.
7. Immediately add 10 μL of 0.5 M EDTA and place on ice and proceed with standard ethanol precipitation procedure briefly outlined below (8–13).
8. Add 5 μL of 5 M NaCl and 300 μL 96 % ethanol. Keep at –80 °C for 2 h or at –20 °C overnight.
9. Centrifuge at 14,000 × *g* for 10 min at 4 °C.
10. Decant supernatant and add to pellet 0.5 mL of cold 70 % ethanol.
11. Centrifuge at 14,000 × *g* for 5 min at 4 °C and decant supernatant.
12. Repeat the ethanol wash step.
13. Decant the supernatant, briefly dry the pellet, dissolve in 15 μL H_2O, and store at –20 °C until use.

3.10 DNase Titration

1. Dilute DNase I in 50 % glycerol/1.5 M NaCl, at 1:10, 1:50, 1:100, 1:250, 1:500, and 1:1,000.
2. For each dilution, prepare a reaction mix, working on ice:
   - 5 μL nick translation buffer.
   - 1 μg genomic DNA (total genomic DNA from various organisms, or large-insert genomic clones).
   - 2 μL DNase I.
   - Distilled water to 50 μL.
3. Place reaction mixes in water bath at 13–14 °C for 30 min.
4. Use conventional agarose gel electrophoresis to ascertain the lengths of the resulting DNA fragments, and select for use in nick translation the dilution producing fragments of 100–500 bp.
5. Store the DNase dilutions at −20 °C; you will need to repeat titration if the fragment size in nick translation reactions is increased, or if the fluorescence background when visualizing FISH signals is too high.

3.11 Preparation of MM 2.1 Hybridization Buffer

1. Mix 0.5 mL 20× SSC, 5.5 mL formamide, and 1 mL H_2O.
2. Add 1 g dextran sulfate, dissolve for 2–3 h at 70 °C, set pH to 7.0 with either HCl or NaOH (dextran sulfate has a pH 6–8, and formamide becomes acidic with time). Store at −20 °C.

3.12 Preparation of Hybridization Mix

Hybridization reaction mix contains labeled DNA probe (or probes), salmon sperm DNA, Cot-I DNA (unlabeled—in the case that it is used as a as blocking agent rather than probe), 1× SSC, 0.1 mg/mL dextran sulfate, 55 % formamide.

1. To prepare 15 μL of hybridization mix with probes of genomic DNA clones, mix:
   - 100 ng labeled DNA probe.
   - 10.5 μL MM 2.1 buffer.
   - 20 μg Cot-I DNA.
   - 10 μg salmon sperm DNA.
   - Adjust volume to 15 μL with distilled water.
     (Scaling up or down is possible.)
2. To prepare 15 μL of hybridization mix with probes of labeled Cot-I DNA, mix:
   - 100 ng labeled Cot-I DNA.
   - 10.5 μL MM 2.1 buffer.
   - 1 μg salmon sperm DNA (optional).
   - Adjust volume to 15 μL with distilled water.
     (Scaling up or down is possible.)

3.13 Probe Denaturation and Start of Hybridization

1. Denature probe at 70 °C (in a water bath) for 10 min.
2. Pre-anneal at 37 °C for 1 h (omit this step when the probe is labeled Cot-I DNA).
3. Pipette 3–4 μL of probe mix onto slide, cover with 9 mm round coverslip, and seal with rubber cement. If a larger coverslip is used, the volume of probe mix can be increased, e.g., up to 30 μL of the mix is used under a 20×20 mm coverslip. As an alternative to glass, plastic coverslips cut from overhead transparency film can be used; they are flexible and easier to remove after hybridization.
4. Incubate overnight at 37 °C in a humid box. A shorter incubation time (down to 1 h) is possible when Cot-I DNA is used as probe.

3.14 Post-hybridization Washes (See Notes 6 and 3)

1. Prepare the following solutions:
   - Three jars with 50 % formamide/2× SSC and two with 2× SSC; warm them to 42 °C in a water bath. (The temperature in the jars will be a degree or two lower than in the water bath; check with thermometer).
   - One jar with 2× SSC and one with 4× SSC, 0.05 % Tween 20; keep these at room temperature.
   - 500 mL wash buffer (WB): 4× SSC, 0.05 % Tween 20.
2. Carefully remove rubber cement and place slides in jar with 2× SSC at room temperature for 10 min or until coverslips have floated off.
3. Transfer slides to 50 % formamide/2× SSC at 42 °C and incubate for 5 min.
4. Repeat incubation for 5 min each in the second and third jars with 50 % formamide/2× SSC.
5. Wash slides for 5 min each in the two jars of 2× SSC at 42 °C.
6. Transfer slides to a jar with 4× SSC, 0.05 % Tween 20 for 10 min at room temperature.

3.15 Preparation of Antibody Detection Solutions (See Note 3)

1. Prepare antibody dilution solution: 4× SSC, 0.05 % Tween 20, with 5 % nonfat milk powder (the volume depends on the number of slides, *see* Subheading 2).
2. Depending on the probe label, prepare detection solutions as described below. The amount of detection solution required per slide for each incubation is given; this should be scaled up according to the number of slides.
   (a) Antibody solutions for detection of probes labeled with biotin:

- Detection solutions 1 and 3: 2 μL of Cy-3 conjugated streptavidin mixed with 98 μL of antibody dilution solution.
- Detection solution 2: 1 μL anti-avidin-D mixed with 99 μL of antibody dilution solution.

(b) Antibody solutions for detection of probes labeled with digoxigenin:

- Detection solution 1: 4 μL of solution 1 from the fluorescent antibody enhancer set for digoxigenin detection mixed with 96 μL of antibody dilution solution.
- Detection solution 2: 4 μL of solution 2 from the fluorescent antibody enhancer set for digoxigenin detection mixed with 96 μL of antibody dilution solution.
- Detection solution 3: 4 μL of solution 3 from the fluorescent antibody enhancer set for digoxigenin detection mixed with 96 μL of antibody dilution solution.

(c) Antibody solutions for simultaneous detection of probes labeled with biotin and probes labeled with digoxigenin in the same hybridization reaction:

- Detection solution 1: 2 μL of Cy-3 conjugated streptavidin and 4 μL of solution 1 from the fluorescent antibody enhancer set for digoxigenin detection mixed with 94 μL of antibody dilution solution.
- Detection solution 2: 1 μL anti-avidin-D and 4 μL of solution 2 from the fluorescent antibody enhancer set for digoxigenin detection mixed with 95 μL of antibody dilution solution.
- Detection solution 3: 2 μL of Cy-3 conjugated streptavidin and 4 μL of solution 3 from the fluorescent antibody enhancer set for digoxigenin detection mixed with 94 μL of antibody dilution solution.

After all components are mixed, spin the tubes down on a bench microcentrifuge, leave for 10 min in a dark place at room temperature, centrifuge for 10 min at $14,000 \times g$ at room temperature, and finally transfer supernatants to fresh tubes. Keep in a dark place before use.

Use the solutions on the day of preparation.

3.16 Signal Detection (See Note 9)

Perform all incubations in a humid box at 37 °C under Parafilm coverslips. Slides should not dry out at any stage of the detection process.

1. Drain slides and apply 100 μL of antibody dilution solution under Parafilm coverslips.
2. Incubate for 30 min at 37 °C in a humid box, remove the Parafilm, and let solution drain onto a tissue.
3. Apply 100 μL of antibody detection solution 1, cover with Parafilm and incubate for 40–60 min at 37 °C in a humid box.
4. Remove Parafilm and wash three times in WB at room temperature.
5. Apply 100 μL of antibody detection solution 2, cover with Parafilm and incubate for 40–60 min at 37 °C.
6. Repeat **steps 4**, **3**, and **4** (in that order).
7. Dehydrate slides in 70, 80, and 95 % ethanol for 5 min each and air-dry.

3.17 Signal Visualization (See Note 9)

1. Stain gels with 20 μL of DAPI prepared in Vectashield (Vector Laboratories).
2. Alternative stains: Propidium iodide (2.5 μg/mL), Hoechst 33258 (0.5 μg/mL), SYBR Gold (0.1 μL/mL) or ethidium bromide (20 μg/mL).
3. Visualize and record the signals using appropriate filters; overlay the recorded images.

4 Notes

1. It is not recommended to use enzyme buffer containing β-mercaptoethanol as this can produce a high background of DNA damage.
2. Cot-I DNA is often supplied in a concentration lower than 10 μg/μL. It should be then concentrated (for example by ethanol precipitation) to 10 μg/μL.
3. Here we provide the protocol for detection of signals with specific detection kits from Roche Applied Science and Vector Laboratories; however, many more detection systems are available and can be used. We recommend following the general protocols from the supplier where possible and adapting them for use with agarose-embedded preparations when necessary (*see* also **Note** 7).
4. Electrophoresis solution should be cooled before use.
5. The voltage of 0.8 V/cm is recommended (calculated on the basis of the distance across the platform).

6. To minimize the risk of agarose being detached, all the steps (i.e., preparation of slides, various stringent washes, detection of signals) should be carried out in the smallest possible volumes. Recommended times in FISH protocols should not be exceeded (and can be shortened when appropriate).
7. Chemical denaturation of comet DNA is used as low melting point agarose would melt at the temperature used for DNA denaturation in standard chromosomal preparations.
8. Hybridization with human metaphase spreads before carrying out experiments with comet DNA is advisable to check that each probe produces low unspecific hybridization.
9. In contrast with standard FISH preparations, comets (and thus signals) are organized in three-dimensional space. This allows investigating the real organization of chromatin in living cells, but on the other hand produces some difficulties in the process of visualizing the signals.

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Chapter 22

Methods to Study Histone Chaperone Function in Nucleosome Assembly and Chromatin Transcription

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Abstract

Histone chaperones are histone interacting proteins that are involved in various stages of histone metabolism in the cell such as histone storage, transport, nucleosome assembly and disassembly. Histone assembly and disassembly are essential processes in certain DNA-templated phenomena such as replication, repair and transcription in eukaryotes. Since the first histone chaperone Nucleoplasmin was discovered in *Xenopus*, a plethora of histone chaperones have been identified, characterized and their functional significance elucidated in the last 35 years or so. Some of the histone chaperone containing complexes such as FACT have been described to play a significant role in nucleosome disassembly during transcription elongation. We have reported earlier that human Nucleophosmin (NPM1), a histone chaperone belonging to the Nucleoplasmin family, is a co-activator of transcription. In this chapter, we describe several methods that are used to study the histone chaperone activity of proteins and their role in transcription.

Key words Chromatin transcription assay, ACF complex, p300, Nucleophosmin

1 Introduction

The eukaryotic genome is packaged into a topologically complex, fibrous superstructure known as chromatin which is compact and repressive in nature. Its fluidity, however, is essential for various cellular processes such as transcription, replication, and repair. Various epigenetic phenomena have been implicated in maintenance of the dynamicity of chromatin structure including the local chromatin composition (presence of histone variants), methylation status of underlying DNA, posttranslational modifications of histones and non-histone proteins, histone chaperones, and ATP-dependent chromatin remodeling.

Nucleosome assembly is one of the key processes that not only contributes to local changes in chromatin allowing access for transcription, replication, and repair machinery but also plays a major role in epigenetic inheritance. Histones and DNA tend to form insoluble

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aggregates when mixed directly at physiological conditions. Proper assembly of histones with DNA to produce chromatin is facilitated, by a group of acidic proteins known as histone chaperones. They bind to free histones and prevent their improper and premature interactions with DNA. They are involved in essential processes of histone metabolism with distinct functions such as histone transport and storage (e.g., NAP1), replication coupled histone deposition (e.g., CAF1), replication independent variant histone deposition (e.g., HIRA, Daxx), histone removal (e.g., Asf1) [1] and transcriptional co-activation (e.g., NPM1) [2], some of the histone chaperones are also known to be maintained abundantly in the cell for quick supply of histones during stress responses [3].

Histone chaperones are known to have various cellular functions apart from nucleosome assembly. Mutation, disruption or deletions of histone chaperones have been known to cause defects in gene expression and genome stability, leading to pathological conditions such as cancer [4]. Although many of the histone chaperones are well characterized, many other chromatin-associated proteins are being assigned histone chaperone activity. The specificity of histone chaperones to distinct histone proteins, the interacting partners/associated complexes of histone chaperones, effects of posttranslational modifications of the chaperones as well as their substrates are active areas of discovery.

Nucleophosmin/NPM1 (also known as Numatrin/B23/NO38) is a highly abundant protein majorly localized to the nucleolus of the cell. It is multifunctional in nature with known roles in ribosome biogenesis [5], genomic stability [6], centrosome duplication [7], molecular chaperone function, nucleocytoplasmic transport, cell proliferation [8], transcription regulation [2] apart from its histone chaperone function [2, 9]. NPM1 is known to undergo several posttranslational modifications such as acetylation [2], phosphorylation [9], ubiquitination and sumoylation. We have earlier shown that it functions as a co-activator in transcription and enhances acetylation-dependent transcription from the chromatin template. Acetylation of NPM1 leads to enhanced histone chaperone activity and interaction ability with acetylated histones [2]. Further, acetylated NPM1 foci colocalizes with RNA Polymearse II foci in the cells implicating its role in transcription regulation. NPM1 was found to be overexpressed and hyperacetylated in oral cancer [10].

This chapter describes methods to characterize histone chaperones in the context of transcription using recombinant human NPM1. In vitro assays of nucleosome assembly and transcription are useful in teasing out specific functions of histone chaperones from the array of complex processes they are involved in. A protein is said to have histone chaperone activity if it has histone binding property and can transfer histones onto a suitable DNA template elucidated by the classical histone transfer assay (HTA).

The methods described in this chapter have aided us in describing the histone chaperone activity of NPM1 and the discovery that acetylated NPM1 is a better histone chaperone and transcription co-activator compared to unmodified NPM1 [2]. Further, with the help of these assays we have characterized NPM3, another member of the Nucleoplasmin and a homolog of NPM1. We showed that although NPM3 binds to histones, it does not have histone chaperone activity. In presence of NPM1, however, NPM3 inhibits the chaperone activity of NPM1 [11].

We describe the histone transfer assay where the chaperoning activity of NPM1 is assessed by visualizing the introduction of negative supercoils due to chaperone mediated assembly of nucleosomes onto a relaxed DNA template. Further, we describe two different methods used to assemble chromatin prior to subjecting to in vitro chromatin transcription. Chromatin assembly mediated by NAP1 alone and NAP1 along with the Drosophila ACF complex, an ATP-dependent chromatin remodeler are compared to show that the chromatin assembled as a result of the combined effect of the histone chaperone activity of NAP1 and the remodeling activity of ACF is more repressive as compared to that of NAP1 alone. In vitro chromatin transcription assays are used to study the effect of histone chaperone on the remodeling events which facilitate transcription from repressed chromatin. By modifying these protocols the effect of posttranslational modifications etc. of the chaperone can also be studied.

2 Materials

2.1 pG$_5$ML Array Template

The pG$_5$ML array template consists of the transcription template flanked on both sides by five repeats of the Sea urchin 5S rDNA nucleosome positioning sequences. The transcriptional cassette consists of five repeats of the Gal4 binding site upstream to the Adenovirus Major Late promoter which controls the transcription of a 380 bp G-less cassette [12].

2.2 Purification of Core Catalytic Domain of Drosophila Topoisomerase I (dTopo I) [13]

1. BL21 (DE3) competent cells.
2. Luria broth.
3. 50 mg/ml kanamycin solution.
4. dTopo I expression construct.
5. 1 M IPTG.
6. Lysis buffer: 50 mM sodium phosphate pH 7.0, 0.5 M NaCl, 15 % glycerol, 15 mM imidazole, 0.1 % (v/v) NP-40 (IGEPAL CA-630), 0.2 mM PMSF, and 0.5 mM benzamidine.
7. Ni-NTA His-Bind Resin (Novagen).
8. End-to-end rotor.

9. Econo-Fast column (Bio-Rad).
10. Elution buffer: Lysis buffer containing 0.5 M imidazole.
11. Dialysis buffer: 25 mM HEPES pH 7.6 and 0.1 mM EDTA, 10 % glycerol, 50 mM NaCl, 0.01 % NP-40, 0.5 mM DTT, 0.2 mM PMSF, and 0.5 mM benzamidine.

2.3 Preparation of HeLa Nuclear Extract and Nuclear Pellet

1. HeLa S3 cell line.
2. Spinner flasks.
3. Joklik's medium (Sigma) supplemented with L-glutamine (Sigma), 1× penicillin–streptomycin–amphotericin B solution, and 10 % fetal bovine serum (FBS).
4. 1× PBS.
5. Hypotonic buffer: 10 mM Tris–HCl pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM PMSF, and 10 mM β-mercaptoethanol.
6. Dounce's homogenizer (Wheaton) with pestle B.
7. Low salt buffer: 20 mM Tris–HCl pH 7.9, 25 % glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM KCl, 0.2 mM PMSF, and 10 mM β-mercaptoethanol.
8. High salt buffer: 20 mM Tris–HCl pH 7.9, 25 % glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1.2 M KCl, 0.2 mM PMSF, and 10 mM β-mercaptoethanol.
9. TGME buffer: 50 mM Tris–HCl pH 7.9, 25 % glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA pH 8.0, 0.2 mM PMSF, and 10 mM β-mercaptoethanol.
10. BC100: 1 M HEPES pH 7.9, 100 mM KCl, 20 % glycerol, and 0.2 mM EDTA pH 8.0.

2.4 Purification of Core Histones

1. HeLa nuclear pellet.
2. Buffer A: 100 mM potassium phosphate buffer pH 6.7, 0.1 mM EDTA, 10 % glycerol, 0.1 mM PMSF, 0.1 mM DTT, and 630 mM NaCl.
3. Dounce's homogenizer (Wheaton) with pestle B.
4. Hydroxyapatite BioGel HTP (Bio-Rad).
5. 10 mM potassium phosphate, pH 6.7.
6. Econo-Fast glass column (Bio-Rad).
7. Buffer B: Buffer A containing 2 M NaCl.
8. Dialysis buffer: 20 mM Tris–HCl pH 7.9, 20 % glycerol, 2 mM β-mercaptoethanol, 0.2 mM EDTA, 2 mM PMSF, 0.1 % NP-40, and 100 mM KCl.
9. Trichloroacetic acid.
10. Acetone.

2.5 Purification of Human NPM1, Mouse NAP1

1. BL21 (DE3) competent cells.
2. Luria broth.
3. 50 mg/ml kanamycin (for NPM1) and 100 mg/ml ampicillin (for mNAP1).
4. His_6-NPM1 and His_6-mNAP1 expression construct.
5. 1 M IPTG.
6. Lysis buffer: 20 mM Tris–HCl pH 7.9, 20 % glycerol, 2 mM β- mercaptoethanol, 0.2 mM EDTA, 2 mM PMSF, 0.1 % NP-40, 30 mM imidazole, and 300 mM KCl.
7. Ni-NTA His-Bind Resin (Novagen).
8. End-to-end rotor.
9. Wash buffer: Same as Lysis buffer.
10. Econo-Fast column (Bio-Rad).
11. Elution buffer: Lysis buffer containing 250 mM imidazole and 100 mM KCl.
12. Dialysis Buffer: 20 mM Tris–HCl pH 7.9, 20 % glycerol, 2 mM β- mercaptoethanol, 0.2 mM EDTA, 2 mM PMSF, 0.1 % NP-40, and 100 mM KCl.
13. Q sepharose Fast Flow (GE Healthcare).
14. BC0-BC500: 20 mM Tris–HCl pH 7.9, 20 % glycerol, 2 mM β-mercaptoethanol, 0.2 mM EDTA, 2 mM PMSF, 0.1 % NP-40, and 0–500 mM KCl.

2.6 Purification of Drosophila ACF Complex

1. *Sf*21 cell line.
2. 150 mm dishes.
3. TC100 media supplemented with 1× penicillin–streptomycin–amphotericin B solution, 40 μg/ml gentamicin, and 10 % FBS.
4. Cell Scrapers.
5. dACF and dISWI virus with known titer.
6. 1× PBS.
7. Dounce's homogenizer (Wheaton) with pestle B.
8. Protease inhibitor cocktail (Sigma).
9. Anti-FLAG M2 agarose beads (Sigma).
10. End-to-end rotor.
11. Lysis Buffer: 20 mM Tris–HCl pH 7.9, 20 % glycerol, 0.5 M NaCl, 4 mM $MgCl_2$, 0.4 mM EDTA, 2 mM DTT, 20 mM β-glycerophosphate, 0.4 mM PMSF, 1× protease inhibitor cocktail (Sigma).
12. Dilution buffer: 20 mM Tris–HCl pH 7.9, 10 % glycerol, 0.02 % NP-40.

13. Wash buffer: 20 mM Tris–HCl pH 7.9, 150 mM NaCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 10 mM β-glycerophosphate, 15 % glycerol, 0.01 % NP-40, 0.2 mM PMSF, 0.5× protease inhibitor cocktail.
14. Elution buffer: 0.2 mg/ml FLAG peptide (Sigma) in Wash buffer.

2.7 Reagents for In Vitro Interaction Studies

1. Recombinant NPM1 protein.
2. HeLa core histones or recombinant core histone octamer.
3. Ni-NTA His-Bind Resin (Novagen).
4. Interaction buffer: 20 mM Tris–HCl pH 7.9, 20 % glycerol, 0.2 mM EDTA pH 8.0, 0.1 % Nonidet P-40, 2 mM PMSF, 300 mM KCl, and 30 mM imidazole (*see* **Note 1**).
5. 2× SDS loading buffer: 100 mM Tris–HCl pH 6.8, 4 % (w/v) SDS electrophoresis grade, 0.2 % (w/v) bromophenol blue, 20 % glycerol, and 200 mM β-mercaptoethanol.

2.8 Histone Transfer Assay

1. pG_5ML plasmid (*see* **Note 2**).
2. HeLa core histones.
3. dTopo I (catalytic domain).
4. Recombinant NPM1 protein.
5. 2× Assembly buffer: 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 200 mM NaCl, and 0.1 mg/ml bovine serum albumin (BSA) (Sigma) (*see* **Note 1**).
6. Proteinase K stop buffer: 200 mM NaCl, 20 mM EDTA pH 8.0, 1 % SDS, 0.25 mg/ml glycogen, and 0.125 mg/ml proteinase K.
7. 1 % Agarose gel.
8. 6× Sample buffer: 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol, 30 % (v/v) glycerol in water.

2.9 Template Relaxation Assay

1. pG_5ML plasmid.
2. dTopo I protein.
3. 2× Template relaxation assay buffer: 100 mM Tris–HCl pH 7.5, 100 mM KCl, 20 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 60 μg/ml BSA.
4. Phenol–chloroform–isoamyl alcohol (25:24:1) pH 8.0.
5. 3 M sodium acetate, pH 5.2.
6. Absolute ethanol.
7. 1 % Agarose gel.

2.10 Buffers and Reagents for Chromatin Assembly by ACF Complex Method

1. Recombinant mNAP1 protein.
2. HeLa core histones or recombinant core histone octamer.
3. 0.5 M KCl.
4. 0.1 M ATP.
5. 0.1 M $MgCl_2$.
6. 0.5 M creatine phosphate.
7. 5 mg/ml creatine Kinase.
8. ACF complex.
9. HEG buffer (25 mM HEPES, pH 7.6, 0.1 mM EDTA, 10 % glycerol).
10. MNase stop buffer (20 mM EDTA, 200 mM NaCl, 1 % SDS, 0.25 mg/ml glycogen).
11. Proteinase K solution (20 mg/ml).
12. MNase (0.5 U/μl) (Sigma).

2.11 Buffers and Reagents for In Vitro Transcription Assay

1. 2× Transcription assembly buffer: 20 mM Tris–HCl pH 8.0, 2 mM EDTA, 300 mM NaCl, and 0.2 mg/ml BSA.
2. 2× HAT buffer: 20 mM HEPES, pH 7.8, 100 mM KCl, 10 mM DTT, 1 mM PMSF, 40 mM sodium butyrate, 0.5 mg/ml BSA, and 10 % glycerol.
3. BC 0: 20 mM Tris–HCl pH 7.9, 20 % glycerol, 0.2 mM EDTA pH 8.0.
4. BC 200: 20 mM Tris–HCl pH 7.9, 20 % glycerol, 0.2 mM EDTA pH 8.0, 200 mM KCl.
5. 20× Buffer: 0.4 M HEPES pH 8.4 and 100 mM $MgCl_2$.
6. 20× NTP mix: 12 mM ATP, 12 mM CTP, 0.5 mM UTP, and 2 mM 3’O-methyl GTP (Amersham).
7. 10 μCi/μl α^{32}pUTP (Board of Radiation and Isotope Technology, DAE, Govt. of India).
8. Transcription loading dye: 8.0 M urea in 1× TBE, xylene cyanol, and 0.005 % bromophenol blue.

3 Methods

3.1 Purification of pG_5ML

DNA templates for in vitro transcription and histone transfer assays are purified by plasmid maxi prep kit as recommended by the manufacturers (Qiagen).

3.2 Purification of dTopoI

His_6-tagged core catalytic domain of the Drosophila topoisomerase I is purified using Ni-NTA affinity chromatography [13].

1. Transform BL21 (DE3) competent cells with the expression vector and incubate the plate containing 50 μg/ml kanamycin overnight in a 37 °C incubator.
2. The following evening inoculate a single colony from this plate into a 100 ml LB containing 50 μg/ml kanamycin and incubate the culture in a 37 °C shaker incubator at 180–200 rpm.
3. The next morning, transfer the 100 ml culture into a 2 l Erlenmeyer flask containing 900 ml of LB supplemented with 50 μg/ml kanamycin.
4. Incubate the culture in a 37 °C incubator under shaking conditions at 200 rpm until OD reaches 0.5.
5. Induce the culture with IPTG to a final concentration of 0.42 mM and incubate at 30 °C for 5 h under shaking conditions at 200 rpm.
6. Harvest the cells by centrifugation at 6,000 rpm (5,000 × *g*) for 10 min at 4 °C.
7. Resuspend the pellet in 30 ml of lysis buffer and sonicate with four burst of 30 s at a setting of 32 % amplitude (Sonics-Vibra cell).
8. Clarify the lysate by centrifugation at 16,000 rpm (30,000 × *g*) for 30 min at 4 °C.
9. Add Ni-NTA beads (pre-equilibrated with the lysis buffer) to the clarified lysate and incubate on an end-to-end rotor at 4 °C for 3 h.
10. Wash the resin five times with the lysis buffer and pack it into an Econo Fast column.
11. Elute the protein with the elution buffer (lysis buffer containing 0.5 M imidazole).
12. Dialyze the purified protein in dialysis buffer (*see* Subheading 2.2) (Fig. 1a).

3.3 Preparation of HeLa Nuclear Extract and Nuclear Pellet

HeLa nuclear extract is prepared from HeLa S3 cell line cultured in spinner flasks [14].

1. Grow the HeLa S3 cells in Joklik's medium (Sigma) supplemented with L-glutamine (Sigma), 1× penicillin–streptomycin–amphotericin B solution, and 10 % FBS.
2. Pellet the cells at 3,000 rpm (1,800 × *g*) for 10 min at 4 °C.
3. Wash the pellet with ice-cold 1× PBS containing 0.2 mM PMSF and 10 mM β-mercaptoethanol.
4. Determine the packed cell volume (PCV).
5. Wash the cells with one PCV volume of the hypotonic buffer and pellet down at 2,500 rpm (1,250 × *g*) for 5 min at 4 °C.
6. Decant the supernatant carefully and resuspend the cells in 2 PCV volumes of the hypotonic buffer and incubate in ice for 15 min.

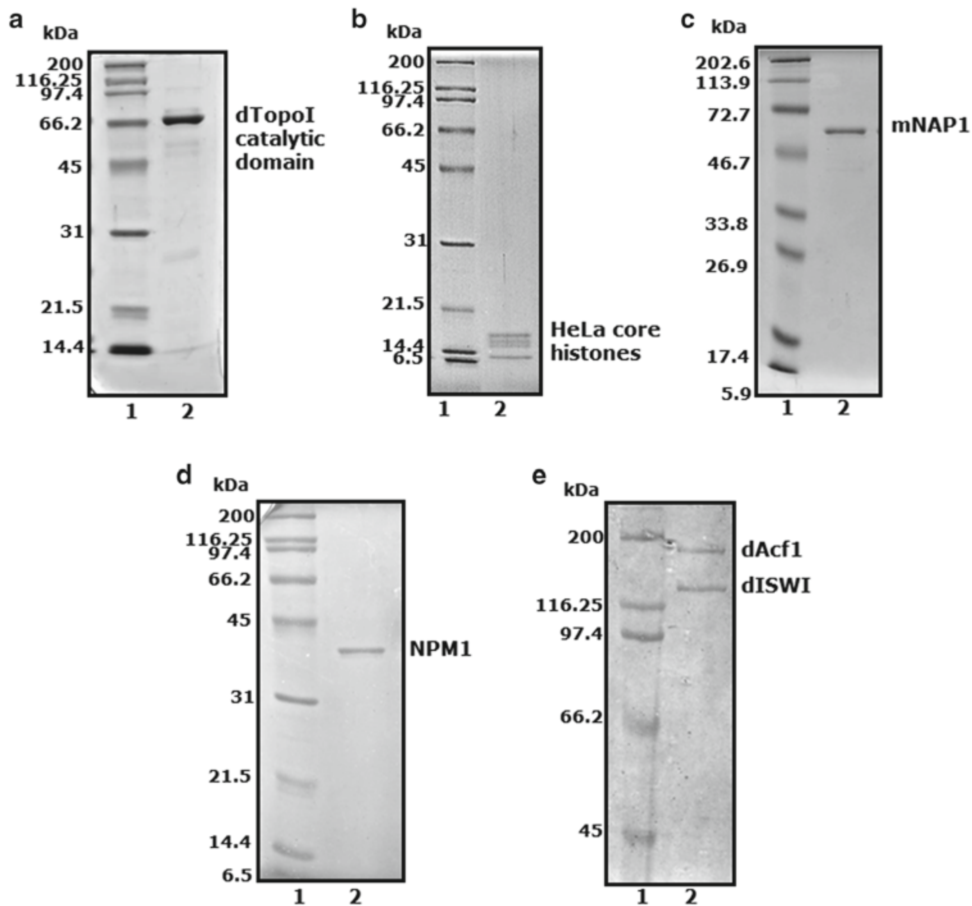

Fig. 1 Protein profile of (**a**) dTopo I catalytic domain (**b**) HeLa core histones (**c**) mNAP1 (**d**) NPM1 and (**e**) *Drosophila* ACF complex

7. After the incubation, homogenize the cell suspension by applying 15 strokes with the pestle B in a Dounce's homogenizer (Wheaton) for 15 min.
8. Centrifuge the homogenate at 4,000 rpm (3,200 × *g*) for 15 min at 4 °C.
9. The resultant supernatant is the cytoplasmic extract (S100 extract), which is stored in a buffer composed of 100 mM NaCl in 5 % glycerol at –80 °C.
10. Determine the pellet volume and record it as the nuclear pellet volume (NPV).
11. Homogenize the nuclear pellet with half the NPV volume of low salt buffer.
12. Transfer the homogenate into a small beaker with a magnetic bead and add an equal volume of high salt buffer dropwise and stir continuously for 30 min.

13. Clarify the resultant homogenate by centrifugation at 14,000 rpm (25,000 × *g*) for 20 min at 4 °C.
14. Dialyze the supernatant against BC100 for 3 h and centrifuge at 14,000 rpm (25,000 × *g*) for 20 min at 4 °C.

The supernatant from **step 13** is the HeLa nuclear extract and the pellet obtained is the HeLa nuclear pellet which is homogenized in TGME buffer and is subsequently used for histone purification.

3.4 Purification of HeLa Core Histones

Human core histones are purified from the HeLa nuclear pellet [14].

1. The evening before the purification swell about 5 g of Hydroxyapatite BioGel HTP (Bio-Rad) overnight by soaking in 10 mM potassium phosphate buffer pH 6.7.
2. The next day thaw and resuspend 5 ml HeLa nuclear pellet in 15 ml buffer A.
3. Homogenize the suspension in a Dounce's homogenizer (Wheaton) with Pestle B for 30 min on ice.
4. Clarify the suspension at 14,000 rpm (25,000 × *g*) for 20 min at 4 °C.
5. Transfer the supernatant to a fresh tube and add 5 g (per 2.4 mg DNA) presoaked Hydroxyapatite BioGel HTP (Bio-Rad).
6. Incubate this at 4 °C for 3 h on an end-to-end rotor.
7. Wash the resin twice with 25 ml of buffer A with 630 mM NaCl on a centrifuge.
8. Pack the hydroxyapatite into a column and wash overnight with 500 ml–1 l buffer A containing 630 mM NaCl (*see* **Note 3**).
9. Elute the core histones in buffer B containing 2 M NaCl. Collect 20 fractions of 0.5 ml each.
10. To detect the presence of histones in the collected fractions, add 25 % trichloroacetic acid to a 20 μl aliquot of eluted protein and allow the precipitation to proceed for 30 min at 4 °C.
11. Centrifuge at 13,000 rpm (16,000 × *g*) at 4 °C for 30 min to collect the precipitate.
12. Wash once with prechilled acetone (~500 μl) and centrifuge again at 13,000 rpm (16,000 × *g*) at 4 °C for 30 min.
13. Discard the supernatant carefully and dry the pellet under vacuum in a Speed-Vac or let it air-dry.
14. Dissolve the resultant pellet in 10 μl of 1× SDS loading buffer and heat at 90 °C for 10 min.
15. Resolve the histones on a 15 % SDS–PAGE. Visualize the bands by staining with Coomassie brilliant blue solution.
16. Pool the elutions with similar concentration of protein and dialyze against dialysis buffer to remove imidazole.
17. Store as 50 μl aliquots in −80 °C (*see* Subheading 2.4) (Fig. 1b).

3.5 Purification of NPM1 and mNAP1

His_6-tagged recombinant NPM1 or mNAP1 is purified using Ni-NTA affinity chromatography followed by Anion exchange chromatography [2].

1. Transform BL21 (DE3) competent cells with either NPM1 or mNAP1 expression constructs and incubate the plate containing the appropriate antibiotic overnight in a 37 °C incubator.
2. The following evening inoculate a single colony from this plate into a 100 ml LB containing 50 μg/ml kanamycin or 100 μg/ml ampicillin and incubate the culture in a 37 °C shaker incubator at 180–200 rpm.
3. The next morning, transfer the 100 ml culture into a 2 l Erlenmeyer flask containing 900 ml of LB supplemented with 50 μg/ml kanamycin or 100 μg/ml ampicillin.
4. Incubate the culture in a 37 °C incubator under shaking conditions at 200 rpm until O.D reaches 0.5.
5. Induce the culture with IPTG to a final concentration of 0.5 mM and grow at 37 °C for 3 h.
6. Harvest the cells by centrifugation at 6,000 rpm (5,000 × *g*) for 10 min at 4 °C.
7. Resuspend the pellet in lysis buffer and sonicate with four burst of 30 s at a setting of 32 % amplitude (Sonics-Vibra cell).
8. Clarify the lysate by centrifuging at 16,000 rpm (30,000 × *g*) for 30 min at 4 °C.
9. Add Ni-NTA beads (pre-equilibrated with the lysis buffer) to the clarified lysate and incubate on an end-to-end rotor at 4 °C for 3 h.
10. Wash the resin five times with the lysis buffer and pack it into an Econo Fast column.
11. Elute the protein with the elution buffer (lysis buffer containing 0.25 M imidazole).
12. Check the eluted proteins by running on a 12 % SDS-PAGE gel.
13. Pool the elution fractions containing similar amount of protein and dialyze against BC0 for 6 h with at least two buffer changes to remove imidazole.
14. To the dialyzed protein, add Q sepharose matrix pre-equilibrated with BC0 and incubate in an end-to-end rotor for about 3 h at 4 °C.
15. Wash the resin thrice with BC0 and pack it into an Econo Fast column.
16. Elute the bound proteins by a salt gradient of 100–500 mM KCl.
17. Electrophorese about 3–5 μl of the elution fractions on a 12 % SDS-PAGE gel to check the purity of the fractions.

18. Pool the elution fractions containing similar amount of protein and dialyze against dialysis buffer containing 100 mM KCl for 6 h with at least two buffer changes.
19. Store at −80 °C in about 50 μl aliquots (*see* Subheading 2.5) (Fig. 1c, d).

3.6 Purification of Drosophila ACF Complex

1. Seed five, 150 mm dishes with 9×10^6 *Sf*21 cells per dish in 20 ml of TC 100 media supplemented with 1× penicillin–streptomycin–amphotericin B solution, 40 μg/ml gentamycin, and 10 % FBS.
2. Filter the dACF and dISWI viruses through a 0.22 μ filter and infect the cells at a multiplicity of infection (MOI) of three each.
3. Check the cells under the microscope after 48 h for visible signs of infection.
4. Harvest the cells after 60 h of infection by gentle scraping and centrifugation at 2,000 rpm (800 × *g*) for 10 min at 4 °C.
5. Wash the cells with 1× PBS and collect by centrifugation at 2,000 rpm (800 × *g*) for 5 min at 4 °C.
6. Resuspend the cell pellet in 8 ml Lysis buffer and homogenize in a Dounce homogenizer by applying ten strokes, three times with a gap of 10 min each at 4 °C.
7. Transfer the resulting homogenate to an oak-ridge tube along with 5 ml dilution buffer and centrifuge at 16,000 rpm (30,000 × *g*), for 30 min at 4 °C.
8. Meanwhile equilibrate 200 μl of anti-FLAG M2-agarose beads (SIGMA) with dilution buffer.
9. Add the equilibrated M2 agarose resin to the clarified homogenate and allow binding to proceed in an end-to-end rotor for 2.5 h at 4 °C.
10. After the incubation, collect the beads by centrifugation at 1,200 rpm (300 × *g*) for 10 min at 4 °C.
11. Wash the beads with 10 ml of wash buffer five times and finally collect the beads in a 1.5 ml Eppendorf tube.
12. Add 200 μl elution buffer to the beads and gently vortex the tube for 30 min for each elution.
13. Electrophorese about 5–10 μl of the elution fractions on a 10 % SDS-PAGE gel to check the purity of the fractions.
14. Store about 20 μl aliquots in −80 °C after flash freezing in Liquid N_2 (*see* Subheading 2.6) (Fig. 1e).

3.7 In Vitro Interaction of NPM1 with Core Histones

In vitro interaction assays are carried out using Ni-NTA pull-down method.

1. Mix 1 μg of the His_6-tagged protein (Full length NPM1) with 4.0 μg HeLa core histones or recombinant core histone octamer

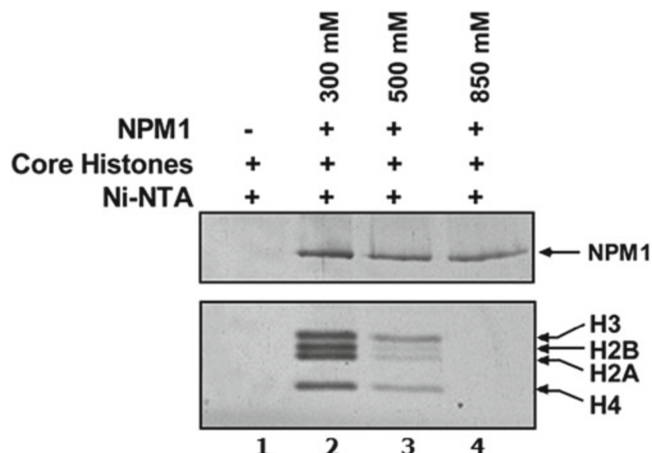

Fig. 2 In vitro interaction of NPM1 with HeLa core histones with increasing salt concentration: Ni-NTA pulldown assays with bead alone (*lane 1*); (*lanes 2–4*) 1 μg His_6-NPM1 incubated with 4 μg of HeLa core histones in presence of 300, 500, and 850 mM KCl concentration in the interaction buffer, respectively. The *top* and *bottom panel* is the Coomassie blue-stained gel showing the amounts of the pulled-down proteins

in the interaction buffer along with the Ni-NTA His-Bind Resin. (Reaction volume: 300 μl).

2. Incubate the mixture for 2 h at 4 °C on an end-to-end rotor.
3. Extensively wash the beads thrice with 1 ml interaction buffer.
4. Resolve the bead bound proteins on a 12 % SDS-PAGE gel (Fig. 2).
5. In order to achieve better sensitivity of this assay, the bound histone could also be subjected to western blotting analysis using highly specific antibodies against individual histones.

3.8 Histone Transfer Assay

The nucleosome assembly activity of NPM1 is assayed by histone transfer assay [15].

1. Relax 200 ng of covalently closed circular plasmid pG_5ML by pre-incubating with 40 ng dTopoI core catalytic domain in 1× assembly buffer at 37 °C for 40 min (*see* **Note 1**).
2. At the same time, separately incubate 5 pmol of NPM1 and 500 ng of core histones in 1× assembly buffer at 37 °C for 40 min.
3. Mix the two reactions together and further incubate at 37 °C for 40 min.
4. Stop the reaction by the addition of the proteinase K stop buffer.
5. At the end of the reaction, extract the plasmid by adding equal volume of phenol–chloroform–isoamyl alcohol (25:24:1)

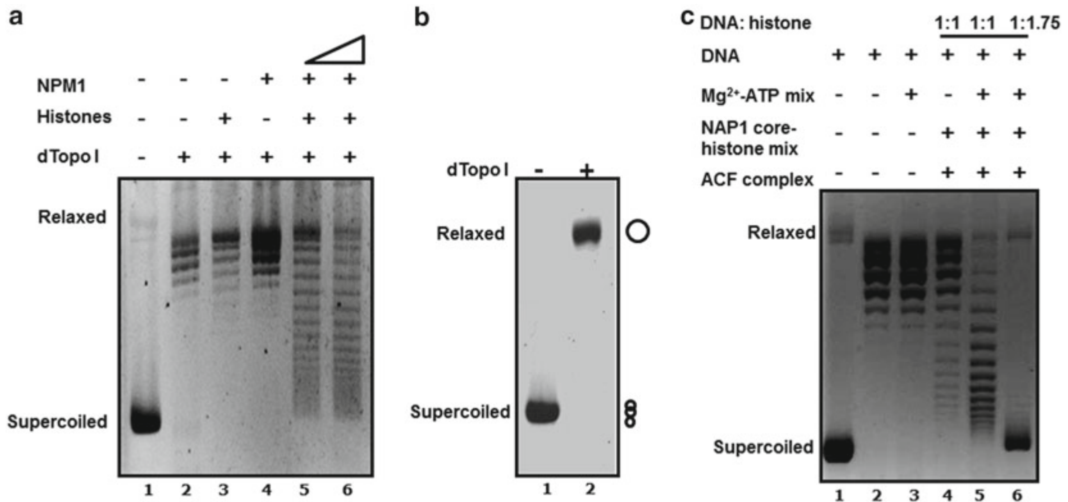

Fig. 3 (**a**) Histone transfer activity of NPM1 was tested with 500 ng of core histones and either 5 (*lane 5*) or 10 pmol of NPM1 (*lane 6*). *Lane 1*, supercoiled DNA; *lane 2*, DNA relaxed by dTopo I; *lane 3*, relaxed DNA with core histones; *lane 4*, relaxed DNA with 10 pmol NPM1; *lane 5* and *6*, relaxed DNA with core histones and 5 and 10 pmol of NPM1, respectively. (**b**) Profile of pG_5ML DNA relaxed by dTopo I (*lane 2*) and supercoiled DNA (*lane 1*). (**c**) Chromatin assembly on pG_5ML template mediated by ACF complex was checked by supercoiling assay. *Lane 1*, supercoiled DNA; *lane 2*, DNA relaxed by dTopo I; *lane 3*, relaxed DNA with Mg^{2+}-ATP mix; *lane 4*, relaxed DNA with NAP1-core histone mix and ACF complex and *lane 5*, relaxed DNA with NAP1-core histone mix and Mg^{2+}-ATP mix added in a DNA: histone ratio of 1:1 and 1:1.75 (*lane 6*)

pH 8.0 followed by precipitation with 0.25 M ammonium acetate and 2.5 volumes of 100 % ethanol at −80 °C for 1 h.

6. Resolve the topoisomers on a 1 % agarose gel (50 V for 12 h) and visualize by staining with ethidium bromide solution (0.5 μg/ml) (Fig. 3a).

3.9 Template Relaxation Assay

1. Incubate 6 μg of supercoiled pG_5ML array DNA with 40 ng dTopo I in 1× relaxation buffer at 37 °C for 2 h.
2. Extract the relaxed DNA with equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) pH 8.0.
3. Precipitate the DNA with one-tenth volume 3 M sodium acetate, pH 5.2 and 2.5 volumes of 100 % ethanol, at −80 °C for 1 h.
4. Dissolve in an appropriate volume of nuclease-free water to obtain a final concentration of 168 ng/μl.
5. Visualize the relaxed DNA on a 1 % agarose gel (run at 50 V for 12 h). Stain with ethidium bromide solution (0.5 μg/ml) (Fig. 3b).

3.10 Chromatin Assembly Using mNAP1

1. Incubate about 250 ng of HeLa core histones with 500 ng of mNAP1 in 22 μl 1× transcription assembly buffer at 37 °C for 15 min [12] (*see* **Note 4**).

2. Add 168 ng of relaxed pG_5ML array template into the reaction mixture and make up the volume to 24 μl using 2× transcription assembly buffer.
3. Incubate at 37 °C for 45 min.

The efficiency of chromatin assembly should be assessed by supercoiling assay and micrococcal nuclease digestion pattern.

3.11 Chromatin Assembly Using mNAP1 and ACF Complex

1. Prepare an NAP1–histone mix by adding 350 ng (3.5 μl) of HeLa core histones (diluted to 0.1 μg/μl in HEG buffer), 2.5 μg of mNAP1, and 1 μl of 500 mM KCl and make up the volume to 48 μl by adding HEG buffer [16].
2. Incubate this mixture on ice for 30 min.
3. Meanwhile prepare Mg^{2+}-ATP mix by adding 2.4 μl 0.1 M ATP, 3.4 μl 0.1 M $MgCl_2$, and 4.7 μl HEG buffer.
4. Prepare a Topoisomerase reaction mixture by adding 5 ng of dTopo I, 0.5 M creatine phosphate and creatine kinase.
5. Add 40 ng of ACF complex to the NAP-1 histone mixture at room temperature followed by addition of 10.5 μl Mg^{2+}-ATP mix, relaxed pG_5ML DNA (100–350 ng) and Topoisomerase mixture and make up the volume to 70 μl by adding HEG buffer (*see* **Note 5**).
6. Incubate the reaction mixture at 27 °C for 3–5 h to allow complete assembly.

The efficiency of chromatin assembly should be assessed by supercoiling assay and micrococcal nuclease digestion pattern.

3.12 Supercoiling Assay

1. After chromatin assembly add equal volume (70 μl) of MNase stop buffer to stop the reaction.
2. Add 50 μg of Proteinase K (2.5 μl from 20 mg/ml stock) and incubate at 37 °C for 1 h.
3. Extract the DNA with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) pH 8.0 followed by precipitation using 0.25 M ammonium acetate and 2.5 volumes of 100 % ethanol.
4. Wash the precipitated DNA with 500 μl of 70 % ethanol to remove the salt and dissolve it in 10 μl of nuclease-free water.
5. Electrophorese the DNA on a 1 % agarose gel in 1× TBE at 50 V for 12 h.
6. Visualize the bands after staining the gel in 0.5 μg/ml ethidium bromide solution (Fig. 3c).

3.13 Micrococcal Nuclease Digestion of Assembled Chromatin

1. Assemble about 1 μg of DNA into chromatin using either of the two methods and make up the volume to 250 μl using HEG buffer.

2. Add $CaCl_2$ to a final concentration of 2 mM and digest with MNase (*see* **Note 6**) at room temperature for 8 min.
3. Stop the reaction by adding 250 μl of MNase stop buffer and add 100 μg of Proteinase K. Incubate this mixture at 37 °C for 1 h.
4. Extract the DNA with equal volumes of phenol–chloroform–isoamyl alcohol (25:24:1) pH 8.0 followed by precipitation by adding 0.25 M ammonium acetate and 2.5 volumes of 100 % ethanol.
5. Wash the precipitated DNA with 500 μl of 70 % ethanol to remove the salt and dissolve it in 10 μl of nuclease-free water.
6. Electrophorese the DNA on a 1 % agarose gel in 1× TBE at 50 V for 12 h.
7. Visualize the bands after staining the gel with 0.5 μg/ml ethidium bromide solution.

3.14 Assessing Histone Acetylation on Chromatin Template

1. Assemble 168 ng of chromatin as mentioned in (*see* Subheading 3.9).
2. Incubate the chromatin in 1× HAT buffer and 180 ng Gal4-VP16 at 30 °C for 20 min.
3. Add 120 ng p300 and 1 ml of 2.5 Ci/mmol ^{3}H-acetyl CoA. Incubate at 30 °C for 15 min.
4. Precipitate the reaction products with 25 % trichloroacetic acid by keeping on ice for 30 min. Centrifuge at 13,000 rpm (16,000 × *g*) at 4 °C for 30 min.
5. Wash once with ice-cold acetone and centrifuge at 13,000 rpm (16,000 × *g*) at 4 °C for 30 min.
6. Pipette out the supernatant and dry the pellet in Speed-Vac.
7. Dissolve the pellet in 10 μl of 1× SDS loading buffer and heat at 90 °C for 10 min. Resolve on 12 % SDS polyacrylamide gel.
8. Visualize the bands by staining with Coomassie Brilliant Blue staining.
9. Equilibrate the gel in Me_2SO (dimethyl sulfoxide, DMSO) for 30 min twice.
10. Perform fluorography using a solution containing 22.5 % 2, 5-diphenyoxazole in Me_2SO for 45 min. Rinse the gel with double-distilled water for 2 h.
11. Vacuum-dry the gel at 60 °C for 90 min, perform autoradiography at −70 °C for 3 days.

3.15 In Vitro Transcription Assay [16]

1. 28 ng of DNA or equivalent amount of chromatinized template (assembled using either mNAP1 or mNAP1 and ACF complex) is incubated with 30 ng of Gal4-VP16 in 0.5× HAT buffer at 30 °C for 20 min (*see* **Note 7**).

2. Add 20 ng of p300 and 1.5 mM acetyl CoA and incubate at 30 °C for 15 min.
3. Optionally add 5 pmol of NPM1 to assess its remodeling or transcriptional coactivation activity.
4. Add 5 μl of HeLa nuclear extract (8.0 mg/ml protein in BC100). Add 0.25 mM DTT in the reaction and incubate at room temperature for 20 min (*see* **Note 5**).
5. Add 0.5 μl of RNaseOUT (Invitrogen), 2.5 μl of 20× NTP mix, and 15 μCi α^{32}P-UTP. Incubate at 30 °C for 45 min.
6. Add 1 μl of (20 U/μl) RNaseT1 (Sigma) and incubate at 30 °C for 20 min.
7. Add 200 μl transcription stop buffer and immediately extract the radiolabeled RNA transcript with 250 μl phenol–chloroform (acid equilibrated pH 4.7).
8. Centrifuge at 13,000 rpm (16,000 × *g*) at room temperature for 15 min.
9. Carefully recover the aqueous phase (230 μl) and precipitate the RNA by adding 23 μl of 3 M sodium acetate, pH 5.2, and 650 μl 100 % ethanol (v/v).
10. Mix briefly by vortexing and incubate at –80 °C for 1 h.
11. Centrifuge at 13,000 rpm (16,000 × *g*) at 4 °C for 30 min.
12. Immediately dry the RNA pellet in Speed-Vac. Dissolve the pellet in 10 μl transcription loading dye.
13. Electrophorese the radiolabeled transcripts on a 7 M urea–5 % polyacrylamide gel in 0.5× TBE for 2 h at 300 V (*see* **Note 8**).
14. Rinse the gel in distilled water for 15 min to remove urea and free ^{32}P-UTP. Vacuum-dry the gel at 80 °C for 40 min.
15. Autoradiograph the gel with an intensifying screen (expose for 12 h) and analyze on Fuji BAS phosphor-imager system (Fig. 4b, c).

4 Notes

1. The optimal salt concentration for the assay needs to be empirically determined.
2. For histone transfer assay any plasmid other than pG_5ML can be used. Presence of Nucleosome Positioning sequence is not essential for this assay. However, in order to assemble chromatin for acetylation-dependent in vitro chromatin transcription assays, a strong nucleosome positioning is important, especially when salt-dependent, NAP1-mediated [14] chromatin assembly is used to reconstitute the chromatin. Nucleosome positioning is facilitated by 5S rDNA sequences flanking the G-less cassette in pG_5ML plasmid.

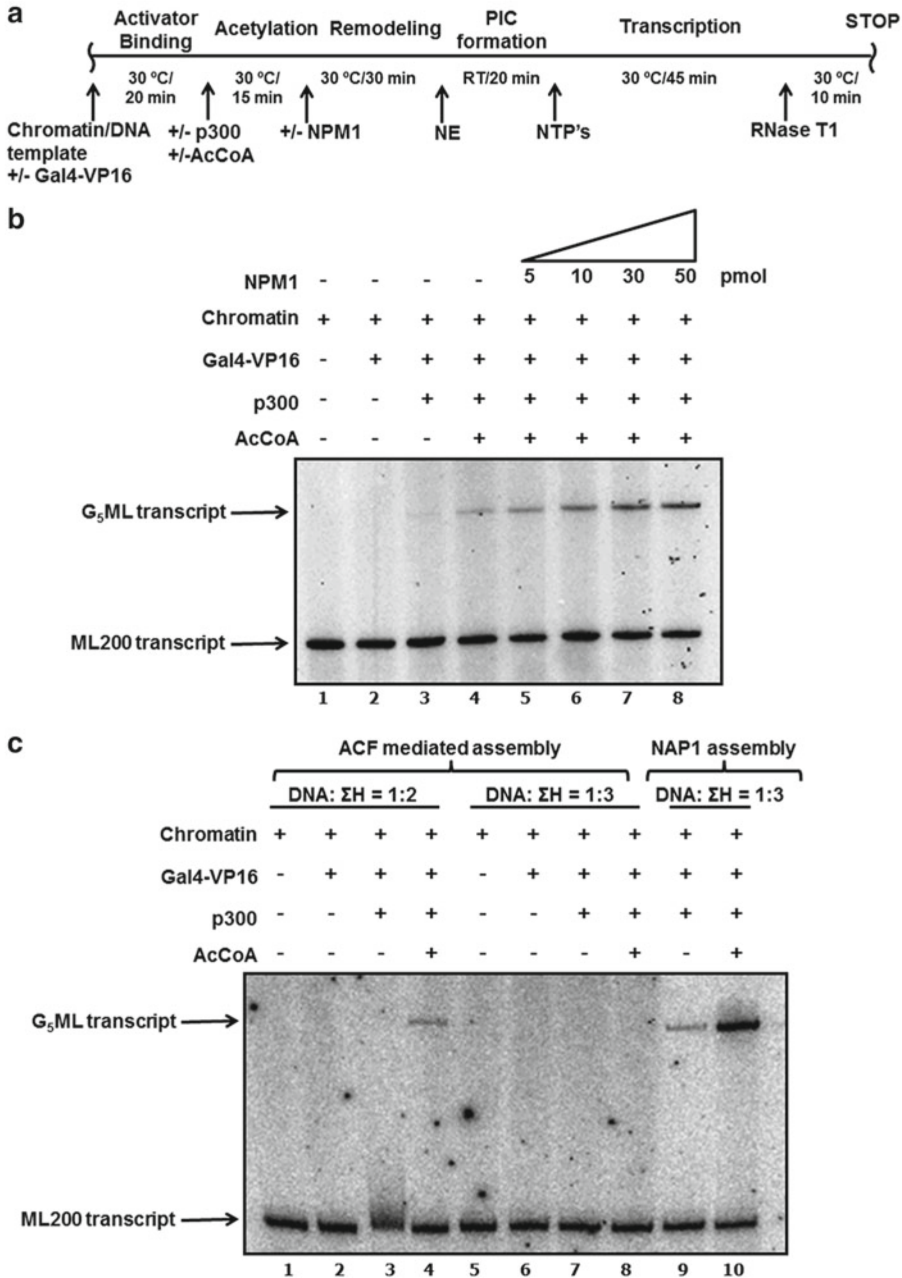

Fig. 4 (**a**) Schematic representation of in vitro transcription protocol. (**b**) 28 ng of freshly assembled chromatin was subjected to the protocol described in panel A with or without Gal4-VP16, full length p300 or 1.5 μM acetyl CoA and in the presence of 5, 10, 30, and 50 pmol of NPM1, respectively (*lanes 5–8*). (**c**) About 28 ng of ACF assembled chromatin (*lanes 1–8*) and NAP1 assembled chromatin (*lanes 9–10*) was subjected to the protocol described in panel A with or without Gal4-VP16, full length p300 or 1.5 μM acetyl CoA with DNA: histone ratio (DNA:ΣH) as indicated

3. During the washing step, when almost 150 ml of buffer A has been passed through the column, it is observed that there is a drastic reduction in the flow rate. In order to maintain the flow rate of the wash step a peristaltic pump may be set up at the rate of 25 ml/h. However, it is necessary to keep the peristaltic pump connected thereafter, until all the washes and elution steps are completed. It is essential to wash the column thoroughly with Buffer A containing 630 mM NaCl as this step ensures the removal of bound H1 from the hydroxyapatite column.
4. Ratio of mNAP1 and HeLa core histones should be standardized for proper assembly into chromatin. This method of assembly does not involve ATP-dependent remodeling and the template is amenable to further remodeling events by exogenously added chaperone. The chromatin template should be close to complete repression in absence of acetylation. Only from this kind of template can several fold activation be brought about by the histone chaperone. This step may need titration with different histone–DNA ratio for chromatin assembly. Chromatin assembly can also be done with reconstituted recombinant core octamer to rule out the effect of in vivo modifications present in HeLa purified core histones. Additionally chromatin assembled with reconstituted core octamer can be further modified by acetylation and methylation for in vitro chromatin transcription assay.
5. The Mg^{2+}-ATP mix and the Topoisomerase reaction mixture must be prepared freshly just before use. It is necessary to bring the NAP-1 histone mixture to room temperature before adding ACF complex. The DNA: histone ratio should be titrated for every new batch of histone octamer/HeLa core histones and relaxed pG_5ML DNA and the efficiency of assembly checked by supercoiling assay and MNase digestion. The ACF assembled chromatin can be used to study chaperone-mediated transcriptional activation.
6. The working concentration (Enzyme activity units) of MNase should be empirically determined by a titration experiment.
7. It is important to gently tap and give a brief spin after each step of transcription protocol. However, never spin down the transcription reaction mixture after addition of HeLa nuclear extract, mix by tapping gently five to six times.
8. To prepare 7 M urea–5 % polyacrylamide gel, slowly dissolve 21 g of urea in 12.5 ml 2× TBE, 6.25 ml of 40 % acrylamide, and 6.25 ml double-distilled water. Finally make up the volume to 50 ml with double-distilled water.

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Chapter 23

Preparation of Mononucleosomal Templates for Analysis of Transcription with RNA Polymerase Using spFRET

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Abstract

Single positioned nucleosomes have been extensively employed as simple model experimental systems for analysis of various intranuclear processes. Here we describe an experimental system containing positioned mononucleosomes allowing transcription by various RNA polymerases. Each DNA template contains a pair of fluorescent labels (Cy3 and Cy5) allowing measuring relative distances between the neighboring coils of nucleosomal DNA using Forster resonance energy transfer (FRET). The single-particle FRET (spFRET) approach for analysis of DNA uncoiling from the histone octamer during transcription through chromatin is described in detail.

Key words Nucleosomes, Chromatin, Transcription, Assembly, Methods, spFRET

1 Introduction

All vital biological intranuclear processes (e.g., DNA replication, repair, recombination, and transcription) occur on DNA organized in chromatin. At the first level of chromatin folding, 147-bp DNA regions are organized in 1¾ superhelical coils on the surface of the histone octamer forming nucleosome cores [1]. Short DNA fragments (150–350 bp) containing single nucleosomes have been used recently for analysis of transcription of nucleosomal templates [2–5] and many other intranuclear processes, including DNA repair in chromatin [6, 7], ATP-dependent chromatin remodeling [8, 9], and analysis of nucleosome structure and thermodynamics [10–13]. In many cases the model templates faithfully recapitulate important aspects of these processes [2, 3, 6, 9, 14, 15]. At the same time, these simple experimental systems can be analyzed with a high resolution. Positioning and histone composition of mononucleosomes

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that are often changed during various processes can be easily monitored by analysis in a native gel [16–19]. Thus, analysis of protein binding to nucleosomes and protein-induced changes in the conformation and/or structure of the complexes are relatively straightforward using mononucleosomal templates.

In the majority of recent studies uniquely positioned nucleosomes were assembled on a DNA fragment containing one of the strong nucleosome-positioning DNA sequences [20, 21]. We routinely use two methods of nucleosome assembly: by dialysis of purified DNA and core histones from 2 M NaCl [22] or by transfer of the histone octamer from “donor” chromatin onto DNA [23]. To avoid any traces of the donor chromatin in the resulting nucleosome preparation, we include an additional step of purification of the templates on Ni-NTA beads [4]. In both protocols DNA or donor chromatin is present in an excess, allowing formation of nucleosomes only on the high-affinity DNA sequences.

Advanced insight into nucleosome structure and dynamics during various intranuclear processes can be achieved with modern experimental approaches based on single particle Forster resonance energy transfer (spFRET) analysis [24, 25]. Thus, spFRET analysis is informative for analysis of alterations in nucleosome structure induced by modification (methylation, acetylation) of DNA or histones [24, 26–28]. spFRET records data from thousands nucleosomes, and each nucleosome is characterized individually. This allows study of structurally different subpopulations of nucleosomes that are often present during various processes of chromatin metabolism.

Here we describe the application of spFRET to analysis of DNA uncoiling from the histone octamer during transcription through chromatin. spFRET is a method of choice because of a large variety and high heterogeneity of chromatin states formed during transcription. We briefly describe basic principles of spFRET and present protocols for preparation of fluorescently labeled nucleosomes and stalled RNAP complexes for spFRET analysis.

2 Materials

2.1 Miscellaneous Items

1. Low adhesion tubes (USA Scientific).
2. Gel extraction kit (Omega Bio-Tek).
3. SpinX centrifuge tube filter (Amicon).
4. Dialysis membranes (Spectra/Por; molecular weight cutoff of 8,000 and 12,000–14,000).
5. Ni-NTA agarose (Qiagen).
6. 8-well Lab-Tek chambered cover glasses (Thermo Scientific).

2.2 Enzymes

1. Restriction enzymes (New England Biolabs, NEB).
2. T4 DNA ligase (NEB).
3. Taq DNA polymerase (NEB).
4. RNA polymerase from *Escherichia coli*.

2.3 Buffers and Solutions

1. TAE buffer: 0.04 M Tris–acetate, pH 8.0, and 1 mM EDTA.
2. TE buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
3. TB (transcription buffer): 20 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and indicated concentration of KCl, mM.
4. EDTA stock solution: 0.5 M Na_2–EDTA, pH 8.0.
5. RLB (RNA loading buffer): 95 % formamide, 10 mM EDTA, 0.1 % SDS, and 0.01 % of each bromophenol blue and xylene cyanol dyes.
6. 2× agarose loading buffer with 8 M urea: 45 mM Tris base, 45 mM Boric Acid, 8 M Urea, 0.01 % bromophenol blue, 0.01 % xylene cyanol, 1 mM EDTA.
7. Annealing buffer: 10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA pH 7.5.
8. TB-PEG buffer for spFRET measurements: TB buffer supplemented with 0.1 % poly(ethylene glycol) (MW 380–420 Da, Aldrich).
9. CRB 1–4 (Core Reconstitution Buffers): All four buffers contain HE, 5 mM β-mercaptoethanol, 0.1 % NP-40 and NaCl at the following concentrations: Buffer 1—1 M; 2—0.8 M; 3—0.6 M; 4—0.01 M.

3 Methods

3.1 Selecting Positions of Fluorophores on the Nucleosomal DNA

FRET arises between two specially selected fluorophores, donor and acceptor, when the distance between them is ca. 10 nm or less. Donor excitation induces a donor fluorescence emission when the donor is far from an acceptor, or an acceptor emission when the donor is close to the acceptor (Fig. 1a, b). The efficiency of FRET (E) is related to the distance r between donor and acceptor according to the formula:

$$E = 1 / \left[1 + (r / R_0)^6\right], \quad (1)$$

where R_0 is the Forster radius. For example, R_0 for fluorophores Cy3 (donor) and Cy5 (acceptor) (labels that are often used in spFRET) is of ca. 60 Å [29], and parameter E is a reliable reporter of changes in the distance between Cy3 and Cy5 in the range of 3–10 nm.

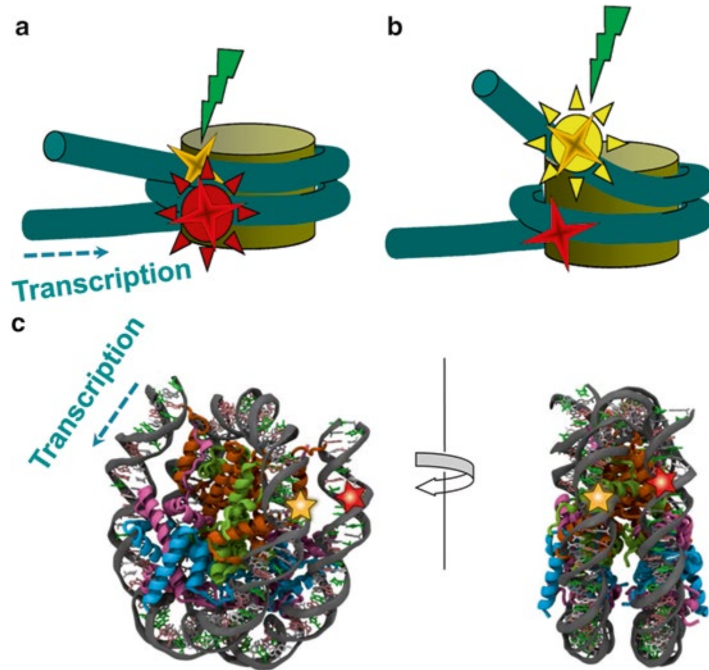

Fig. 1 Experimental system for analysis of DNA uncoiling from the histone octamer. Fluorescent donor (*yellow star*) and acceptor (*red star*) (Cy3 and Cy5 fluorophores, respectively) in a mononucleosome are shown. The direction of transcription is shown by *dashed arrow*. (**a**) A donor is close to an acceptor, and donor excitation results in acceptor emission (high FRET efficiency). (**b**) A donor is far from an acceptor, and donor excitation results in donor emission (no FRET). (**c**) Thymidines in positions +56 and +135 from the promoter-proximal boundary of the 603 s nucleosome can be labeled with Cy3 and Cy5, respectively, to have inter-label distance of approximately 1.9 nm

For spFRET analysis fluorescent labels (one donor and one acceptor) are introduced in the neighboring superhelical coils of DNA on a nucleosome. A distance between labels should be $\leq R_0$. In a case of the 603 DNA template used in our study the labels were incorporated in the positions +56 and +135 bp from the promoter-proximal boundary of the nucleosome (Fig. 1c).

Choice of these positions for DNA labeling is based on the following considerations. According to the structure of the nucleosome assembled on a high-affinity DNA sequence [13], both probes are incorporated into nucleosomal DNA in positions where DNA helix faces solution and the probes are in a close proximity to each other (Fig. 1c). This positioning minimizes the probability of interaction between the fluorescent probes and histones and maximizes the FRET signal characteristic for the intact nucleosome.

In the example described here the probes were incorporated to monitor uncoiling of the promoter-distal region of nucleosomal DNA; however they can be incorporated into different positions on nucleosomal DNA to monitor uncoiling of other DNA regions from the histone octamer.

3.2 Assembly of Mononucleosomal Templates Containing Cy5/Cy3 Probes for In Vitro Transcription

In the protocol described below nucleosomal DNA is obtained after annealing and ligation of overlapping DNA oligonucleotides. Alternatively, nucleosomal DNA can be obtained by PCR using shorter DNA oligonucleotides. DNA fragment containing the T7A1 promoter is amplified separately using a pair of primers. After digestion of both nucleosomal and promoter DNA with *Tsp*RI, the promoter fragment is ligated to the 147-bp 603 template through complementary *Tsp*RI sticky ends [30, 31], and the ligated template is gel-purified.

3.2.1 Purification of Oligonucleotides with Cy5/Cy3 Distal Probes

1. Add 45 μl of water and 75 μl of 2× agarose loading buffer with 8 M urea to 30 μl of 100 μM solutions of the following oligonucleotides (*see* **Note 1**):

   6035AFP 5′-CCCGGTTCGCGCGCCCGCCTTCCGTGTG TTGTCTCCTCTCGG 3DW_Cy3_603-5A2 5′ GCGT CTAAGTACGCTTAGCGCACGGTAGAGC GCAATCCAAGGCTAACC

   ACCGTGCATCGATGTTGAAAGAGGCCCTCCGTCCTT ATTACTTCAAGTCCCTGG GGT 5DW_Cy5_603-5A2

   5′-ACCCCAGGGACTTGAAGTAATAAGGACGGAGG GCCTCTTTCAACATCG

   ATGCACGGTGGTTAGCCTTGGATTGCGCTCTAC CGTGCGCTAAGCGTA

   6035ARP

   5′-CTTAGACGCCCGAGTGACGACTTCACTCGGC AGGCGGGCGCGCGAACCGGG CCCAGTGCC
2. Heat samples at 95 °C for 5 min and separate DNA fragments by denaturing PAGE [2].
3. Cut pieces of the gel, containing primers, and transfer them to 500 μl tubes with a hole in the bottom.
4. Insert tubes into the 1.5 ml tubes and spin on Eppendorf microcentrifuge at 16,000 × *g* for 10 s.
5. Add 5 volumes of TE buffer to the samples after centrifugation and incubate overnight at 4 °C.
6. Collect supernatant by filtering through SpinX centrifuge tube filter by centrifugation at 16,000 × *g* on Eppendorf microcentrifuge for 10 min.

7. Precipitate DNA by isopropanol, wash with 70 % ethanol, and dissolve pellets in 100 μl of TE buffer.
8. Estimate DNA concentration by measuring absorption (A) at the wavelength 260 nm.

3.2.2 Assembly of DNA Template Containing Cy5/Cy3 Probes

1. Mix 2 primers: 3DW_Cy3_603-5A2 and 5DW_Cy5_603-5A2 in equimolar amounts in the annealing buffer, heat at 95 °C for 3 min, and slowly cool down from 95 to 16 °C during 2 h.
2. Extract the annealed product by phenol, then precipitate with ethanol and wash with 70 % ethanol.
3. Dissolve pellet in the annealing buffer and then add a mixture of 6035AFP and 6035ARP primers in equimolar amounts.
4. Heat the mixture of four primers to 45 °C and slowly cool down from 45 to 16 °C during 1 h.
5. Extract the annealed product by phenol, precipitate with ethanol and wash with 70 % ethanol.
6. Dissolve pellet in 15 μl of 1× T4 ligase buffer (NEB), add 4 μl of T4 ligase and incubate overnight at 16 °C.
7. Cut the promoter and the nucleosomal Cy5/Cy3-labeled fragments by *Tsp*RI enzyme in NEB4 buffer (New England Biolabs) for 3 h at 65 °C.
8. Resolve the obtained fragments in a 1.5 % (w/v) agarose gel containing 4 M urea, 0.5 mg/ml ethidium bromide and 0.5× TBE buffer at 4–6 V/cm for 1.5–2 h.
9. Excise the ~140-bp promoter fragment and the ~150-bp nucleosomal fragment avoiding UV illumination (*see* **Note 1**).
10. Purify both fragments by gel extraction kit (Omega Bio-Tek) according to the manufacturer's instructions.
11. Ligate 1–2 μg of the purified promoter DNA fragment [2] and the nucleosomal Cy5/Cy3-labeled fragments at molar ratio 1.15:1 in T4 ligase buffer for 1.5–2 h at room temperature.
12. Resolve the ligated from non-ligated contaminant fragment in a 1.8 % (w/v) agarose gel containing 0.5 mg/ml ethidium bromide and 0.5× TBE buffer at 4–6 V/cm for 2–2.5 h depending on the resolution required for clear band separation.
13. Excise the desired ligated ~300-bp fragment avoiding UV illumination (*see* **Note 1**).
14. Purify the ligated ~300-bp fragment by Gel Extraction kit (Omega Bio-Tek) according to the manufacturer's instructions.
15. Measure DNA concentration on A_{260} wavelength, using $A_{260} = 20$ for 1 mg/ml DNA.

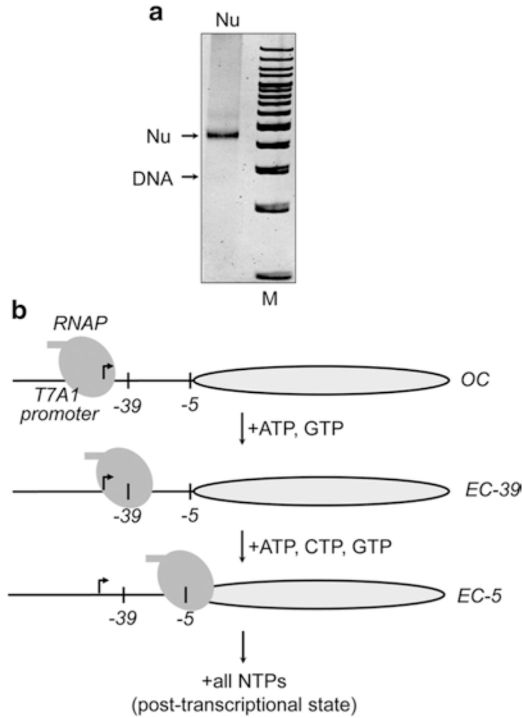

Fig. 2 The experimental approach for stalling RNAP at unique positions on the 603 nucleosome. (**a**) The sequence of the 603 template allows formation of the open initiation complex (OC) and stalling of RNAP ECs at the −5 or −39 positions (relative to the promoter-proximal nucleosome boundary) upon addition of different partial combinations of NTPs. Corresponding complexes are characterized and analyzed using DNase I footprinting or spFRET. (**b**) Analysis of 603 mononucleosomal template containing Cy5/Cy3 fluorophores by native PAGE. *Arrows* indicate positions of DNA and the nucleosome (Nu). M—100-bp DNA ladder (Fermentas). The gel was stained with ethidium bromide

3.2.3 Nucleosome Assembly

Nucleosomes are assembled on the template described above by dialysis against decreasing concentrations of NaCl and using long chromatin without histone H1 as a donor of the histone octamer (Fig. 2a).

1. Cool 500 ml each of CRB1 to CRB4 buffers to 4 °C.
2. Mix 1–3 μg of the DNA fragment with long –H1/H5 donor chromatin at a ratio of 1:5 (w:w) in 0.04–0.1 ml of CRB1 buffer.
3. Dialyze successively against CRB2 and CRB3, each for 2 h at 4 °C. Then dialyze the sample against CRB4 for 3 h or overnight.
4. Transfer the reconstitute to a siliconized Eppendorf tube and store at 4 °C (do not freeze).

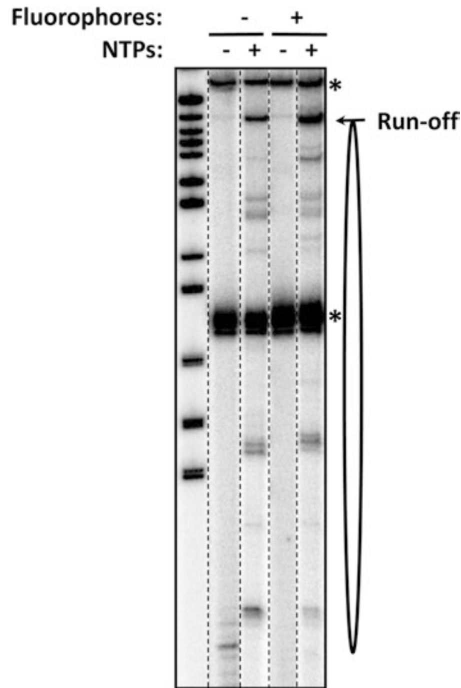

Fig. 3 Analysis of transcription through 603 nucleosome containing intact and fluorophore-labeled DNA. Transcription by the RNAP was conducted in the presence of NTPs for 30 s at 150 mM KCl. The locations of the 603 nucleosome (*oval*), labeled DNA (*asterisk*) and the run-off transcript are shown. No additional pausing was detected on fluorophore-labeled DNA, suggesting that fluorophores do not interfere with progression of the enzyme

5. Check the samples by analysis by native PAGE (see below).
6. Utilize the prepared mononucleosomal templates for the transcription experiments in vitro described below.

3.3 Preparation of Stalled Nucleosome Complexes with RNAP: Open Complex and Elongation Complexes Stalled at the Positions −39 and −5

The "minimal" 603 nucleosomal template for transcription by the RNAP contains a strong T7A1 promoter region with the start site localized 50 bp upstream of promoter-proximal boundary of one of the strong 147-bp 603 nucleosome-positioning sequences [30]. Nucleosome positions on these templates are unique and were mapped with high resolution [32]. The sequence of the template is designed to allow stalling of RNAP at different locations along the templates [30]. The 603 template described here as an example; it allows stalling at the positions −39 and −5 (the numbers indicate positions of the active center of RNAP relative to promoter-proximal nucleosome boundary) using different combinations of NTPs (Fig. 2b [31, 33]).

1. All protein and DNA-protein complexes (*E. coli* RNAP and – H1 donor chromatin) were purified as described [2].
2. To form active initiation complexes, incubate 200 ng of nucleosomal templates or histone-free DNA with fivefold

molar excess of the RNAP in 20 μl of the TB buffer containing 40 mM KCl (TB40) at 37 °C for 10 min.

3. Take a small aliquot (2–3 μl) for FRET measurements (it contains the open complex, OC).
4. Add ApUpC RNA primer to 20 μM, and ATP and GTP to 1 μM to allow formation of the 11-nt labeled RNA transcript (EC-39 complex where the active center of the enzyme is positioned 39 bp upstream of promoter-proximal nucleosomal boundary) at 37 °C for 10 min.
5. Add 20 μl of Ni^{2+}-NTA agarose beads (50 % suspension in alcohol) that have been previously washed three times with 0.5 ml of TB40, incubated in the presence of 0.5 mg/ml of acetylated BSA (Sigma-Aldrich) for 10 min, and washed two times with 0.5 ml TB40.
6. Incubate obtained EC-39 with Ni^{2+}-NTA agarose beads at room temperature for 5 min with gentle pipetting every 40–50 s.
7. Spin the samples at 240×*g* for 1 min at room temperature. Carefully take the supernatant.
8. Wash beads with the immobilized EC-39 complexes once with TB40, twice with TB300 and twice with TB40. Centrifuge after every wash on Eppendorf microcentrifuge at 240×*g* for 1 min at room temperature.
9. Incubate the washed beads in 30 μl of TB150 buffer with 100 mM imidazole at room temperature for 5 min with gentle pipetting every 30–40 s.
10. Centrifuge on Eppendorf microcentrifuge at 240×*g* for 1 min at room temperature. Carefully collect the supernatant.
11. Take a small aliquot (3–4 μl) for FRET measurements; it contains the elongation complex stalled at −39 position (EC-39).
12. Adjust volume to 20 μl. Incubate EC-39 with 1 μM CTP, 20 mg/ml rifampicin, 0.5 mg/ml acetylated BSA, 150 mM KCl at 37 °C for 10 min to advance EC-39 to form EC-5 at the end of the −T DNA track on the upper DNA strand.
13. Take a small aliquot (3–4 μl) for FRET measurements; it contains the elongation complex stalled at −5 position (EC-5).
14. Add 4 mM rNTPs to 100 mM final concentration to start the chase reaction, incubate at room temperature for 10 min. The resulting reaction mixture contains post chase sample for FRET measurements (*see* **Note 2**).

3.4 spFRET Measurements

spFRET analysis is performed in a diluted solution of nucleosomes or stalled elongation complexes using an experimental installation with key modules and elements shown in Fig. 4. Besides home-built experimental installations [24, 34, 35] any commercially available spectrometer for fluorescence correlation spectroscopy

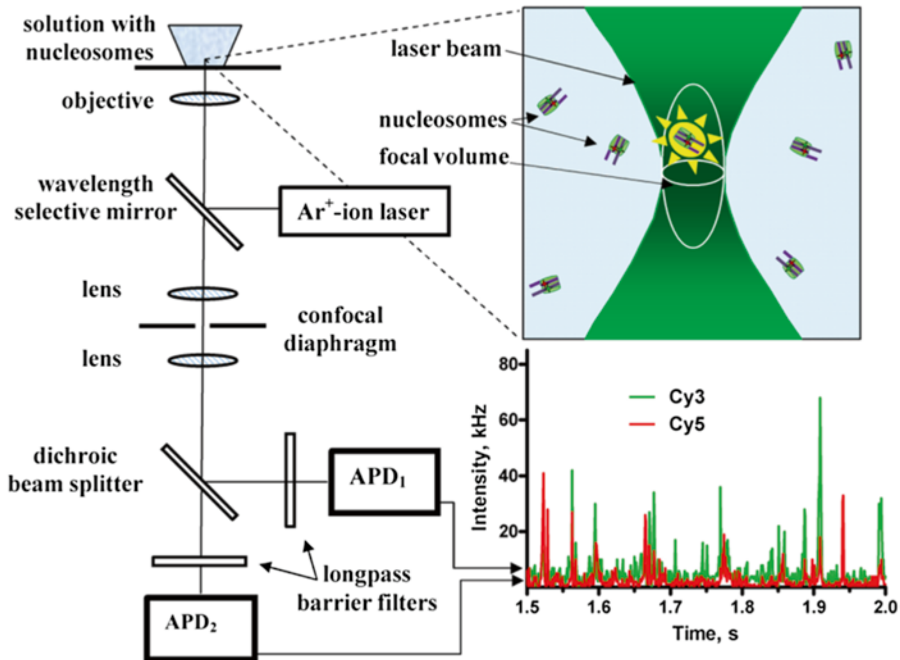

Fig. 4 Experimental setup for the spFRET study of freely diffusing nucleosomes and their complexes. Laser beam is directed to specimen through the objective using a wavelength selective mirror, which reflects light with the laser emission wavelength and transmit other wavelengths. Fluorescence of a specimen collected with the objective is filtered with the confocal diaphragm to reject signals coming from specimen layers situated above and below the focal point. As a result, the confocal diaphragm transmits a specimen signal coming from an ellipsoidal focal volume with main axes of ca. 0.3 and 1.5 μm (*see insert at the top right* showing the principle of single particle detection). Freely diffusing single nucleosomes labeled with donor and acceptor fluorophores pass through the focused laser beam (shown by *green color*), where their fluorescence is excited (shown by *yellow star*). Spatially filtered fluorescence is separated into two spectral parts (corresponding to donor and acceptor fluorescence) with the dichroic beam splitter, additionally spectrally filtrated with the longpass barrier filters (or band-pass filters) and registered with avalanche-photodiodes (APD). *Insert at the bottom right*: an example of fluorescence intensity dependences on time measured with two APDs. *Green* and *red traces* describe bursts of donor and acceptor fluorescence intensities, respectively, which arise when single nucleosomes diffuse through the focal volume. Intensities of these bursts are used to calculate FRET efficiencies (E) for each measured nucleosome

(FCS) or confocal microscope with the module for FCS can be used for spFRET measurements. FCS modules are usually equipped with avalanche-photodiodes (APDs), which surpass conventional photomultipliers installed in commercial confocal microscopes in sensitivity and suit ideally for spFRET measurements.

We utilize the Confocor-3 module of the LSM710-Confocor3 laser scanning confocal microscope (Zeiss, Germany) to measure freely diffusing single nucleosomes and their complexes with RNAP. Measurements are performed using the C-Apochromat water-immersion 40× objective with the 1.2 numerical aperture (*see* **Note 3**). Fluorescence is excited with Ar^+-ion laser using the 514.5 nm wavelength, allowing efficient absorption by Cy3. Intense absorption

by Cy3 at the excitation wavelength allows reducing laser power to 2 μW (*see* **Note 4**). In our measurements a confocal diaphragm aperture is equal to the diameter of Airy disk in the projection of fluorescence emitting point object on the diaphragm. We utilize the 635 nm dichroic beam splitter and the 530 and 580 nm longpass barrier filters (Fig. 4) from a set of standard filters installed in Confocor3. Accordingly, fluorescent signals of Cy3 and Cy5 are collected within the 530–635 and 635–800 nm wavelength ranges, respectively.

1. To study freely diffusing nucleosomes and their complexes with RNAP using spFRET technique, specimens are diluted with TB-PEG buffer to 0.1–0.3 nM in order to have concentration of 0.1–0.3 particles per the focal volume (ca. 1 fL) (*see* **Note 5**).
2. Initiate measurement of Cy3 and Cy5 fluorescence intensities as a function of time (Fig. 4) (*see* **Note 6**).
3. Save the result of a measurement into a file.
4. Discriminate signals associated with nucleosomes, which are observed as a set of sharp spikes against a low intensity background in the measured traces (Fig. 4). Sharp fluctuations of signal intensity are assigned to fluorescence of single nucleosomes when they meet to the following threshold criterion: either Cy3- or Cy5-signal intensity is 5–10 times higher than the corresponding background level. Very intense signals should be rejected also, since they correspond to several nucleosomes which accidentally diffuse through the focal volume simultaneously (*see* **Note 7**).
5. Create a new file, where each identified nucleosome is characterized by the registered fluorescence intensities of Cy3 and Cy5.
6. Recalculate the set of Cy3 and Cy5 signals assigned to single nucleosomes into a set of FRET efficiencies (E) with a formula

$$E = \left(I_{\text{Aa}} - \alpha \times I_{\text{Dd}}\right) / \left(I_{\text{Aa}} + \left(1 - \alpha\right) \times I_{\text{Dd}}\right), \quad (2)$$

where I_{Aa} is intensity of Cy5 fluorescence in the acceptor channel, I_{Dd} is intensity of donor fluorescence in the donor channel (both corrected for background contribution), α is the contribution of Cy3 fluorescence in the channel for Cy5 detection (spectral cross talk) calculated as

$$\alpha = I_{\text{Da}} / I_{\text{Dd}}, \quad (3)$$

where I_{Da} is intensity of Cy3 fluorescence in the Cy5 detection channel corrected for background contribution (*see* **Note 8**).
7. Present the sets of FRET efficiencies calculated for the measured nucleosome samplings graphically as frequency distribution histograms and fit them with a sum of bands having a Gaussian shape (Fig. 5) (*see* **Note 9**).

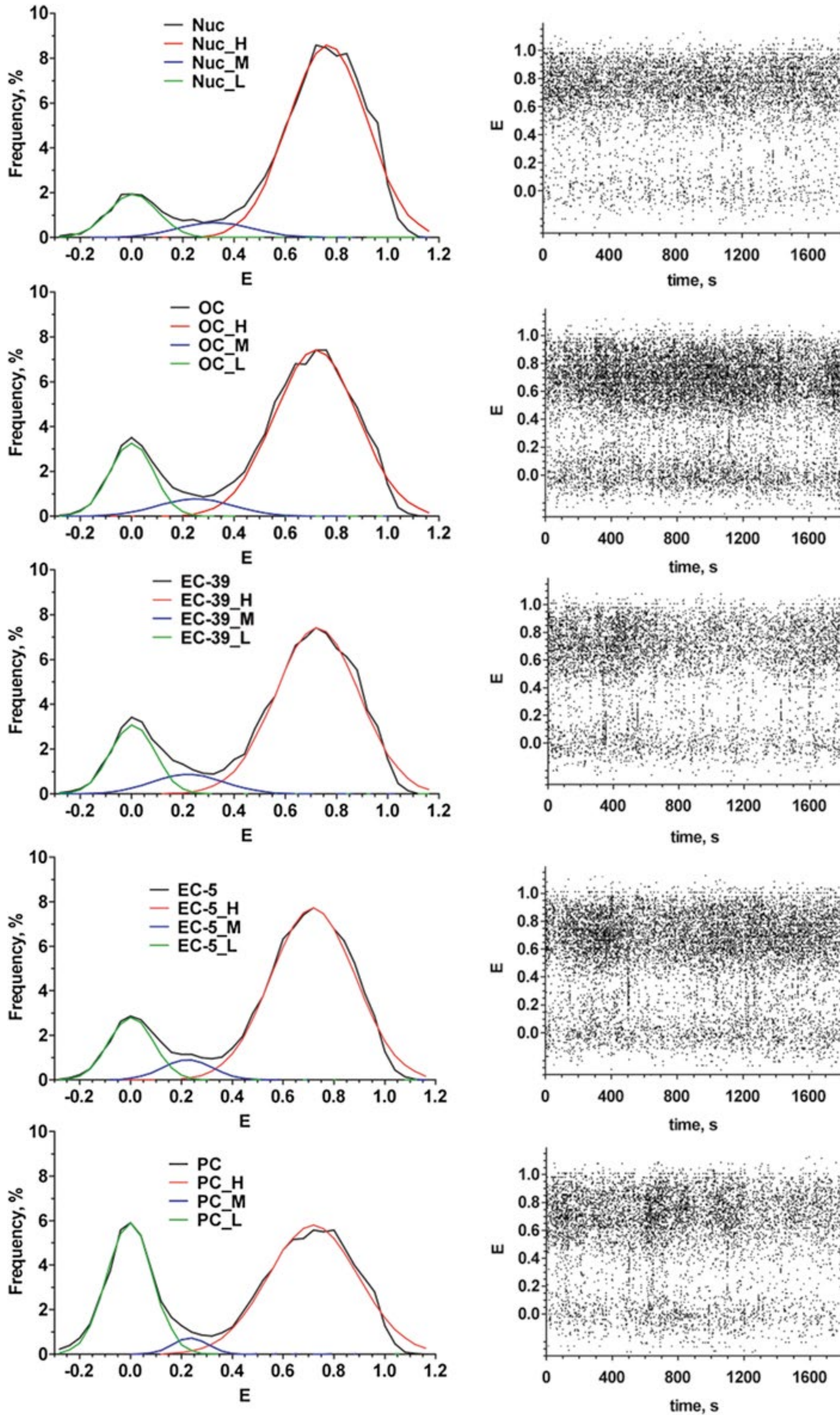

Fig. 5 spFRET analysis of nucleosomes and their complexes with stalled RNAP. *Left column*—frequency histograms of FRET efficiency (*E*) for nucleosomes (Nuc), open complex (OC), elongation complexes −39 (EC-39), −5 (EC-5), and post chase state (PC). *Colored plots* are the result of histogram deconvolution into three states:

4 Notes

1. All operations with fluorescently labeled oligonucleotides should be accompanied by minimal exposure to visible and UV light because the fluorophores are highly sensitive to light. The oligonucleotides 3DW_Cy3_603-5A2 and 5DW_Cy5_603-5A2 were labeled by Cy3 and Cy5 at the positions 15 and 13, respectively.
2. The labeled RNA present in the sample should be characterized before and after transcription by denaturing PAGE (Fig. 3) to make sure that transcription is completed on the majority of the transcribed templates.
3. High numerical aperture of the objective is required to reduce the focal volume from which the fluorescent signal is recorded. Smaller focal volume enables higher concentration of nucleosomes to be used for spFRET analysis, which in turn promotes nucleosome structure stability. Signal intensity collected from a single nucleosome is proportional to the fourth power of the objective numerical aperture.
4. The lower laser power is important to diminish interference of Rayleigh scattering with Cy3 fluorescence and reduce probability of Cy5 photobleaching. High laser power can distort the E profile in frequency diagrams. This distortion is observed as a laser power-dependent shift of a high FRET value in an E-histogram to lower values.
5. Dilution should be performed directly in the well of a chambered cover glass to minimize the loss of nucleosomes because of their adsorption to the walls of the reaction tube. At the recommended concentration nucleosomes diffuse through the focal volume one by one, and signals from single nucleosomes coming to APDs are well separated in time (Fig. 1). Higher concentrations of nucleosomes result in an increase of the background signal and thus affect accuracy of FRET calculations. At lower concentrations the fraction of nucleosomes that are adsorbed on well walls and does not participate in the measurement is considerably increased.
6. It is recommended to perform recording of the signal as a function of time with 3–5 ms dwell time. Dwell time is selected to be roughly equal to an average time of nucleosome diffusion through the focal volume. Total measurement time is restricted by nucleosome structure stability and Cy5 photostability.

Fig. 5 (continued) high FRET (H), middle FRET (M), and low FRET (L). *Right column*—time courses of FRET for nucleosomes and stalled complexes shown on the *left*. *Dots* represent *E* values measured for each single nucleosome (stalled complex) diffused through the focal volume in different moments of an observation period. High structural stability is an obvious property of the nucleosomes and stalled complexes

Under the described conditions measurements can be performed during at least 30 min (Fig. 5). Measurements during 10 min are sufficient to provide statistically reliable sampling (4,000–8,000 particles).

7. In our case, fluorescence spikes are assigned to single nucleosomes when either Cy3- or Cy5-dependent signal intensities are higher than 10 or 5 kHz, respectively, and lower than 80 kHz. Signals having intensity higher than 80 kHz originate from two or sometimes several nucleosomes diffusing through the focal volume simultaneously. Spikes with intensities lower 10 and 5 kHz are enriched with Rayleigh scattering spikes and/or noise. Typical intensities of the background signals are ca. 0.9–1.1 and 0.4–0.6 kHz for Cy3 and Cy5 channels, respectively.
8. Fluorescence spectra of Cy3 and Cy5 overlap in the 635–700 nm region. Therefore fluorescence of Cy3 contributes to the measured signal of Cy5, and this contribution should be taken into account. Under our experimental conditions the spectral cross-talk α is equal to 0.19. Spectral cross-talk for Cy5 fluorescence in the 530–635 nm region as well as direct Cy5 excitation with the 514.5 nm wavelength are negligible.
9. Analysis of E histograms shows that both nucleosomes and stalled complexes OC, EC-39, and EC-5 contain three subpopulations of particles which differ in E (Fig. 5 and Table 1). These subpopulations can be described by E-values that correspond to the maxima of Gaussian bands utilized to decompose the histograms into particular states. The main subpopulation of particles (76–82 %) is characterized by high FRET (high average E value): 0.77 for nucleosomes and 0.72 for OC, EC-39, and EC-5 (Table 1). These particles can be assigned to intact nucleosomes with the promoter-distal DNA (containing Cy3 and Cy5 labels) fully

Table 1
Efficiencies of FRET and relative fractions (in parentheses) for three subpopulations (low-FRET, middle-FRET, and high-FRET) observed in nucleosomes and their complexes with RNAP

	Low-FRET	Middle-FRET	High-FRET
Nuc[a]	0.00[b] (12 %)	0.32[b] (6 %)	0.77[b] (82 %)
OC	0.00 (18 %)	0.25 (6 %)	0.72 (76 %)
EC-39	0.00 (17 %)	0.22 (7 %)	0.72 (76 %)
EC-5	0.00 (14 %)	0.22 (6 %)	0.72 (80 %)
PC	0.00 (33 %)	0.23 (3 %)	0.72 (64 %)

[a]Free nucleosomes
[b]The reported E values were calculated by fitting the histograms (Fig. 6) to a sum of three Gaussian distributions and finding their maxima

coiled on the surface of the histone octamer (Fig. 1c). The high values of FRET are in agreement with the expected short average distance between Cy3 (position +56) and Cy5 (position +135) in a fully assembled nucleosome (ca. 19 Å). The second subpopulation of particles has low FRET value ($E=0.00$), is observed in each specimen and contains 12–18 % of particles (Table 1). This subpopulation most likely consists of nucleosomes containing DNA that is partially uncoiled from the octamer and histone-free DNA. Minor subpopulation of particles (6–7 %) is characterized by intermediate average FRET efficiencies: 0.32 for nucleosomes and 0.22–0.25 for OC, EC-39, and EC-5 (Table 1). It has been suggested that these FRET efficiencies are characteristic for nucleosomes formed after dissociation of an H2A/H2B histone dimer [36]. These are so called hexasomes [37]; they have partially unwrapped DNA end and, as a result, are characterized by the increased distance between Cy3 and Cy5.

A remarkable feature of the studied nucleosomes, OC, EC-39, and EC-5 is their stability. Even at a high ionic strength (i.e., in the buffer containing 150 mM KCl) the equilibrium between high, middle, and low FRET states is preserved during at least 30 min of measurements (Fig. 5). Thus, we do not observe the instability detected for nucleosomes formed on similar 601 DNA sequences in single-molecule experiments at NaCl concentrations higher 100 mM because of rapid nucleosome dissociation [36]. Both intact nucleosomal templates and their complexes with RNAP have comparable high stability.

The high FRET states of OC, EC-39, and EC-5 are very similar, but they are slightly different from the high FRET state of free nucleosomes (Table 1, Fig. 6a). Thus, RNAP binding to the templates outside of the nucleosome core affects DNA packing in the region of Cy3 and Cy5 location. Bulk RNAP molecule likely sterically interferes with the binding of distal DNA end to the octamer and induces slight DNA unwrapping, thus increasing the distance and decreasing the efficiency of FRET between Cy3 and Cy5. The stalled complexes are also characterized by a small increase in the low FRET fraction and proportional decrease in the high FRET subpopulation (Table 1, Fig. 6a).

EC-5 complexes subjected to transcription are converted by RNAP into post chase (PC) state which is characterized by the presence of three similar subpopulations: low ($E=0.00$), middle ($E=0.23$), and high ($E=0.72$) FRET (Fig. 4, Table 1). According to biochemical data more than 95 % of EC-5 complexes are transcribed (not shown). spFRET analysis reveals that ca. 16 % of nucleosomes are converted from high to low FRET state after transcription (Fig. 6b). The fraction of middle FRET nucleosomes is also decreased (Table 1). The remaining 64 % of nucleosomes preserve compact DNA packing which is similar to that of the EC-5. The data suggest that, consistent with the biochemical data [31], a large fraction of nucleosomes survives during transcription.

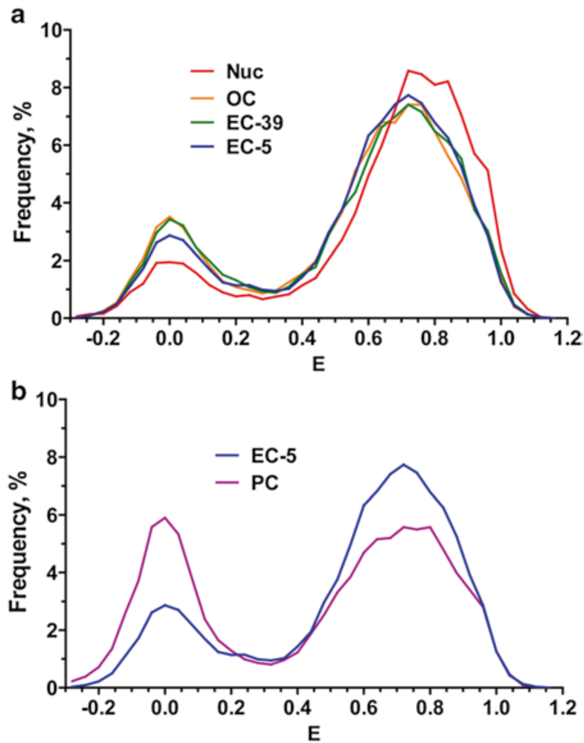

Fig. 6 Comparison of frequency histograms of FRET efficiency (*E*) for (**a**) nucleosomes (Nuc), open complex (OC), elongation complexes EC-39 and EC-5; (**b**) elongation complex EC-5 and post chase state (PC)

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Chapter 24

Transcriptome-Wide Identification of In Vivo Interactions Between RNAs and RNA-Binding Proteins by RIP and PAR-CLIP Assays

Edgar González-Buendía, Ricardo Saldaña-Meyer, Karin Meier, and Félix Recillas-Targa

Abstract

Comprehensive genomic and computational studies in the era of high-throughput sequencing revealed that the major proportion of the human genome is transcribed. This novel insight confronted the scientific community with new questions concerning the expanded role of RNA, especially noncoding RNA (ncRNA), in cellular pathways. In recent years, there has been mounting evidence that ncRNAs and RNA binding proteins (RBPs) are involved in a wide range of biological processes, such as developmental transitions, cell differentiation, stress response, genome organization, and regulation of gene expression. In particular, in the chromatin field long noncoding RNAs (lncRNAs) have drawn increasing attention to their function in epigenetic regulation due to the fact that they were found to interact with multiple chromatin regulators and modifiers. Recently, techniques to study the extent of RNA–protein interactions have been developed in many research laboratories. Here we describe protocols for RNA Immunoprecipitation-Sequencing (RIP-Seq) and Photoactivatable-Ribonucleoside-Enhanced Cross-linking and Immunoprecipitation combined with deep sequencing (PAR-CLIP-Seq) to identify RNA targets of RNA-binding proteins (RBPs) on a transcriptome-wide level, discussing advantages and drawbacks.

Key words Noncoding RNA, lncRNA, RNA-binding protein, RIP, PAR-CLIP, Cross-linking, Immunoprecipitation

1 Introduction

1.1 The Noncoding Transcriptome

The Encyclopedia of DNA Elements (ENCODE) project, amongst other studies, owing to the development of high-throughput-sequencing techniques, has uncovered that a vast majority of the eukaryotic genome is transcribed [1–5]. Whereas only a minor fraction of the transcriptome encodes proteins, the majority appears to lack protein-coding capacity. For this reason, these transcripts were termed noncoding RNAs (ncRNA).

NcRNAs represent a family of multifunctional RNA molecules that comprises well-known classes, such as transfer RNAs (tRNAs),

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ribosomal RNAs (rRNAs), and small nuclear RNAs (snRNAs), in addition to recently identified ncRNAs, such as small ncRNAs (<200 nt) and long ncRNAs (lncRNA) (>200 nt). Moreover, active enhancers are pervasively transcribed, commonly in a bidirectional manner, into another novel class of ncRNAs, so called enhancer RNAs (eRNAs) [6].

Both small and lncRNAs need to interact with RNA binding proteins (RBPs) proteins and assemble into functional ribonucleoprotein complexes in order to fulfill their regulatory tasks [7]. The better-characterized subfamilies of small ncRNAs are microRNAs (miRNAs) and small interfering RNAs (siRNAs) that are primarily involved in the posttranscriptional inhibition of gene expression. Although both are loaded into AGO family members as part of RNA-induced silencing complexes (RISCs) to do so, the mechanistic modes of silencing by miRNAs and siRNAs differ (reviewed in [7]). While miRNAs promote shortening of Poly(A) deadenylation, mRNA decay and translational repression, siRNAs induce target RNA cleavage.

Initially, specific enhancer transcription was described in 1992 for the locus control region (LCR) of the β-globin gene cluster [8]. However, recent advances in high-throughput sequencing have discovered active enhancers to be globally transcribed into eRNAs, revealing a positive correlation between eRNA transcription and expression of adjacent protein-coding genes [6]. Several reports that used either targeted degradation of eRNA or reporter plasmid-based tethering assays supported the idea that eRNAs functionally contribute to enhancer activity [9, 10]. Furthermore, the direct role of eRNA was backed up by the findings that eRNA promotes chromatin looping between enhancer and promoter [9] and contributes to RNA Polymerase II (RNA PolII) loading at target gene promoters [11].

In recent years, particularly lncRNAs have emerged as crucial regulators of cellular processes, such as X-chromosome inactivation (e.g., XIST; [12–14]), imprinting (e.g., Air; [15]), cell differentiation (e.g., ANCR or TINCR; [16, 17]) and developmental patterning (e.g., HOTAIR; [18, 19]). Furthermore, several studies support the idea that one of the major functions of lncRNAs is the regulation of gene expression at the level of chromatin, being both involved in epigenetic silencing and activation. Thus, multiple lncRNAs were found to interact with epigenetic regulators of chromatin. For instance, XIST interacts directly with the polycomb repressive complex 2 (PRC2) and is required for proper recruitment of PRC2 and H3K27 trimethylation activity to the inactive X-chromosome [12, 20]. In analogy, HOTAIR lncRNA specifically associates via two separate RNA domains, functioning like a scaffold, with PRC2 and the LSD1 repressor complex and directs their chromatin-modifying activities to the HOXD locus [21]. In contrast to these repressive mechanisms, other lncRNAs, also

termed ncRNAs-activating (ncRNA-a), possess enhancer-like or co-activator functions. For instance, ncRNA-a7 and 5 bind to the Mediator complex to activate neighboring genes in a *cis*-regulatory mechanism including chromatin looping [22]. HOTTIP and Mistral activate target genes in the HOXA locus by associating with the mixed lineage leukemia (MLL) complex [23, 24].

Considering the importance of lncRNA in regulation of gene expression and other biological processes, it is therefore not surprising that aberrant expression of a subset of lncRNAs (e.g., HOTAIR, MALAT1, ANRIL) has been implicated in human diseases, including cancer. To only give one example, HOTAIR expression is deregulated in several types of cancer, including colorectal and breast cancer [25–29]. Importantly, its upregulation is generally associated with elevated invasiveness and metastasis, as well as poor prognosis.

The examples mentioned above illustrate that RNAs are involved in the modulation of all kind of cellular processes to a much higher extent than previously appreciated. Therefore, techniques that help unraveling the RNA–protein interactome are indispensable to gain a deeper insight in the widespread role of RNA in cell regulation.

1.2 Identification of Protein–RNA Interactions in Non-treated Cells by RIP-Seq

The RNA-binding protein immunoprecipitation (RIP) is an antibody-based method [30] that works analogous to the more frequently used chromatin immunoprecipitation (ChIP), which detects genomic DNA targets of DNA- or chromatin-binding proteins in a cellular context. RIP can be applied to capture RNA molecules of various types (nuclear, cytoplasmic, messenger RNA, noncoding RNA, viral RNA, etc.) that are associated with an RBP on a transcriptome-wide level. In brief, cell extracts are incubated with an antibody against the protein of interest and bound to ProteinA/G beads (immunoprecipitation). Co-bound RNA is isolated from immunoprecipitates and can be analyzed by different strategies: (1) In case one specific RNA molecule is of interest, one can analyze the enrichment of this target by quantitative PCR (qRT-PCR) [19]. (2) Previously, RNA isolated by RIP has also been analyzed using microarray technologies (RIP-ChIP) [31], a method that is naturally limited by array design and coverage. (3) Therefore, RIP has recently been combined with RNA-Sequencing (RIP-Seq) [32]. Initially, HOTAIR one of several HOX ncRNAs encoded in the HOXC locus was found to co-immunoprecipitate with PRC2 in RIP and assist its recruitment to the HOXD locus [19]. A transcriptome-wide level dataset obtained by RIP-Seq suggested that the PRC2 complex subunit Ezh2 interacts with more than 9,000 RNAs [33].

In contrast to the alternative methods that include a cross-linking step to stabilize in vivo protein–RNA interactions (*see* Subheading 1.3), RIP is carried out under native conditions and

therefore permits only the detection of kinetically stable interactions. Consequently, the stringency of washing and purification steps is restricted, as native RNA–protein interactions are desired to stay intact. For this reason, it is indispensable, to include negative controls that will help uncover false-positive results. These can include a mock control, omitting the antibody, as well as an IgG control, both to detect background binding of RNA to the beads, and determine RNA enrichment in the actual RIP sample. The gold standard of controls, however, is knockout cells or tissues that are devoid of the protein that is immunoprecipitated. Moreover, results of RIP assays are commonly known to exhibit a certain degree of variability (with a reproducibility of 60 % or more) [34]. Thus, it is worthwhile to include multiple biological replicates.

Taken together, RIP assays have successfully identified a plethora of RBP RNA targets using samples from various organisms [30, 32, 33, 35].

1.3 Mapping RNA Target Binding Sites of RNA-Binding Proteins (RBPs) by PAR-CLIP-Seq

As RIP, Cross-linking and Immunoprecipitation (PAR-CLIP) is an antibody-based method that has been successfully applied to study RNA–protein interactions [36, 37]. In contrast to RIP, cells or tissues are cross-linked by ultraviolet (UV) irradiation (e.g., wavelength of 254 nm) to covalently cross-link direct in vivo RNA–protein contacts. To put it in a nutshell, after UV cross-linking, immunoprecipitations of RNA–protein complexes are carried out from cell extracts using a specific antibody against the protein of interest. Importantly, covalent cross-linking permits stringent washing of immunoprecipitates, which contributes to lower background binding of RNAs. Furthermore, the treatment with UV light allows a partial digest with RNase that preserves the core RNA sequence directly involved in protein interaction. Upon ligation of a 3′ linker and radiolabeling of the highly purified RNA, RNA–protein complexes are separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane that will only retain RNA–protein complexes but not free RNA molecules. Radioactively labeled complexes are excised from the membrane, protein is Proteinase K digested and a linker is ligated to the 5′ ends of RNA. Ligated linkers are utilized to reversely transcribe the RNA into cDNA and amplify the latter by PCR. The resulting cDNA library can be analyzed by RT-PCR focusing in specific RNA targets or in an unbiased manner by high-throughput sequencing.

Furthermore, CLIP exist in different versions: (1) High-Throughput Sequencing-CLIP (HITS-CLIP) [38]; (2) Individual-nucleotide resolution CLIP (iCLIP) [39, 40]; and (3) Photoactivatable-Ribonucleoside-Enhanced CLIP (PAR-CLIP) [41]. HITS-CLIP basically refers to the classical CLIP protocol combined with high-throughput sequencing and was used in several studies to identify RBP RNA-targets [38, 42–44]. Despite

this, iCLIP and PAR-CLIP introduced modifications to the method in order to improve it and circumvent some inherent drawbacks.

The CLIP protocol requires the reverse transcriptase to pass through the cross-link site, to which amino acids remain attached even after Proteinase K digest, to complete cDNA synthesis to the end of the 5′ linker. However, primer extension assays revealed that the majority of the cDNA population truncates prematurely at the cross-link site nucleotide [39, 45]. Therefore, iCLIP introduces an adapter at the 5′ end through the primer used for reverse transcription by cDNA circularization and subsequent linearization. Thus, both truncated and read-through cDNAs are captured [39]. Importantly, iCLIP also provides information about the cross-link site that permits precise mapping of RNA–protein contacts at a nucleotide resolution. Besides, insertion of a random barcode sequence to the adaptor marks individual cDNA molecules and helps eliminating PCR replicates.

Another problem with the CLIP assay is the low efficiency of RNA–protein UV cross-linking. In addition, the position of the cross-link site cannot be readily identified within the cross-linked fragments, making it more difficult to distinguish between specific RNA-targets and nonspecific RNA background. However, nucleotide substitutions and small deletions are frequently introduced by the reverse transcriptase at the site of cross-linking [46, 47]. The development of the cell-based PAR-CLIP approached these issues by feeding living cells with photoreactive ribonucleoside analogs, such as 4-thiouridine (4-SU) and 6-thioguanosine (6-SG) that are incorporated into nascent RNA transcripts (*see* Fig. 1). In contrast to CLIP, UV light at 365 nm leads to efficient cross-linking between photoreactive ribonucleoside containing RNAs and RBPs. Another advantage of using photoreactive ribonucleosides is that cross-link sites are characterized by point mutations in the sequenced cDNA. Accordingly, 4-SU or 6-SG results in a thymidine to cytidine or a guanosine to adenosine transition, respectively. Despite, the obvious advancements there are also problems associated with photoreactive ribonucleosides treatment. At concentrations that are used for RNA labeling, 4-SU treatment can have cytotoxic effects, inhibit rRNA synthesis, and trigger nucleolar stress response, which could influence the outcome of PAR-CLIP results [31, 48]. However, a direct comparison of PAR-CLIP and HITS-CLIP data of RBPs HuR and Argonaute 2, that bind low- and high-complexity sequences, respectively, yielded similar results for high-affinity global binding sites [46]. As with RIP, the reliability of CLIP results benefits greatly from performing biological replicates, yet recently it was demonstrated that covalently cross-linked background binding is common and reproducible between replicates of PAR-CLIP experiments and a bioinformatic background correction is necessary to identify novel protein–RNA binding sites and to improve motif recognition [49].

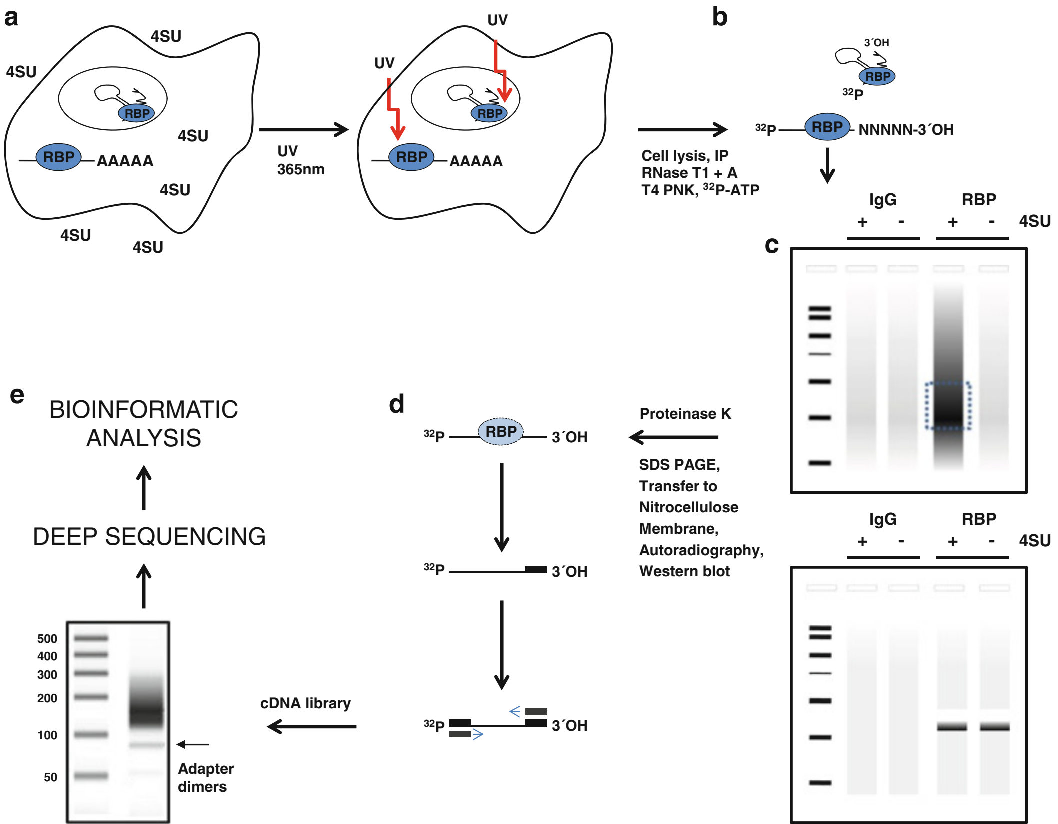

Fig. 1 Flowchart describing the PAR-CLIP methodology: (**a**) 4-SU incorporation and UV cross-linking of cell cultures. (**b**) RBP immunoprecipitation and RNA radioisotope labeling. (**c**) *Upper panel*: Autoradiography of RNA–RBP complexes and IgG control. Frame indicates the excised membrane fragment corresponding to the molecular weight of the RBP. *Lower panel*: Western-blot assay used as a control using an specific antibody against the RBP. (**d**) Elution and reverse transcription of RBP-cross-linked RNA after Proteinase K digestion. (**e**) Gel electrophoresis of cDNA library isolated from RNA–RBP complexes. Resulting cDNA library is submitted to high-throughput sequencing and subsequent bioinformatic analysis. For more details see text

A prerequisite for both RIP and CLIP applications is a well-characterized and specific antibody against the protein of interest that exhibits high immunoprecipitation efficiency. Preferably, proteins are immunoprecipitated at endogenous levels. However, both RIP and CLIP assays have been directed against recombinantly expressed and tagged proteins [41, 47, 50, 51]. Nonetheless, one should keep in mind that an imbalance in the equilibrium between RNA and protein by overexpressing either one might disturb the in vivo stoichiometry and induce nonspecific binding events [52].

In conclusion, CLIP has evidently proven to be a powerful tool to unravel novel RNA–protein networks [42–44, 53–56]. In contrast to RIP, that is prone to detect indirect or nonphysiological associations [31, 57], CLIP methods were developed to precisely map protein contact sites within direct RNA targets.

In summary, techniques that investigate associations between proteins and RNAs, have already tremendously contributed to our current knowledge about the density of RNA–protein networking and they will further be of great value in gaining a deeper insight into their biological roles and mechanisms. In the following section, we provide detailed protocols for RIP-Seq and PAR-CLIP-Seq that have been successfully applied to identify RNA targets of the multifunctional CCCTC-binding factor (CTCF) in human cells [53]. These protocols facilitate identification of RNA-targets of a given protein on a transcriptome-wide level.

2 Materials

2.1 Materials for RIP-Seq

Nuclear isolation buffer: 1.28 M sucrose, 40 mM Tris–HCl pH 7.5, 20 mM $MgCl_2$, 4 % Triton X-100.

RIP buffer: 150 mM KCl, 25 mM Tris–HCl pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5 % NP40, 100 U/ml RNase inhibitor SUPERaseIn (add freshly each time), protease inhibitors (add freshly each time).

Wash Buffer: BC200 + 0.05 % IGEPAL.

Dilution buffer: Wash buffer + 10 mM EDTA + 1:600 murine RNase inhibitor.

RIP wash: Wash buffer + 1 mM of $MgCl_2$.

2.2 Materials for PAR CLIP-Seq

BTE: 10 mM Bis-tris pH 6.7, 1 mM EDTA.

IP_{50}: 20 mM Tris–HCl pH 7.9, 2 mM EDTA, 50 mM KCl, 10 % glycerol 0.05 % IGEPAL-630 (NP-40).

Buffer A: 10 mM Tris–HCl pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF.

CLIP Buffer: 20 mM Hepes-NaOH pH 7.4, 5 mM EDTA pH 8.0, 0.15 M NaCl, 2 % Empigen, DNase I (1/100) and RNase inhibitors (1/200).

PK Buffer: 50 mM Tris–HCl pH 7.3, 50 mM NaCl, 10 mM EDTA.

2× PK Buffer: 50 mM HEPES pH 7.0, 250 mM NaCl, 20 mM EDTA, 1 % SDS.

RIP Buffer: CLIP Lysis Buffer + 10 mM EDTA and RNAsin.

Nuclease Reaction 20 μl: 17 μl IP_{50}, 2 μl Turbo DNase buffer, 1 μl Turbo DNase (2 U).

Dephosphorylation Reaction: 17 μl IP_{50}, 2 μl Antarctic phosphatase buffer, 1 μl (5 U) Antarctic phosphatase (NEB # M0289).

3′ Linker Ligation: 13 μl IP_{50}, 2 μl 3′ linker (100 μM), 2 μl T4 RNA ligase buffer, 2 μl 10 mM ATP, 1 μl T4 RNA ligase 1 (NEB #M0204).

5′ Labeling Reaction: 17.3 μl IP_{50}, 2 μl PNK buffer, 0.5 μl PNK (5 U), 0.2 μl/2 μCi [^{32}P]-γATP (3,000 Ci/mmol, 10 mCi/ml); use more if the isotope is old.

Urea/PK Buffer: 0.42 g of urea in 687 μl of PK Buffer for a final volume of 1 ml.

3 Methods

3.1 RIP-Seq

3.1.1 Cell Culture and Nuclei Isolation

1. Grow 1×10^7 cells of interest to confluence (*see* **Note 1**).
2. Harvest cells by trypsinization.
3. Wash cells twice with cold PBS.
4. Pellet cells by centrifugation at 1,000 × *g* and keep on ice.
5. Resuspend cells in 2 ml freshly prepared nuclear isolation buffer.
6. Pellet nuclei by centrifugation at 2,500 × *g* for 15 min.
7. Resuspend nuclear pellet in 1 ml freshly prepared RIP buffer.
8. Pellet nuclear membrane and debris by centrifugation at 16,000 × *g* for 10 min.
9. Recover supernatant.

3.1.2 Immunoprecipitation (IP) of RNA–Protein Complexes

1. Start with 1 mg of nuclear extracts (*see* **Note 2**).
2. Dilute the protein extract in dilution buffer to a final volume of 1 ml.
3. Spin at 10,000 × *g* for 10 min. The supernatant was subjected to IP.
4. Add antibody and rotate at 4 °C for 3 h to overnight (*see* **Note 3**).
5. Pre-wash 5 μl of Dynabeads Protein G per μg of antibody 3× with 1 ml of dilution buffer each.
6. Resuspend beads with dilution buffer in the same original volume.
7. Add beads to the sample and incubate for an additional hour at 4 °C with rotation.
8. Wash twice with 1 ml RIP wash by pipetting.
9. Resuspend in 50 μl RIP Wash + TurboDNase (1 μl = 2 U) and incubate for 10 min at room temperature.
10. Wash two times with 1 ml RIP wash and during the last wash divide into two tubes, 100 μl for a WB to test if the IP was efficient and 900 μl to purify the RNA.

3.1.3 Purification of RNA-Bound

1. Add 1 ml of TRIzol to RNA samples and isolate RNA (*see* **Note 4**).
2. Resuspend RNA in 20 μl of ß-TE and add 1× TurboDNase Buffer and 1 U of TurboDNase.

3. Incubate for 10 min at RT and isolate RNA by TRIzol again.
4. Resuspend in 50 μl ß-TE (*see* **Note 5**).

3.1.4 Quality Control of RNA

1. Use 1 μl to measure RNA concentration using NanoDrop and run 40 ng in a denaturing formaldehyde agarose gel to confirm RNA integrity.

   If you obtained a sufficient amount of RNA (>800 ng) of high integrity you can start with the library construction for deep sequencing or the generation of primer specific cDNA for RT-qPCR (*see* **Note 6**).

3.2 PAR-CLIP-Seq

DAY 1.

3.2.1 Cell Culture and UV Cross-Linking

1. Plate 20×10^6 cells per condition (*see* **Note 7**).
2. Add 400–500 μM 4-SU (Sigma) to the cells and incubate for 2–16 h at 37 °C to allow its incorporation into newly synthesized RNA (*see* **Note 8**).
3. Remove media. Add 10 ml cold PBS.
4. Cross-link with 150–500 mJ/cm^2 of UV (365 or 312 nm) without the lid of the cell culture dish using a Stratalinker UV Crosslinker (Stratagene) (*see* **Note 9**).
5. Remove the PBS and harvest the cells by scraping.
6. Pellet cells by centrifugation at $1{,}000 \times g$ for 3 min at 4 °C (*see* **Note 10**).

3.2.2 Cell Lysis and DNase Treatment (Preparation of Nuclear Lysates)

1. Resuspend 100 μl of cell pellet in 500 μl of Buffer A. Incubate for 5 min on ice and allow cell swelling.
2. Spin down at $2{,}500 \times g$ for 5 min at 4 °C and discard supernatant.
3. Add another 500 μl of Buffer A containing 0.2 % NP-40.
4. Lyse 5 min on ice.
5. Spin nuclei down at $2{,}500 \times g$ for 5 min at 4 °C and discard supernatant (cytoplasm).
6. Resuspend pellet in CLIP Buffer (with RNasin and Turbo DNase).
7. Incubate for 10 min at 37 °C.
8. Centrifuge at $20{,}000 \times g$ for 5 min at 4 °C, discard insoluble materials, and keep the supernatant (nuclear lysate).
9. Measure protein concentration and proceed immediately.

3.2.3 Immunoprecipitation (IP), RNase A Digestion, and Dephosphorylation

1. Prepare IP under RNA-preserving conditions.
2. Dilute 1 mg of extract in RIP Buffer to a final volume of 1 ml and incubate for 5 min on ice.
3. Keep 1 % as input to use in a control WB.

4. Add antibody to each condition.
5. Incubate at 4 °C for 3 h with rotation.
6. Add Dynabeads Protein G (*see* **Note 11**).
7. Incubate at 4 °C for 1 h with rotation.
8. Add RNase cocktail and incubate 37 °C for 5 min (*see* **Note 12**).
9. Place tube(s) in a magnetic separator and remove supernatant.
10. Wash beads once with 1 ml RIP Buffer.
11. Wash beads once with 1 ml IP_{50}.
12. Resuspend beads in 20 μl Nuclease Reaction for 10 min at 37 °C, shake gently.
13. Wash once with RIP Buffer.
14. Wash once with IP_{50}.
15. Resuspend beads in 10 μl of Dephosphorylation Reaction for 15 min at 37 °C, shake gently.
16. 3′ Linker Ligation for 1 h at room temperature.
17. Wash once with CLIP Buffer.
18. Wash once with IP_{50}.

3.2.4 Radiolabeling of RNA Cross-Linked to Immunoprecipitated Proteins

1. Resuspend in 10 μl of 5′ Labeling Reaction.
2. Incubate for 15 min at 37 °C, shake gently.
3. Wash once with 1 ml of RIP BUFFER and transfer to new tubes, take a 100 μl aliquot for a control WB and 900 μl to continue the protocol.

Bis-Tris 8 % PAGE

1. Resuspend beads in 30 μl BTE (10 mM Bis-tris and 1 mM EDTA pH 6.7), 1:40 2-mercaptoethanol and 1× LDS loading buffer (NuPAGE LDS NP0007).
2. Incubate at 70 °C for 10 min, shake gently.
3. Run samples on a 10-well Bis-tris gel kept on ice (4 °C) at 150 V for 1 h (*see* **Note 13**).

Semidry Transfer

Transfer at 20 V for 40 min (*see* **Note 14**).

Exposure

Expose using a high sensitivity film for 2 h or overnight (*see* **Note 15**).

DAY 2.

3.2.5 Library Construction

1. Cut desired radiolabeled bands from membrane (*see* **Note 16**), cut into small pieces, transfer into a tube and spin down.
2. Add 180 μl PK Buffer and 20 μl Proteinase K (Ambion), pre-warm the mixture for 10 min at 37 °C.
3. Incubate for 30 min at 37 °C, shake gently.

4. Add 200 μl 7 M Urea/PK Buffer and incubate for 30 min at 55 °C.
5. Add 80 μl of H_2O and 480 μl of phenol–chloroform–isoamyl alcohol and vortex until the mixture is white, spin at 20,000 × *g* for 10 min.
6. Recover upper aqueous phase and add 1 μl glycogen, 50 μl 3 M sodium acetate, and 1 ml of 100 % EtOH. Incubate for 30 min at −80 °C.
7. Precipitate by spinning at 20,000 × *g*, 4 °C for 30 min.
8. Wash pellet once with 70 % EtOH and spin at 20,000 × *g* for 5 min.
9. Wash once with 80 % EtOH spin at 20,000 × *g* for 5 min.
10. Resuspend in 16 μl BTE (PAR-CLIP-RNA).

PNK Treatment

1. Prepare following reaction mixture: 2 μl 10× PNK Buffer, 1 μl 300 mM ATP, 1 μl T4 PNK, 16 μl PAR-CLIP-RNA.
2. Incubate at 37 °C for 10 min.
3. Add 460 μl of H_2O and 480 μl phenol–chloroform–isoamyl alcohol and vortex until white, spin at 20,000 × *g* for 10 min.
4. Add 1 μl glycogen, 50 μl 3 M sodium acetate, and 1 ml of 100 % EtOH. Incubate for 30 min at −80 °C or overnight at −20 °C.
5. Spin for 30 min, at 20,000 × *g*, 4 °C.
6. Wash pellet once with 70 % EtOH and spin for 5 min at 20,000 × *g*, 4 °C.
7. Wash 1× with 80 % EtOH spin for 5 min at 20,000 × *g*, 4 °C.
8. Resuspend in 5 μl BTE.

5′ Linker Ligation

1. Denature 5′ linker at 70 °C for 2 min and keep on ice for 1 min.
2. Assemble and incubate: 1 μl 10× T4 RNA ligase buffer, 1 μl 10 mM ATP, 1 μl T4 RNA ligase 1, 2 μl pretreated 5′ RNA linker, 5 μl PAR-CLIP-RNA (PNK treated; *see* Subheading “PNK Treatment”).
3. Incubate for 1 h at room temperature.
4. Add 470 μl of H_2O and 480 μl phenol–chloroform–isoamyl alcohol and vortex until white, spin at 20,000 × *g* for 10 min.
5. Add 1 μl glycogen, 50 μl 3 M sodium acetate, and 1 ml of 100 % EtOH.
6. Incubate for 30 min at −80 °C.
7. Spin at 20,000 × *g* at 4 °C for 30 min.
8. Wash pellet once with 70 % EtOH and spin for 5 min at 20,000 × *g*.

9. Wash once with 80 % EtOH spin for 5 min at 20,000 × *g*.
10. Resuspend in 8 μl BTE and transfer to a PCR tube.

cDNA Synthesis Reverse Transcriptase PCR

1. Prepare the following RT-PCR reaction mixture: 8 μl PAR-CLIP-RNA (+5′ linker; *see* Subheading "5′ Linker Ligation," 1 μL 10 μM RT-Primer (anneals with 3′ linker), 1 μl 10 mM dNTPs.
2. Run the following program: 75 °C 5 min/50 °C 5 min/25 °C 5 min.
3. Add 10 μl Reverse Transcriptase Mix: 4 μl 5× RTIII buffer, 2 μl 100 mM DTT, 1 μl RTIII enzyme, 0.2 μl RNasin (NEB), 2.8 μl H_2O.
4. Run the following program: 50 °C 45 min/55 °C 15 min/90 °C 5 min/4 °C hold.
   DAY 3.

First LM-PCR for Library Construction

1. Assemble: 20 μl PAR-CLIP cDNA (*see* Subheading "cDNA Synthesis Reverse Transcriptase PCR"), 10 μl 5× Q5 buffer, 1 μl 10 μM Primer Mix (complementary to both linkers), 1.5 μl dNTPs, 0.5 μl Q5 hotstart (NEB) 17 μl H_2O.
2. Run following PCR program: 98 °C 30 s//98 °C 10 s/65 °C 30 s/72 °C 30 s//×10 cycles/72 °C 5 min/4 °C hold.
3. Size selection using a 2 % agarose gel.
4. Cut the gel between 100 and 250 bp.
5. Purify DNA using QIAquick gel-extraction kit (Qiagen).
6. Elute in 32 μl of TE.

Amplification of Libraries for Submission to Sequencing

1. Assemble: 30 μl libraries, 10 μl 5× Q5 buffer, 1 μl Amplification primers (Illumina primers for sequencing), 1 μl 10 mM dNTPs, 0.5 μl Q5 HOTSTART, 0.25 μl UDG (heat labile, Roche 1 U/μl), 7.25 μl H_2O.
2. Run the following program:

   37 °C 15 min/

   98 °C 30 s//98 °C 10 s/65 °C 30 s/72 °C 30 s//× # of cycles/72 °C 3 min/4 °C hold (*see* **Note 17**).
3. Purification with QIAquick PCR-extraction kit (Qiagen) (*see* **Note 18**).

Quantification of Amplified Libraries

1. Prepare two independent 1:1,000 dilutions for each library and do a combined 1:10 dilution for qPCR (*see* **Note 19**).
2. Run qPCR as in Subheading "First LM-PCR for Library Construction" (*see* **Note 20**).
3. Adjust the samples to 10 nM concentration with TE.

4. Verify product size by loading 2 µl of the final 10 nM libraries on a vertical 9 % PAA (29:1), 0.5× TGE gel.
5. Submit to sequencing.

4 Notes

RIP-Seq

1. Increasing the number of cells improves the final results. Try to plate at least 1×10^7 cells (on a 1×15 cm plate) per condition.
2. One or more negative controls should be maintained throughout the experiment, e.g., Mock sample or immunoprecipitation from knockout cells or tissue.
3. The amount of antibody needs to be optimized by doing a titration.
4. TRIzol RNA extraction reagent (1 ml) is used according to manufacturer's instructions.
5. Purified RNA can be stored at −80 °C.
6. If the objective of the experiment is to survey new RBP-RNA interactions, libraries for deep sequencing can be constructed with any standard RNA-Seq protocol depending on the type of RNAs of interest. Otherwise, if the target is known primer specific cDNA can be used as template for qPCR.

PAR-CLIP-Seq

7. Increasing the cell number improves the final results. Try to plate at least 10×10^6 cells (on 1×15 cm plate) per condition.
8. Use a 100 mM stock in DMSO.
9. This will depend on the cell type and protein of interest.
10. Optionally, you can flash-freeze in liquid nitrogen and store at −80 °C.
11. Typically 5 µl per 1 µg of antibody.
12. RNase cocktail (A 0.5 U/µl + T1 20 U/µl, Ambion #AM2286). The concentration needs to be optimized experimentally.
13. For example for CTCF an 8 % Bis-tris gel was prepared.
14. This will vary depending on the protein size and gel %.
15. If the signal is overexposed on the film, use shorter exposure times.
16. For example between 100 and 150 kDa for CTCF.
17. The number of cycles in the PCR should be calculated to obtain a 20 nM final concentration. The total number of molecules going into the amplification should be at least the same

as number of reads that are desired. Amplifying a few molecules with too many cycles will lead to duplicate reads.

18. Add 5 μl 3 M sodium acetate pH 5.2 per sample and elute in 31 μl EB.
19. For example transfer 1 μl of each 1:1,000 dilution to 18 μl of H_2O.
20. Accuracy is critical because the cluster density depends on the library concentration.

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Chapter 25

Chromatin Immunoprecipitation Assays: Analyzing Transcription Factor Binding and Histone Modifications In Vivo

Smitha Pillai, Piyali Dasgupta, and Srikumar P. Chellappan

Abstract

Studies in the past decade have shown that differential gene expression depends not only on the binding of specific transcription factors to discrete promoter elements but also on the epigenetic modification of the DNA as well as histones associated with the promoter. While techniques like electrophoretic mobility shift assays could detect and characterize the binding of specific transcription factors present in cell lysates to DNA sequences in in vitro binding conditions, they were not effective in assessing the binding in intact cells. Development of chromatin immunoprecipitation technique in the past decade enabled the analysis of the association of regulatory molecules with specific promoters or changes in histone modifications in vivo, without overexpressing any component. ChIP assays can provide a snapshot of how a regulatory transcription factor affects the expression of a single gene, or a variety of genes at the same time. Availability of high quality antibodies that recognizes histones modified in a specific fashion further expanded the use of ChIP assays to analyze even minute changes in histone modification and nucleosomes structure. This chapter outlines the general strategies and protocols used to carry out ChIP assays to study the differential recruitment of transcription factors as well as histone modifications.

Key words Chromatin immunoprecipitation, Histone modification, Methylation, Acetylation

1 Introduction

Chromatin immunoprecipitation is a powerful tool for identifying proteins, including transcription factors and histone proteins, associated with specific regions of genome, using specific antibodies that recognize the protein of interest. Chromatin immunoprecipitation assays provide an unbiased glimpse into the changes occurring on chromatin in vivo, in response to extracellular signals or during differentiation and development [1–3]. It has long been established that differential gene expression correlates with the recruitment of specific and general transcription factors on relevant promoters; in addition, it has also become clear that covalent modification of histones in the promoter region also contributes

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significantly to the process [1, 4]. Indeed, the pattern of acetylation and methylation of histones in the promoter region is so vital to modulating gene expression that it is thought that these processes code for the gene expression profiles per se [5–7]. While modifications of histones and recruitment of transcription factors are dynamic events, it is possible to freeze the process chemically and examine the changes [8, 9]. ChIP assays provide an efficient, cost-effective means to achieve this. Studies from the laboratories of Doug Dean, Peggy Farnham and others in the 1990s established many of the protocols currently in use. Our lab has utilized ChIP assays to study the recruitment of E2F1, Rb, Raf-1, prohibitin, or transcriptional co-repressors like HDAC1, HP1γ to promoters, in response to specific signaling events. The protocol described here is an updated and modified version of an earlier protocol [10, 11].

Generally, ChIP assays can be done on tissue culture cells or from tissue samples. The basic assay protocol used to study histone modifications is shown in Fig. 1; this has been modified in certain situations. The initial step in the ChIP assay is to cross-link DNA to the associated proteins by treating the cells with formaldehyde [12–14]; in certain situations, investigators have bypassed this step to prevent chemical modifications of the chromatin [4, 15, 16]. The second step is to isolate the DNA–protein complexes; the isolated DNA is then fragmented into 1–2 Kb fragments, either by sonication [17–19] or treatment with micrococcal nuclease [4, 20, 21], in the third step. The DNA fragments are then immunoprecipitated using specific antibodies of interest—antibodies to transcription factors, modified histones, co-activators or co-repressors, etc., which is the fourth step in the ChIP assay. The immunoprecipitated DNA–protein complexes are de-cross-linked in the fifth step; lastly, this is followed by PCR using specific primers to the promoters of interest in the sixth step. Alternately, the DNA can be used for probing microarrays of promoter regions to get an idea of global changes in factor recruitment and histone modifications [1, 13, 22–28] (*see* **Note 1**). Protocols for conducting such a ChIP on chip experiment and analyzing the data are described in the next two chapters.

2 Materials

1. *37 % Formaldehyde (Molecular Biology Reagent Grade):* Formaldehyde is supplied as a 37 % solution in water and can be purchased from Sigma Aldrich (catalog # F-8775) or other sources. Formaldehyde is a biohazard and needs to be handled inside a fume hood.
2. *1 M Glycine:* Dissolve 3.75 g of tissue culture grade glycine (Fischer Biotech, catalog # BP381-1) in 50 ml of water to obtain a 1 M solution.

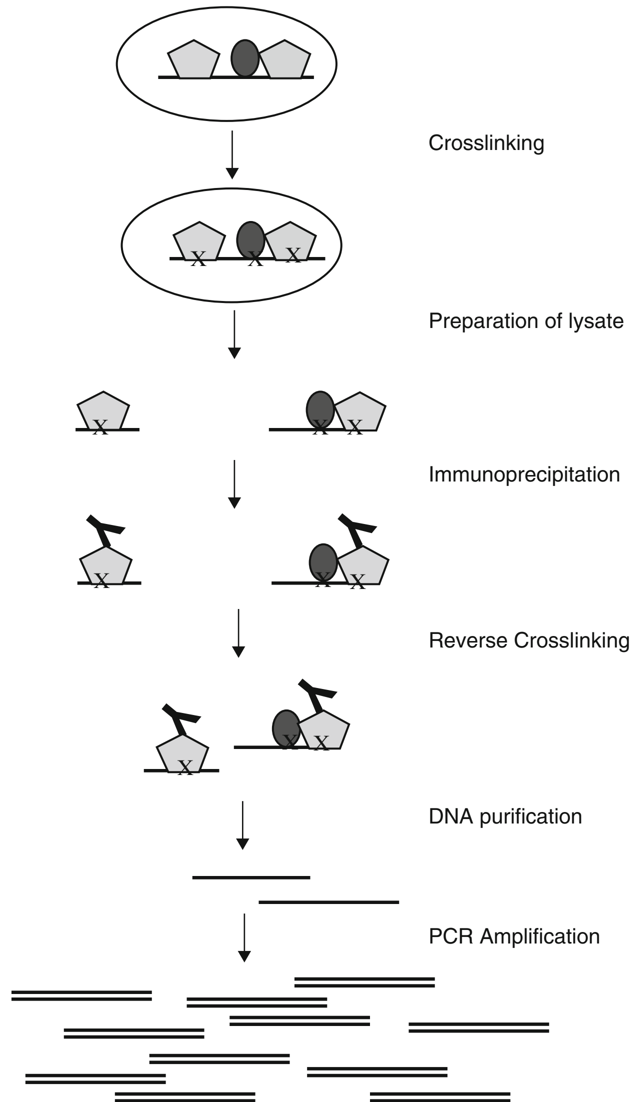

Fig. 1 The various steps involved in conducting a basic ChIP assay. Details of each step are provided in the text

3. *20× PBS:* Dissolve 160 g NaCl, 4 g KCl, 2.88 g Na_2HPO_4, 4.8 g KH_2PO_4 in 1 L Milli-Q water. Adjust the pH of the buffer to 6.4 to obtain 20× PBS stock solution. Take 50 ml of this buffer and make it up to 1 L with Milli-Q water to obtain 1× PBS.

4. *BSA (10 mg/ml):* Dissolve 100 mg of BSA (Sigma-Aldrich, catalog # A3059-50G) in 10 ml of Milli-Q water. Aliquot in 100 μl and store at −20 °C.

5. *Salmon sperm DNA:* Salmon sperm DNA is obtained from Sigma-Aldrich (catalog # D7696) as a 10 mg/ml solution. Use 10 μl for pre-clearing steps.

6. *Herring sperm DNA:* Some labs use herring sperm DNA as a substitute for salmon sperm DNA. Herring sperm DNA is obtained from Sigma-Aldrich (catalog # D7290) as a 10 mg/ml solution. Use 10 μl for pre-clearing.
7. *Lysis Buffer:* 44 mM Tris–HCl (pH 8.1), 1 % SDS, 10 mM EDTA, 10 mM sodium butyrate (for histone acetylation ChIP assays). Add the following protease inhibitors fresh to the lysis buffer just before use: 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin.
8. *100 mM PMSF:* Dissolve 0.174 g of PMSF (Sigma-Aldrich) in 10 ml of absolute ethanol. Aliquot into 1 ml in microcentrifuge tubes and store at −20 °C. Remember that the half-life of 1 mM PMSF (after it has been added to the lysis buffer) is 30 min, so add it just before use.
9. *Dilution buffer:* 16.7 mM Tris-HCl, pH 8.1, 250 mM NaCl, 0.01 % SDS, 1 % Triton X-100.
10. *Wash Buffer:* 16.7 mM Tris–HCl, pH 8.1, 375 mM NaCl, 0.01 % SDS, 1 % Triton X-100.
11. *Elution Buffer:* 0.1 M $NaHCO_3$, 1 % SDS, 5 mM NaCl.
12. *Resuspension Buffer:* 50 mM Tris–HCl, pH 8.0, 2 mM EDTA, 0.2 % Sarkosyl.
13. *IP Elution Buffer:* 50 mM $NaHCO_3$, 1 % SDS.
14. *Proteinase K:* Dissolve 50 mg of Proteinase K (Fischer Biotech, cat# BP-1700-100) in 2 ml of Milli-Q water. Aliquot into 100 μl and store at −20 °C.
15. *5× PK Buffer:* 50 mM Tris–HCl, pH 7.5, 25 mM EDTA, 1.25 % SDS.
16. *3 M sodium acetate, pH 5.2:* Dissolve 20.4 g of sodium acetate in 50 ml of water. Adjust the pH to 5.2.
17. *Phenol–chloroform–isoamyl alcohol:* Phenol–chloroform–isoamylalcohol is obtained from Fischer Biotech (catalog # BP1752-100). Phenol is a highly corrosive chemical and should be handled with extreme care.
18. *1× TE:* 10 mM Tris, pH 8.1, 1 mM EDTA.
19. *Glycogen:* Glycogen is supplied as a 20 mg/ml solution from Roche (catalog # 901393).
20. *RNase A:* Dissolve 0.1 g of RNase A (Fischer Biotech, catalog # BP2539-250) in 10 ml of water. Store at 4 °C.
21. *5 M NaCl:* Dissolve 58.4 g NaCl in 200 ml of Milli-Q water.
22. *MNase Enzyme:* MNase is obtained from Amersham Biosciences (catalog # E70196Y). Dissolve in Milli-Q water containing 20–50 % glycerol at a concentration of 1 mg/ml. Aliquots (10–20 μl) are stored at −20 °C and used only once after thawing on ice.

23. *MNase Digestion Buffer:* 0.32 M sucrose, 50 mM Tris–HCl, pH 7.5, 1 mM $CaCl_2$, 4 mM $MgCl_2$, 0.1 mM PMSF.
24. Protein A (Amersham Biosciences, catalog # 17-0872-05) Protein G (Amersham Biosciences, catalog # 17-0618-01), Protein A/G beads (Santa Cruz Biotechnology, catalog # sc-2003) or Staph A (American Type Culture Collection) cells.

3 Methods

3.1 Chromatin Immunoprecipitation (ChIP) Assay Protocol

3.1.1 Preparation of ChIP Cell Lysate

1. ChIP assays to analyze multiple factors or modifications will require a substantial amount of cells as the starting material. It is recommended that at least 2×10^7 adherent or suspension cells be used per sample [29–32] (*see* **Notes 2** and **3**). Add formaldehyde directly to the tissue culture media to a final concentration of 1 %. This corresponds to 280 μl of the commercially available 37 % Formaldehyde per 10 ml of tissue culture media. Incubate the cells with the formaldehyde on an orbital shaker for 20 min at room temperature for efficient cross-linking of DNA to the bound proteins. For histone acetylation studies, addition of histone deacetylase inhibitor sodium butyrate (20 mM) to the culture medium to prior to fixation has been recommended, to preserve histone lysine acetylation [33].
2. Terminate the cross-linking reaction by adding glycine to a final concentration of 0.125 M, using the 1 M stock solution. Incubate the cells on the orbital shaker for additional 5 min.
3. If the assay is performed on adherent cells, scrape the cells in 5 ml of 1× ice-cold PBS and transfer to a fresh 15 ml centrifuge tube. In case of non-adherent cells, collect the cells along with the media and transfer to a 15 ml tube. Centrifuge the cells at 800×*g* (1,000 rpm) for 5 min in a table-top centrifuge (ThermoForma, General purpose centrifuge) to collect the cells. Wash the cell pellet twice with 10 ml of ice-cold PBS. Resuspend the pellet carefully in 1 ml 1× cold PBS and transfer it to an eppendorf tube. Use a sheared 1 ml tip for the resuspending the cells. Spin at 800×*g* (3,000 rpm) for 5 min in a refrigerated tabletop microcentrifuge ar 4 °C (Beckman-Coulter, Microfuge R or an equivalent model).
4. Aspirate the supernatant carefully and resuspend the cell pellet in ice-cold lysis buffer containing protease inhibitors (for 75 μl cell pellet, add 200 μl lysis buffer). Mix well using a pipetman to obtain a uniform homogenate; avoid clumping of the cells. Do not vortex the lysate. The efficiency of lysis can be checked by trypan blue staining; if cells are not lysed they can be homogenized on ice using a Dounce homogenizer with B type pestle. Certain studies have used a glass beads disruption

method to prepare ChIP lysates [30], as described in Alternate Protocols, Subheading 3.1.

5. Sonicate the lysate to shear the chromatin to below an average length of 1 kb. The time and the number of pulses vary according to cell type, the sonicator used and extent of cross-linking (*see* **Note 4**). However, we have found for most cells, three cycles of sonication at power 4 for 30 s on a Fisher Sonic Dismembrator (Model 100), followed by incubation on ice for 30 s, is sufficient to shear the chromatin properly. As an alternative to sonication, digestion with micrococcal nuclease [4, 15, 20] can be also done to cleave the chromatin into oligonucleosomes (refer to Alternate Protocols, Subheading 3.2). This method is referred to as "native chromatin immunoprecipitation". However, this technique is incompatible with formaldehyde cross-linking since cross-linked chromatin is inefficiently cleaved by nucleases. The protocol for cell lysis and nuclei preparation in native ChIP assays is described in refs. 4, 15, 16, 20.
6. Spin at 15,000 rpm in a tabletop refrigerated microcentrifuge for 15 min. Carefully transfer the supernatant into a new microcentrifuge tube. At this point the chromatin can be snap-frozen in liquid nitrogen and stored at −70 °C, if needed.

3.1.2 Setting Up the Immunoprecipitation (IP) Reaction

1. If previously frozen, thaw out the ChIP lysate on ice. Save 20 % of the lysate as the input control. Keep the input control aside and do not use it for the immunoprecipitation reaction. The rest of the lysate is equally divided into three microcentrifuge tubes. Set up the following immunoprecipitation reactions:
   (a) Irrelevant antibody.
   (b) Positive control antibody.
   (c) The antibody that is specific to the protein of interest.
2. Use 3 μg of antibody per immunoprecipitation reaction. Add 300 μl of dilution buffer and rotate the mixture overnight at 4 °C. Pre-clearing ChIP lysates is a good method to reduce nonspecific backgrounds and eliminate false positives during the assay (for pre-clearing protocols *see* Support Protocols, Subheading 3.2.2). While it may not be necessary to pre-clear in all cases, it should be carried out if nonspecific background bands are observed in PCR (*see* **Note 5**).
3. ChIP assays can be performed with Protein A, Protein G, Protein A/G beads or Staph A cells. The choice of beads depends on the isotype of the antibody to be used for the immunoprecipitation. Add the 120 μl of 1:1 Protein G slurry or 400 μl of Protein A slurry (for preparation of beads or Staph A, refer to support protocols 3.2.1–3.2.3). Thereafter, make

up the volume to 700 μl with dilution buffer and rotate for 2 h at 4 °C. If you are using Staph A beads, incubate each sample with 10 μl of preblocked Staph A beads [31, 32, 34, 35] (*see* Support Protocols, 3.2.2 and 3.2.3). Rotate on a nutator at 4 °C for 15 min.

3.1.3 Extraction of Immunoprecipitated DNA

1. Spin the tubes from **step 3**, Subheading 3.1.2 at (600 × *g*) 2,500 rpm at room temperature for 30 s, in a table-top microcentrifuge at room temperature. Carefully aspirate the supernatant and discard.
2. Efficient washing is critical to reduce background. Wash the beads three times with dilution buffer and twice with wash buffer. It has been found that transferring the beads to a new tube after the first wash helps reduce nonspecific bands. Remove the supernatant and discard each time; care should be taken not to lose beads during the wash steps. Carefully remove all the supernatant using a gel-loading tip in the final wash (*see* **Note 6**).
3. Add 300 μl of elution buffer. Rotate for 15 min at room temperature.
4. Spin the tubes at 2,000 × *g* (5,000 rpm) for 2 min at room temperature, in a table-top microcentrifuge.
5. Transfer the supernatant to fresh tubes and incubate 65 °C overnight to reverse formaldehyde cross-linking. The tube containing the input DNA (Subheading 3.1.2, **step 1**) should also be incubated at 65 °C overnight.
6. Add 0.1 volumes of 3 M sodium acetate, pH 5.2, followed by 2½ volumes of ice-cold 100 % Ethanol to the tubes and incubate at −70 °C for 10 min to precipitate the DNA.
7. Centrifuge the samples at 17,000 × *g* (14,000 rpm) for 15 min at 4 °C, in a refrigerated microcentrifuge.
8. Wash the pellet with 2.5 volumes of ice cold 70 % ethanol. Spin the tubes at 17,000 × *g* for 15 min at 4 °C. Air-dry the pellet.
9. Resuspend the DNA in 200 μl of Milli-Q water. Add 50 μg of Proteinase K (2 μl from a stock solution of 25 mg/ml Proteinase K), to each sample and incubate at 37 °C for 30 min.
10. Extract the DNA by adding equal volumes of phenol–chloroform–isoamyl alcohol. Vortex well to mix the layers. Spin the tubes at 17,000 × *g* (14,000 rpm) for 15 min at 4 °C, in a refrigerated microcentrifuge to separate the layers. Collect the upper layer carefully using a pipetman, transfer to a fresh tube, and repeat **steps 6–8** to extract the DNA. Alternately, use a QiaQuick spin column (Qiagen Corporation) to extract DNA and elute the DNA in 50 μl of TE.

11. Resuspend the immunoprecipitated DNA samples in 50 μl of water. The input DNA is resuspended in 250 μl water. The DNA of interest is amplified by PCR using specific primers to the sequence of interest. Use 5 μl of immunoprecipitated DNA for PCR analysis; 1 μl of the input DNA would be sufficient for PCR. PCR conditions vary according to the primers used; generally 30 cycles of amplification will result in visible bands. Analyze the DNA fragments by agarose gel electrophoresis (*see* **Notes 7–9**).
12. The ChIP protocol described in this chapter can be modified to examine the interaction of multiple proteins associated to specific genomic regions as a complex (*see* **Note 10**).

3.2 Support Protocols for ChIP Assay

3.2.1 Preparation of Protein A, G, A/G Beads or Staph A Cells

Appropriate beads are chosen depending on the isotype of the antibodies used and their affinity for protein A, protein G, Protein A/G. Protocols from the Farnham lab use Staph A cells for immunoprecipitation reaction. We use Protein G or Protein A for most applications in our laboratory.

1. Protein A: Weigh out 60 mg of Protein A and resuspend it in 2 ml of dilution buffer. Incubate for 30 min on ice. Subsequently, use 400 μl of the above slurry for each ChIP reaction.
2. Protein G: We use Protein G slurry containing 20 % ethanol. The required volume of beads are taken and washed three times with dilution buffer to remove the ethanol preservative. Subsequently, the beads are resuspended in equal volume of dilution buffer and 120 μl of beads are used per sample. The same procedure can be also used for preparation of Protein A/G beads.
3. Staph A cells: Resuspend 1 g of Staph A cells in 10 ml of 1× resuspension buffer [8, 35]. Centrifuge at 10,000 rpm for 5 min at 4 °C. This step is repeated once again. Thereafter resuspend in 3 ml of 1× PBS containing 3 % SDS and 10 % β-ME. Boil the beads for 30 min and collect by centrifugation at 1,000 rpm for 5 min. Wash twice in 1× resuspension buffer and repeat centrifugation at 10,000 rpm for 5 min. Resuspend in 4 ml of resuspension buffer, aliquot in 100 μl volumes in microcentrifuge tubes and snap-freeze.

3.2.2 Preclearing of ChIP Lysates

Preclearing lysates is a good method to eliminate nonspecific background in ChIP assays. ChIP assay protocols using Staph A cells require an initial preblocking step to reduce nonspecific binding [31, 36].

Preblocking Staph A Beads

For every tube (100 μl) of Staph A cells add 10 μl herring sperm DNA (10 mg/ml) and 10 μl BSA (10 mg/ml). Incubate on a nutator at 4 °C for at least 3 h or more (overnight incubation can also be done). Centrifuge at 10,000 rpm for 3 min. Remove supernatant and wash twice with 1× resuspension buffer. Resuspend the

cells in equal volume of resuspension buffer and use 10–15 μl for each pre-clearing step.

3.2.3 Pre-clearing Protocols with Protein A, Protein G or Protein A/G

Add 120 μl of 1:1 Protein G slurry (or 400 μl of Protein A) to the ChIP lysate (obtained from 2×10^7 cells as mentioned previously in Subheading 3.1.1) in a microcentrifuge tube. Add 15 μl of salmon sperm DNA (10 mg/ml) and 10 μl of BSA (10 mg/ml). Rotate on a nutator for 30 min at 4 °C. Thereafter spin the tubes at 14,000 rpm for 30 s in a table-top centrifuge at 4 °C. Collect supernatant fraction and discard the beads. This supernatant fraction is used for the immunoprecipitation reaction described in Subheading 3.1.2.

Pre-clearing Protocols Using Staph A Cells

Use the preblocked Staph A cells as described in subheading “Preblocking Staph A Beads” to preclear the ChIP lysate. 10–15 μl of preblocked Staph A beads are added to the ChIP lysate and incubated on a nutator for 15 min at 4 °C. Centrifuge the tube at 14,000 rpm for 5 min and transfer the supernatant to a fresh tube. Use this pre-cleared lysate for the subsequent immunoprecipitation reaction (Subheading 3.1.2).

3.2.4 DNA Extraction from Immunoprecipitates Using Staph A Beads

1. The immunoprecipitation reaction is set up as detailed in Subheading 3.1.2, **steps 2** and **3**.
2. Centrifuge the samples at 14,000 rpm for 3 min at room temperature.
3. Wash the pellets twice with 1.4 ml of 1× resuspension buffer followed by four washes with IP buffer. The washes are done by first dissolving the Staph A pellet in 200 μl of the appropriate buffer and adding 1.2 ml of the same buffer. The tubes are incubated with the wash buffer on a nutator for 3 min at room temperature. Thereafter, the tubes are centrifuged at 14,000 rpm for 3 min. The wash buffer is carefully aspirated off. After the last wash, the entire buffer is removed using a gel-loading tip.
4. Elute the immune-complex by adding 150 μl of IP elution buffer. Mix very well for about 15 min. Microcentrifuge the tubes at 14,000 rpm for 3 min. Transfer the supernatants to fresh tubes.
5. Repeat the elution step. Both the elution fractions are pooled. Spin the tubes at 14,000 rpm for 5 min at room temperature. Transfer the supernatants to fresh tubes. Add 1 μl of 10 mg/ml RNaseA and NaCl to a final concentration of 0.3 M. Incubate samples at 67 °C for 4–5 h (or overnight) to reverse cross-linking between protein and DNA. Take the input DNA sample and incubate at 65 °C for 4–5 h (or overnight) to reverse cross-linking.
6. Precipitate the DNA by adding 2.5 volumes of ice-cold ethanol to each of the tubes and incubate at –20 °C overnight.

7. Centrifuge the DNA at 14,000 rpm for 15 min at 4 °C. Remove the residual ethanol and air-dry the DNA pellet.
8. Dissolve the pellet in 100 μl TE. The sample may be viscous and may have to be dissolved in a larger volume. Add 25 μl of 5× PK buffer and 1.5 μl of Proteinase K to each sample. Incubate for 1–2 h at 45 °C.
9. Add 175 μl of TE to each sample. Extract with 300 μl of phenol–chloroform–isoamyl alcohol.
10. Add 30 μl of 5 M NaCl, 5 μg of tRNA and 5 μg of glycogen to each sample. Mix well and then add 750 μl of ice-cold ethanol. Incubate at −20 °C overnight.
11. Next day, centrifuge the tubes at 14,000 rpm for 20 min at 4 °C. Aspirate the supernatant carefully and air-dry the DNA pellet. Resuspend the DNA pellet in 30 μl of TE. Use 2–3 μl for PCR analysis. For the total input sample, dilute the sample 300 fold and then perform the PCR reaction [31, 32].

3.3 Alternate Protocols for ChIP Assay

3.3.1 Preparation of ChIP Lysate

1. Perform **steps 1–3** as detailed in Subheading 3.1.1.
2. Resuspend the pellet in equal volume 200 μl of ChIP lysis buffer
3. Add an equal volume of glass beads.
4. Vortex at the maximum setting six times for 30 s at 4 °C each with 3 min pauses between each vortex.
5. Pierce the bottom of the microcentrifuge tube with a needle and place the tube carefully in another microcentrifuge tube. Spin at 700 × *g* for 2 min. The liquid should move through the first microcetrifuge tube into the bottom tube.
6. Add 200 μl of ChIP lysis buffer and sonicate the samples as described in Subheading 3.1.1, **steps 5** and **6**.

3.3.2 Fragmentation of Chromatin Using Micrococcal Nuclease (MNase)

1. Prepare nuclear pellet from cells as detailed in refs. 9 and 36.
2. Resuspend the nuclear pellet in 1 ml MNase digestion buffer and place on ice
3. Aliquot two 1.5 ml eppendorf tubes with 500 μl of resuspended nuclei.
4. Add 1 μl of micrococcal nuclease (Mnase) enzyme to each tube and mix gently.
5. Incubate the tubes in a 37 °C water bath for 10 min. The incubation times as well as MNase concentrations have to be optimized to generate mainly trinucleosomes, dinucleosomes, and mononucleosomes.
6. Add 20 μl of stop solution. Chill on ice.
7. The suspension was centrifuged at 13,000 × *g* for 20 s. The supernatant comprised of the second soluble fraction S2 (containing larger fragments of chromatin), whereas the pellet

contained the smaller fragment S1 fraction. The pellet was resolubilized in lysis buffer and pooled with the S2 fraction in a fresh clean tube.

8. Continue the ChIP assay as described in Subheading 3.1.1 from **step 6** onwards.

3.4 ChIP Assay on Tissues

1. Chop the tissues with a razor blade or scalpel into small pieces and resuspend them in serum free media. Add 1 % formaldehyde (some studies have used 0.4 % formaldehyde) and rotate the tubes for 10 min at room temperature. ChIP assays can also be performed on frozen tissues after dry pulverization using liquid nitrogen [37–39].
2. Stop the cross-linking reaction by adding glycine to a final concentration of 0.125 M. Rotate at room temperature for 5 min.
3. Centrifuge the samples at 250 × *g* for 5 min and discard the supernatant. Wash the sample twice in PBS. Resuspend the chopped tissue in 1–2 ml PBS. Homogenize the tissue on ice using a Dounce homogenizer or Polytron Homogenizer. Centrifuge the sample at 250 × *g* to collect all the cells; discard the supernatant.
4. Follow the standard ChIP protocol as detailed in Subheading 3.1.1, **step 5** onwards [34, 40, 41].

4 Notes

1. Chromatin immunoprecipitation is a powerful approach that allows the elucidation of interactions between endogenous proteins and their native chromatin sites, thereby providing insights into physiological transcription [42–47] and transactivation mechanisms [3, 48, 49]. ChIP assays have been extensively used to study histone acetylation and methylation patterns at a variety of genomic loci [2, 5, 9, 36, 48, 50]. In addition, ChIP methodology has been coupled to microarrays (ChIP-chip) containing genomic regions to facilitate genome wide binding patterns of transcription factors to specific promoters [1, 2, 26]. Procedures by Ren et al. involve immunoprecipitation of the DNA by ChIP assays is blunt-ended by T4 DNA polymerase to allow ligation of a universal linker (LM-PCR) [13, 51]. The ChIP-enriched DNA and input DNA are then labeled with different fluorescent dyes and hybridized to a single DNA microarray containing promoter sequences (for detailed description of the method *see* refs. [9, 13, 23, 24, 26, 51]).

   Despite the many advantages of the ChIP assay, it can be fraught with many obstacles. The first important thing to keep in mind is to make the lysates using large number of cells

(at least 2×10^7) to achieve a good signal to noise ratio in conventional ChIP assays. However, recent studies report use of very small cell numbers (as low as 100 or 1,000) in ChIP assays to successfully profile histone modifications from embryonal carcinoma cells and biopsies [52]. This method, referred to as micro (μ) ChIP enables us to study DNA–protein interactions from rare cell samples and small tissue biopsies (for detailed protocols *see* refs. 53, 54). Efficient lysis of cells is another important parameter for a successful ChIP assay. It may be a good idea to check the efficacy of cell lysis using trypan blue exclusion method. In case cells are not being efficiently lysed, use a different lysis buffer. A list of commonly used lysis buffers are given below.

Other lysis buffers which have been reported in literature

(a) /http://genomecenter.ucdavies.edu/farnham/farnham/protocols/chips.html [31].
5 mM PIPES, pH 8.0, 85 mM KCl, 0.5 % NP-40, protease inhibitors

(b) 50 mM Tris, pH 8.1, 150 mM NaCl, 5 mM EDTA, 0.1 % sodium deoxycholate, 1 % Triton X-100, protease inhibitors [30].

(c) /http://www.biochem.northwestern.edu/ibis/morimoto/Protocols/
50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 % SDS, 2 % Triton X-100, 1 mM EDTA, protease inhibitors [55].

(d) 250 mM sucrose, 10 mM Tris–HCl, pH 7.4, 10 mM Na-butyrate, 4 mM magnesium chloride, 0.1 M PMSF, 0.1 M benzamidine, 0.1 %w/v Triton X-100, protease inhibitors [15].

2. Moreover, cells can be homogenized on ice after addition of cell lysis buffer to facilitate the release of nuclei. In case of heterogeneous samples, it is essential that the cells be sorted to enrich the cell population of interest before performing the ChIP assay. To analyze histone acetylation, add 10 mM sodium butyrate to the lysis buffer, dilution and elution buffers. Sodium butyrate prevents the loss of histone acetylation during sample preparation by inhibition of histone deacetylase activity.
3. It is essential to perform all steps of cell lysis and immunoprecipitation on ice or at 4 °C, wherever indicated to prevent chromatin degradation by endogenous nucleases. Similarly, protease inhibitors should be added wherever mentioned to preserve the integrity of proteins bound to the chromatin.
4. Another crucial step in the ChIP assay is the sonication of DNA. It is essential that sonication should cause efficient shearing of the

genomic DNA to about 1 kb in size. The efficiency of sonication should be checked by agarose gel electrophoresis when the ChIP assays are being standardized with new cell lines or sonicators. It must be remembered that sonication of cross-linked chromatin gives rise to randomly sheared DNA fragments, which may not produce small enough chromatin fragments at the region of interest. Furthermore, cross-linking the DNA to the protein by formaldehyde may mask critical protein epitopes resulting in poor efficiency of immunoprecipitation. On the other hand, a disadvantage of native ChIP is that certain protein–DNA interactions are transient or intrinsically unstable, precluding detection unless the chromatin–DNA complexes are trapped by cross-linking. The selection of conventional ChIP assay versus native ChIP assay depends upon the nature of the experimental system. Whereas the native ChIP is particularly suited for proteins like core histones that are tightly bound to chromosomes, the conventional ChIP assay works better for studying linker histones, transcription factors, and proteins which may relocalize or dissociate during sample preparation [16, 56].

5. Although the use of PCR ensures a high degree of signal amplification, a low efficiency in the immunoprecipitation reaction can give rise to nonspecific signals or no signal. Hence, it is essential to use 2–4 μg of a high-affinity, high specificity antibody for the immunoprecipitation reaction. Pre-clearing the lysates before immunoprecipitation is a good way to improve specificity and minimize nonspecific background in the ChIP assay (Methods, Subheading 3.2.2). However, we have found that even in the absence of pre-clearing, the ChIP assay works provided all other safeguards are carefully followed. Furthermore, one may have to try different antibodies to optimize the ChIP assay because certain antibodies may efficiently immunoprecipitate the native protein but may be not be effective in immunoprecipitating proteins cross-linked to chromatin. The exact conditions of the immunoprecipitation may have to be further optimized to ensure a high recovery of target DNA during the IP. It is crucial to incorporate a positive control as well an irrelevant antibody in the ChIP assay to facilitate critical interpretation of data.
6. After the immunoprecipitation reaction, efficient and stringent washing is critical to reduce nonspecific background. The number of washes can be increased if necessary. The wash buffer can be varied according to the nature of antibody or conditions of immunoprecipitation. Some laboratories use 2× PBS as the wash buffer, others wash the immune complexes sequentially with 1 ml of the following buffers: Low-salt wash buffer, High-salt wash buffer, LiCl wash buffer, and 1× TE.

A list of wash buffers used by different laboratories is given below

(a) /http://genomecenter.ucdavies.edu/farnham/farnham/protocols/chips.html [31].
100 mM Tris–HCl, pH 9.0 (8.0 for monoclonal antibodies), 500 mM LiCl, 1 % NP-40, 1 % sodium deoxycholate.

(b) 250 mM sucrose, 10 mM sodium butyrate, 4 mM magnesium chloride, 0.1 mM PMSF [15].

(c) *Low-Salt Wash Buffer:* 20 mM Tris–HCl, pH 8.1, 150 mM NaCl, 0.1 % SDS, 1 % Triton X-100, 2 mM EDTA [31, 57].

(d) *High-Salt Wash Buffer:* 20 mM Tris–HCl, pH 8.1, 500 mM NaCl, 0.1 % SDS, 1 % Triton X-100, 2 mM EDTA.

(e) *LiCl Wash Buffer:* 10 mM Tris–HCl, pH 8.0, 0.25 mM LiCl, 1 % NP-40, 1 % deoxycholate, 1 mM EDTA.

7. It is important to be extremely careful and meticulous during this assay. The amplification by PCR is highly sensitive; it will amplify even minimal nonspecific signals. We recommend all possible precautions to prevent contamination from other DNA sources: amplification in a dedicated space, use of fresh gloves, sterile filter tips, and clean microfuge tubes for the assay.

8. Recent studies involving ChIP assays have used real-time PCR for detecting as little as twofold changes in protein–DNA interactions. Real-time PCR allows data to be collected during cycles when amplification is occurring exponentially [58]. It increases the precision of ChIP measurements allowing accurate detection of small variations in protein–DNA interactions [33].

   Although quantitative real-time PCR is a very sensitive and fast method that can be employed to analyze immunoprecipitated DNA fragments obtained after ChIP assay, ChIP-qPCR data needs to be normalized for sources of variability including amount of chromatin, efficiency of immunoprecipitations and DNA recovery.

   There are two commonly used methods to normalize ChIP-qPCR data: Percent input method and fold enrichment method

   (a) Percent input method

   The percentage of immunoprecipitations of a particular genomic locus can be calculated from qPCR data and represented as a percentage of starting material or input. This can be measured using the following formula:

$$\%(\text{ChIP} / \text{Total input}) = 2\,\hat{}\left[\left(\text{Ct}(\text{x\%input}) - \log(\text{x\%}) / \log 2\right) - \text{Ct}(\text{ChIP})\right] \times 100\%$$

   where “2” is the amplification efficiency (Amplification efficiency (AE) of the primer set which can be calculated by using tenfold serial dilutions of input DNA in qPCR

and using the following formula AE = 10^(−1/slope)); Ct (ChIP) and Ct (x%input) are threshold values obtained from exponential phase of qPCR for the immunoprecipitated DNA sample and input sample respectively; the compensatory factor (logx%/log2) is used to take into account the dilution 1:x of the input. The recovery is the % (ChIP/Total input).

(b) Fold enrichment method

This method of interpretation of the ChIP-qPCR data is also called "signal over background" or "signal over noise." In this method, the ChIP signals (Ct(ChIP)) are divided by the no-antibody signals Ct(mockIgG), representing the ChIP signal as the fold increase in signal relative to the background signal [59]. This can be measured using the following formula:

$$\text{Fold enrichment} = 2 \wedge -\left(\text{Ct}\left(\text{mockIgG}\right) - \text{Ct}\left(\text{ChIP}\right)\right)$$

This method is not suitable in many ChIP experiments since background signal levels do vary between primer sets, samples, and experiments. For example, in ideal ChIP experiments there should not be any detectable level of signal from the mock IgG IP, in such cases this method cannot be followed for calculating fold enrichment.

It is recommended to determine the occupancy of specific factors on negative as well as positive genomic regions to confirm the specific recruitment of the transcription factor to a particular genomic region. Percentage of enrichment of specific factors or epigenetic signatures at specific locus can be normalized based on the enrichment on a known/positive control region [60].

9. Another possibility is to perform duplex amplification PCR [61, 62], which involves co-amplification of a fragment from the region of interest along with a control fragment. Applications, in particular for allelic studies, or on genomic imprinting in mammals, one can apply SSCP [63, 64] or hot-stop PCR [65], especially to differentiate silent alleles from the active alleles. The different PCR-based approaches have been reviewed in refs. 3, 4, 66.

10. Sequential ChIP or ChIP-re-ChIP methods have been employed to examine the interaction of two or more proteins probably as a complex in a specific genomic region. Although this method detects probable physical interaction of proteins, it does not imply direct protein–protein interaction. For sequential ChIP, one of the chromatin associated factor is first immunoprecipitated using a specific antibody and the eluted material is then subjected to another round of immunoprecipitation using a second antibody. If the proteins recognized by both

the antibodies are co-occupied in the same genomic region, the recovered DNA can then be subjected to PCR amplification or high throughput sequencing methods [67–69]. Our lab has successfully employed ChIP-re-ChIP to identify association of acetylated E2F1 on angiogenic promoters FLT1 and KDR upon VEGF stimulation of endothelial cells [70].

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Chapter 26

ChIP on Chip and ChIP-Seq Assays: Genome-Wide Analysis of Transcription Factor Binding and Histone Modifications

Smitha Pillai and Srikumar P. Chellappan

Abstract

Deregulation of transcriptional activity of many genes has been causatively linked to human diseases including cancer. Altered patterns of gene expression in normal and cancer cells are the result of inappropriate expression of transcription factors and chromatin modifying proteins. Chromatin immunoprecipitation assay is a well-established tool for investigating the interactions between regulatory proteins and DNA at distinct stages of gene activation. ChIP coupled with DNA microarrays, known as ChIP on chip, or sequencing of DNA associated with the factors (ChIP-Seq) allow us to determine the entire spectrum of in vivo DNA binding sites for a given protein. This has been of immense value because ChIP on chip assays and ChIP-Seq experiments can provide a snapshot of the transcriptional regulatory mechanisms on a genome-wide scale. This chapter outlines the general strategies used to carry out ChIP-chip assays to study the differential recruitment of regulatory molecules based on the studies conducted in our lab as well as other published protocols; these can be easily modified to a ChIP-Seq analysis.

Key words Whole-genome amplification, Transcription factors, Histone modifications, Promoter arrays, Next-generation sequencing

1 Introduction

DNA-binding proteins perform a variety of important functions in cells, including transcriptional regulation, chromosome maintenance, replication, and DNA repair. The interactions between transcription factors and their DNA binding sites are an integral part of transcriptional regulatory networks. These interactions control the coordinated expression of thousands of genes during normal growth and in response to external stimuli. Studies on the sequence-specific binding of transcription factors to promoter DNA were provided a major boost by the development of electrophoretic mobility shift assays (EMSAs or gel-shift assays) three decades ago [1, 2]. While this technique provided the capability to analyze the binding of proteins to DNA in vitro, it could not provide a snapshot of the binding status in the intact cells.

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Development of chromatin immunoprecipitation assays (ChIP assays), where DNA bound proteins are cross-linked to DNA in the living cells and analyzed by PCR provided the ability to examine the binding of proteins to specific targets in vivo in response to specific signals or during normal development. Further, these assays also enabled the analysis of histone modifications and contributed significantly to the elucidation of the histone code and the epigenetic regulation of gene expression.

Given the ability of chromatin immunoprecipitation assays to provide a picture of the promoter occupancy of protein in an in vivo situation, attempts were made to extend this technology to genomewide analysis of DNA binding and histone modifications [3, 4]. Recent advances in DNA microarray technology as well as the availability of whole-genome sequences make it possible to map the entire spectrum of interactions of a particular protein with specific DNA sequences across the whole genome. This is achieved by an immunoprecipitation of the protein of interest cross-linked to its target DNA sequences; the bound fragments are identified by a microarray based analysis of enriched DNA fragments. This technique, referred to as ChIP on chip or genome-wide location analysis is one of the widely used methods for identifying in vivo transcription factor binding sites in a high-throughput manner [5–9]. In conventional ChIP assays, the cells are treated with formaldehyde to covalently cross-link protein and the bound DNA [10, 11]; the cells are then lysed and the chromatin is sheared into 200–1,000 base pair fragments by sonication [10] or treatment with micrococcal nucleases [12, 13]. An antibody specific for a protein of interest is then used to immunoprecipitate protein bound DNA fragments; the associated DNA is de-cross-linked by incubating in a low pH buffer, protease treated and the associated DNA is examined for the presence of the promoter or DNA sequence of interest using appropriate PCR primers [14]. This technique, as can be imagined, used to analyze the association of specific transcription factors, or modified histones with DNA. The ChIP-on-chip version of this protocol differs only in the post IP steps. In this case, the immunoprecipitated DNA is amplified using a random primed PCR or the immunoprecipitated DNA is blunt ended by T4 DNA polymerase to allow ligation of a universal linker (LM-PCR) or whole-genome amplification method (WGA) and subsequently labeled and hybridized into DNA microarrays [15–18].

The extent of ChIP-chip application depends, in part, on the development of microarray technology, especially the availability of arrayed slides for DNA from human, mouse, or other model organisms. There are two different types of DNA microarrays available; the first type is created by spotting DNA onto polylysine modified glass slides using pin spotting robots [19]. The density of spotted

arrays is limited to 20,000–30,000 features since the feature sizes below 100 μm are difficult to achieve [20]. A different type of oligonucleotide array is available which is created by synthesizing oligomers in situ on the array by photolithography. These chips marketed by Affymetrix can be of higher density containing up to several million features [21]. Today, arrays representing promoter regions, CpG islands, entire chromosomes or entire genomes are used for ChIP-chip assays [22–25]. High-density oligonucleotide arrays that tile the entire human nonrepetitive genomic sequences are available from Affymetrix, NimbleGen Systems, and Agilent Technologies (*see* **Notes 1** and **2**).

The major applications of ChIP-chip assays are the identification of binding targets for transcription factors and identification of posttranslational modifications on DNA-associated proteins including specific histone modifications. Initial studies using ChIP-chip technology were directed to detect transcription factor–chromatin binding in the yeast *S. cerevisiae* [26–29]. ChIP-chip technology was later applied to mammalian systems to study the recruitment of transcription factors such as E2F, GATA1, and Rb, but these studies surveyed only a small fraction of the genome due to the limitations in the microarray technology and also the incomplete information on genome annotation [24, 30, 31]. The recent advances in DNA microarray synthesis technology and the completion of human genome project revealing more promoter regions has allowed extensive screening of the genome for the binding position of protein factors. In recent years ChIP-chip has been expanded to investigate posttranslational modifications of DNA binding proteins. Different strategies can be employed to study histone modifications using ChIP-chip technology. One is to detect the distribution of histone modifications using antibodies specifically targeting the modifications [7] and the other is using ChIP-chip to locate the enzymes that catalyze the histone modification reactions [32]. Another useful application of these kinds of studies would be to identify genes silenced by histone modification in carcinogenesis [23]. It is also possible to combine the ChIP-chip data with expression profiling to establish the correlation of histone markers with transcriptional activity [7, 33].

ChIP-on-chip assays can be done on tissue culture cells or from tissue samples. The assay involves multiple steps, each of which might require standardization based on the specific experimental conditions and antibody used (Fig. 1). We have utilized ChIP-chip assays to study the global analysis of binding of proteins of interest to promoter regions in response to specific signaling events, using Affymetrix promoter arrays (GeneChip human promoter 1.0R array, Affymetrix, cat. no. 900775). The protocol we have successfully used is as follows:

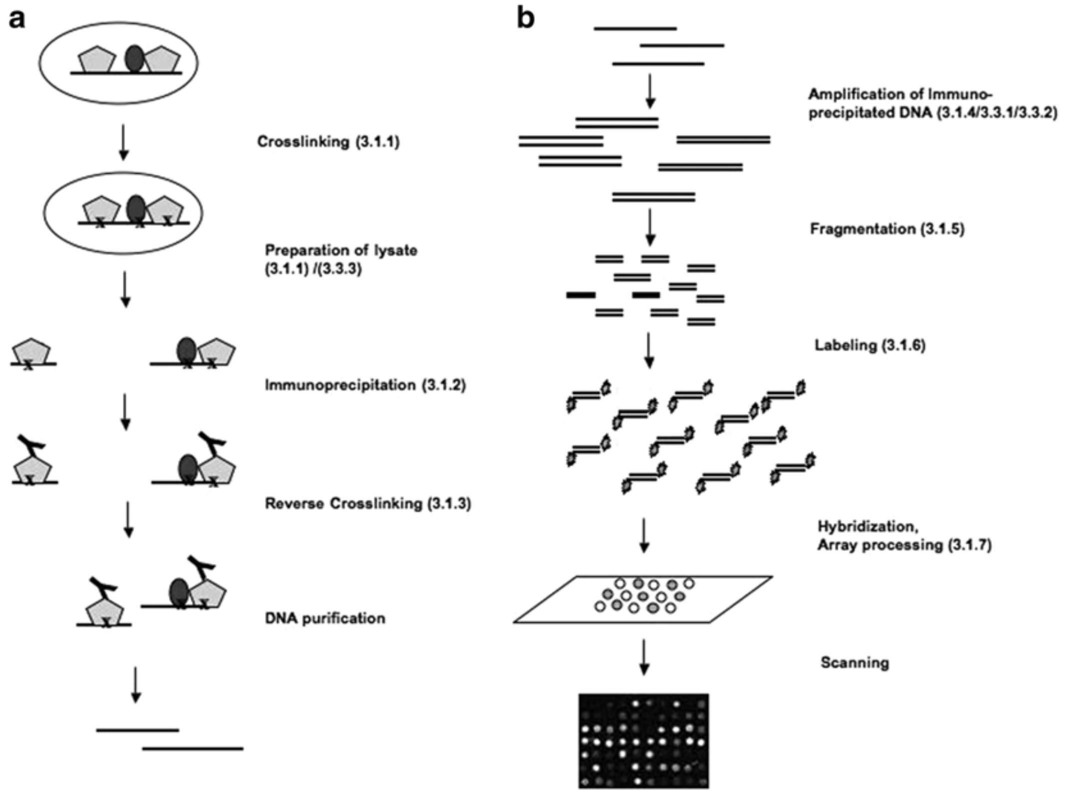

Fig. 1 Schematic of ChIP on chip workflow. (**a**) Chromatin immunoprecipitation. Cells are grown under the desired experimental conditions and protein–DNA cross-links are created by treating the cells with formaldehyde. Cells are lysed, DNA is sheared either by sonication or by micrococcal nuclease and immunoprecipitated with antibody against protein of interest. Cross-links are reversed and DNA is purified. (**b**) ChIP-chip experiment. The immunoprecipitated DNA is amplified either by random primed PCR or Ligation mediated PCR or Whole-Genome Amplification. The amplified DNA is fragmented, labeled with a fluorophore and hybridized to a microarray and analyzed

2 Materials

1. Formaldehyde (37 %) (Molecular Biology Reagent Grade; Sigma-Aldrich).
2. Glycine, 1 M: 3.75 g of tissue grade glycine (Fischer Biotech) in 50 ml of water.
3. 20× Phosphate buffered saline (PBS): 160 g NaCl, 4 g KCl, 2.88 g Na_2HPO_4, 4.8 g KH_2PO_4 in 800 ml of water. Adjust the pH of the buffer to 6.4 and make up the volume to one liter.
4. IGEPAL CA-630 (Sigma-Aldrich).
5. 100 mM PMSF: 0.174 g of PMSF (Sigma-Aldrich), in 10 ml of absolute ethanol. Aliquot into microcentrifuge tubes and

store at −20 °C. Add PMSF to the lysis buffer just before use as the half-life of 1 mM PMSF in the buffer is 30 min.

6. Protease Inhibitor Stock: Prepare a 25× stock by dissolving 1 protease inhibitor tablet (Roche, cat. no. 11873580001) in 2 ml of nuclease-free water.
7. Proteinase K (Fischer Biotech, cat. no. BP-1700-100).
8. LiCl (10 M): 42.39 g LiCl (Sigma-Aldrich cat. no. L-8895) in 100 ml of water.
9. Protein G Sepharose 4 Fast Flow (GE healthcare, cat. no. 17-0618-01).
10. Sequenase Version 2.0 DNA polymerase (USB, cat. no. 70775Y).
11. Primer A: 200 μM GTTTCCCAGTCACGGTC$(N)_9$ (HPLC Purified).
12. Primer B: 100 μM GTTTCCCAGTCACGGTC (HPLC Purified).
13. Taq Polymerase 5 U/μl (Invitrogen, cat. no. 10342-020).
14. 100 mM dNTPs (Promega, dATP—cat. no. U120-B, dCTP—cat. no. U121B, dGTP—cat. no. U122B, dTTP—cat. no. U123B).
15. 100 mM dUTP (Promega, cat. no. U119A).
16. BSA (20 mg/ml): 100 mg of BSA (Sigma-Aldrich cat. no. A3059-50G) in 5 ml of water. Aliquot 100 μl into 1.5 ml tubes and store at −20 °C.
17. 1 M DTT (Dithiothreitol): 1.54 g DTT (Fisher Biotech cat. no. BP172-5) in 10 ml of water. Aliquot 100 μl into 1.5 ml tubes and store in −20 °C.
18. 5 M NaCl: 58.4 g NaCl in 200 ml of water.
19. 1 M $MgCl_2$: 20.3 g of $MgCl_2 \cdot 6H_2O$ in 100 ml of water.
20. 1 M $CaCl_2$: 14.7 g of $CaCl_2 \cdot 2H_2O$ in 100 ml water.
21. Deoxycholate (sodium salt) (Sigma-Aldrich cat. no. 21115).
22. GeneChip WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix, cat. no. 900812).
23. GeneChip sample cleanup module (Affymetrix, cat. no. 900371).
24. Control oligonucleotide B2, 3 nM (Affymetrix, cat. no. 900301).
25. GeneChip hybridization, wash and stain kit (Affymetrix, cat. no. 900301).

2.1 Buffers

1. Lysis Buffer: 10 mM Tris–HCl (made from stock 1 M Tris–HCl pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 4 % IGEPAL, 1 mM PMSF (add fresh). Store at room temperature.

2. IP Dilution Buffer: 20 mM Tris–HCl (made from stock 1 M Tris–HCl pH 8), 2 mM EDTA, 1 % Triton X-100, 150 mM NaCl, Protease Inhibitor Stock (add fresh). Store at room temperature.
3. Protease Inhibitor Stock: Prepare a 25× stock by dissolving 1 protease inhibitor tablet (Roche, cat. no. 11873580001) in 2 ml of nuclease-free water. Store the aliquots at −20 °C.
4. ChIP Wash 1: 20 mM Tris–HCl (made from stock 1 M Tris–HCl pH 8), 2 mM EDTA, 1 % Triton X-100, 150 mM NaCl, 1 mM PMSF (add fresh). Store at room temperature.
5. ChIP Wash 2: 20 mM Tris–HCl (made from stock 1 M Tris–HCl pH 8), 2 mM EDTA, 1 % Triton X-100, 0.1 % SDS, 500 mM NaCl, 1 mM PMSF (add fresh). Store at room temperature.
6. ChIP Wash 3: 10 mM Tris–HCl (made from stock 1 M Tris–HCl pH 8), 1 mM EDTA, 0.25 M LiCl, 0.5 % IGEPAL, 0.5 % Deoxycholate (sodium salt). Store at room temperature.
7. TE (10 mM Tris–HCl pH 8, 1 mM EDTA).
8. Elution Buffer: 25 mM Tris–HCl (made from stock 1 M Tris–HCl pH 7.5), 10 mM EDTA, 0.5 % SDS. Store at room temperature.
9. Buffers for Hybridization:
   (a) 12× MES stock buffer (1.22 M MES, 0.89 M Na+): MES hydrate (64.61 g), MES sodium salt (193.3 g), MilliQ Water (800 ml). Adjust the pH between 6.5 and 6.7. Adjust the volume to 1,000 ml. Do not autoclave. Filter-sterilize by passing through 0.2 μm filter. Store at 2–8 °C away from light.
   (b) 2× hybridization buffer (100 mM MES, 1 M (Na+), 20 mM EDTA, 0.01 % Tween-20): 12× MES stock buffer (8.3 ml), 5 M NaCl (17.7 ml), 0.5 M EDTA (4 ml), 10 % Tween 20 (100 μl). Make up the volume to 50 ml with water and store at 2–8 °C away from light.
   (c) Wash buffer A: Non-stringent wash buffer (6× SSPE, 0.01 % Tween 20): 20× SSPE (300 ml), 10 % Tween 20 (1 ml). Make up the volume to 1 l and filter-sterilize using 0.2 μm filter.
   (d) Wash buffer B (100 mM MES, 0.1 M Na+, 0.01 % Tween 20): 12× MES stock buffer (83.3 ml), 5 M NaCl (5.2 ml), 10 % Tween 20 (1 ml). Make up the volume to 1 l, filter through 0.2 μm filter and store at 2–8 °C protected from light.
   (e) 2× stain Buffer (100 mM MES, 1 M Na+, 0.05 % Tween 20): 12× MES stock buffer (41.7 ml), 5 M NaCl (92.5 ml),

10 % Tween 20 (2.5 ml). Make up the volume to 250 ml, filter through 0.2 μm filter and store at 2–8 °C protected from light.

2.2 Instruments and Software Required

1. Genechip Scanner 3000 7G.
2. GeneChip Operating Software (GCOS) v1.3 or higher.
3. GeneChip Fluidics Station 400 or 450.
4. Tiling array software for data analysis.

3 Methods

3.1 Preparation of ChIP Lysate from Cells

1. ChIP assays require large amounts of cells as the starting material. Usually, for each IP reaction 50–200 million cells are recommended [34–36]. For a ChIP on chip experiment at least two IP reactions should be done; an experimental sample where IP is done using antibody against the protein of interest and a negative control with a nonspecific antibody or a mock IP with no antibody. Add formaldehyde directly to the tissue culture plate to a final concentration of 1 % (280 μl of 37 % formaldehyde/10 ml of media) and incubate in an orbital shaker for 10 min for cross-linking DNA to the protein (*see* **Note 3**).
2. Add glycine to a final concentration of 0.125 M using a 1 M stock solution and inoculate for 5 min in the orbital shaker to terminate the cross-linking.
3. Aspirate the formaldehyde and media into a waste container, and wash the cells twice with 10 ml of ice-cold 1× PBS. In case of adherent cells, scrape the cells in 3–5 ml of ice-cold 1× PBS and transfer into a 15 ml centrifuge tube. If the cells are non-adherent, collect them along with the media in a 15 ml centrifuge tube. Centrifuge the cells at 800×*g* (1,000 rpm) for 5 min in a tabletop centrifuge with a swing bucket rotor (ThermoForma, General purpose centrifuge) at 4 °C to collect the cells. Wash the cell pellet twice with ice-cold 1× PBS and centrifuge at 800×*g* (1,000 rpm) for 5 min in a refrigerated tabletop centrifuge at 4 °C. Discard the supernatant and proceed to the next step or snap-freeze the pellet and store at −80 °C.
4. Resuspend the pellet in 1.5 ml ChIP lysis buffer containing 60 μl PMSF. To the tube add 40 μl of 100 mM PMSF, 100 μl of 25× protease inhibitor stock, 460 μl of Lysis buffer, 100 μl of 20 % SDS, 80 μl of 5 M NaCl and 220 μl of nuclease free water to bring the total volume to 2.5 ml. Mix the cells using a pipetman to obtain a uniform homogenate. Transfer the lysate equally (800 μl) into 1.5 ml centrifuge tubes.

5. Sonicate the samples to lyse the cells and shear the DNA to 100–1,000 bp fragments. The time and number of pulses required to shear DNA depends on the cell type, extent of cross-linking and the instrument used for sonication. We have observed that for most adherent cells three to four cycles of sonication at power four for 30 s on a Fisher Sonic Dismembrator (Model 100), followed by incubation on ice for 30 s is sufficient to shear the chromatin. However, number of pulses needed to shear DNA depends on cell density and cell type. Some cells are resistant to sonication treatment; micrococcal nuclease treatment may improve chromatin shearing in such cell lines [12, 14, 37–39]. Refer alternate protocol for micrococcal nuclease treatment.
6. Transfer the sonicated samples into 1.5 ml tubes and centrifuge at 17,000 × *g* for 15 min in a refrigerated microcentrifuge (Beckman-Coulter, Microfuge R, or an equivalent model) to remove the cell debris. The samples can be snap-frozen and stored at −80 °C or proceed directly to the immunoprecipitation step.
7. Sonication efficiency can be checked by de-cross-linking 100 μl of the sample and loading in a 1 % agarose gel.

3.2 Setting Up Immunoprecipitation Reaction

1. Thaw the frozen ChIP lysates on ice. Transfer lysate from three 1.5 tubes into one 15 ml tube and add 5 volumes of IP dilution buffer containing freshly added protease inhibitors (Roche).
2. Use 10–15 μg of antibody per immunoprecipitation reaction. The amount of antibody to be added depends on quality, affinity and specificity of the antibody (*see* **Note 4**). Usually a negative control is performed using the same number of cells with a nonspecific IgG or no antibody control (*see* **Note 5**) Preclearing ChIP lysate is recommended to reduce the nonspecific backgrounds and eliminate false positives. For preclearing protocols *see* **step 1**.
3. After adding the appropriate amount of antibody, rotate the reaction overnight at 4 °C.
4. The antibody bound protein–DNA complexes can be recovered using Protein A (Amersham Biosciences), Protein G (Amersham Biosciences), ProteinA/G (Santa Cruz Biotechnology), or Staph A (ATCC) cells. The choice of the beads depends on the isotype of the antibody to be used for the immunoprecipitation and their affinity for protein A, protein G or protein A/G. Protein G is used for most of the applications in our laboratory. Add the required volume of protein G beads containing 20 % ethanol (GE healthcare) to a microcentrifuge tube and centrifuge at 800 × *g* (300 rpm) for 1 min. Discard the

supernatant, add 1 ml of wash buffer to the tube, mix well and centrifuge. Discard the supernatant and repeat the washings for three times with wash buffer to remove the ethanol preservative. Add 200 μl of 1:1 Protein G slurry or 400 μl of Protein A slurry to the IP reaction.

5. Add PMSF to the IP reaction to a final concentration of 1 mM. Incubate IP reaction with the beads at room temperature for 2–4 h.
6. Centrifuge the tubes at 800 × *g* (3,000 rpm) at 4 °C (Beckman-Coulter, Microfuge R or an equivalent model) for 1 min. Discard the supernatant by aspirating carefully to avoid losing beads while aspirating.
7. Add 700 μl of wash buffer 1 containing freshly added PMSF, mix well and transfer to a new microcentrifuge tube. Rotate the tube in a nutator at room temperature for 1 min. Centrifuge at 800 × *g* for 1 min and discard the supernatant.
8. Repeat the **steps** 7 and **8**.
9. Add 700 μl of wash buffer 2 containing PMSF, rotate in a nutator for 5 min and centrifuge at 800 × *g* for 1 min.
10. Discard the supernatant, add 700 μl of wash buffer 3, rotate the tubes in nutator for 5 min and centrifuge at 800 × *g* for 1 min.
11. Discard the supernatant; wash the beads with 700 μl of TE.
12. Rotate the tubes in nutator for 1 min. Centrifuge at 800 × *g* for 1 min; discard the supernatant.
13. Repeat washing with TE, centrifuge at 800 × *g* for 1 min, discard the supernatant.
14. Add 200 μl of elution buffer to the beads and incubate at 65 °C for 30 min.
15. Centrifuge at 2,000 × *g* (5,000 rpm) for 2 min. Transfer the supernatant into a new tube. This is the immunoprecipitated sample.

3.3 Reverse Cross-Linking

1. Add 10 μl of proteinase K (20 mg/ml) to 200 μl of the sample.
2. Incubate overnight in heat block at 65 °C.
3. Clean up the de-cross-linked samples using Affymetrix cDNA cleanup columns. Elute twice with 25 μl Elution buffer.

3.4 Amplification of Immunoprecipitated DNA

A single chromatin immunoprecipitation sample does not provide enough DNA for hybridization into a promoter array (*see* **Notes 6–9**). Commonly used techniques for amplifying the immunoprecipitated DNA are random primed PCR amplification, LM-PCR (*see* Alternate protocol 3.10.1) and WGA (*see* Alternate protocol 3.10.2).

Random primed PCR amplification of immunoprecipitated DNA.

1. Use sequenase enzyme for linear amplification of immunoprecipitated DNA. Dilute sequenase stock with sequenase dilution buffer to 1.3 U/μl. Four microliters of the 1.3 U/μl working stock will be needed for each sample being amplified. For initial round of linear amplification use 10 μl of immunoprecipitated DNA.

   Set up eight reactions (four with immunoprecipitated sample and four with the control IP) with the following components (volume shown is for one reaction) in 200 μl PCR tubes.

Purified DNA	10 μl
5× sequenase reaction buffer	4 μl
Primer A (200 μM)	4 μl
Total volume	18 μl

2. Cycle conditions for Random priming.
   (a) 95 °C for 4 min.
   (b) Snap cool samples on ice.
   (c) 10 °C on ice.
3. Prepare the cocktail using the following components (volume shown is for one reaction).

1. BSA (20 mg/ml)	0.1 μl
2. DTT (0.1 M)	1 μl
3. dNTPs (25 mM)	0.5 μl
4. Diluted sequenase (1/10 from 13 U/μl stock)	1 μl
5. Total volume	2.6 μl

   Prepare cocktail for eight reactions.
   (a) Add 2.6 μl of the cocktail to each sample; mix well by pipetting give a brief spin to bring down the components and keep the samples back in thermocycler.
   (b) 10 °C for 5 min.
   (c) Ramp from 10 to 37 °C over 9 min.
   (d) 37 °C for 8 min.
   (e) 95 °C for 4 min.
   (f) Snap cool on ice for 5 min.
   (g) 10 °C for 5 min.
   (h) Add 1 μl of diluted sequenase (1.3 U/μl) to each sample.
   (i) 10 °C for 5 min.
   (j) Ramp from 10 to 37 °C over 9 min.

(k) 37 °C for 8 min.

(l) Repeat from **steps 7** to **13** for two more cycles.

(m) 4 °C hold.

4. For each IP purify with Microspin S-300 HR (GE Healthcare) columns.
5. Set up PCR for incorporating dUTP into the immunoprecipitated DNA

   Prepare a dNTP mixture containing dUTP at concentrations indicated below.

dCTP	10 mM
dATP	10 mM
dGTP	10 mM
dTTP	10 mM
dUTP	10 mM

This mixture containing dNTP + dUTP can be stored at −20 °C.

Set up PCR using the following components:

Random primed DNA from the previous step	20 μl
10× PCR buffer	10 μl
50 mM $MgCl_2$	3 μl
10 mM dNTPs + dUTP	3.75 μl
100 μM Primer B	4 μl
Taq polymerase (5 U/μl)	2 μl
Nuclease free water	58.75 μl
Total volume	100 μl

Cycle conditions:

A

95 °C for 30 s.

45 °C for 30 s.

55 °C for 30 s.

72 °C for 60 s.

B

95 °C for 30 s.

45 °C for 30 s.

55 °C for 30 s.

72 °C for 60 s, Add 5 s for every subsequent cycles.

4 °C hold.

6. After PCR amplification, check amplified DNA on 1 % gel.
7. Purify PCR samples with Affymetrix cDNA cleanup columns, elute with 20 μl elution buffer.
8. Quantitate DNA using spectrophotometer or NanoDrop. Ideally, more than 5 μg of amplified.

DNA is obtained from each IP reaction. To check the differences between the IP and negative control samples, Real Time PCR should be performed using primer sets designed for DNA regions that are known to be specifically immunoprecipitated using the antibody used in the experiment.

3.5 Fragmentation of Amplified DNA

Components for fragmentation are available in GeneChip WT Double stranded DNA terminal labeling Kit (P/N 900812). The conventional method of fragmentation of DNA molecules utilizes DNase 1 to digest the DNA molecules, which produces fragments with 3′ –OH termini compatible for terminal labeling by TdT. However, a serious issue in using DNase1 in fragmenting DNA is that it may result in fragments with less than desired lengths. Recently, uracyl DNA glycosilase (UDG) in combination with apurinic or apyrimidinic endonuclease 1 (APE 1) has been successfully employed to generate DNA fragments. UDG specifically recognizes uracil and removes it by hydrolyzing the N-Cl′ glycosylic bond linking the uracil base to the deoxyribose sugar. The loss of the uracil creates an abasic site (also known as an AP site or apurinic/apyrimidinic site) in the DNA [40, 41]. Treatment of the sample DNA molecule with APE 1, will cause controlled breaks in the DNA at the abasic site. An apurinic/apyrimidinic endonuclease (APE1) can cleave the DNA molecule at the site of the dU residue yielding fragments possessing a 3′-OH termini, thus allowing for subsequent terminal labeling. The amount of dU incorporation may be regulated to determine the average length of fragments after UDG/APE 1 treatment. The ratio of dUTP to dTTP may be, for example, about 1–4, or about 1–5, 1–6, 1–10 or 1–20. The amount of dUTP incorporated depends on the ratio of dUTP to dTTP which in turn will decide the average size of fragments. The higher the ratio of dUTP to dTTP the more uracil incorporated and the shorter the average size of the fragments.

1. Set up the fragmentation reaction as follows (volume shown is for one reaction):

Double stranded DNA from the previous step	5.5–7.5 μg
10× cDNA fragmentation buffer	4.8 μl
UDG (10 U/μl)	1.5 μl
APE (100 U/μl)	2.25 μl
Nuclease free water	up to 48 μl

Mix the components and give brief spin.

2. Incubate the reactions at 37 °C for 1 h, followed by 93 °C for minutes and 4 °C for at least 3 min. Flick-mix the tubes and spin down briefly and transfer 45 μl to a new tube.
3. The remaining sample can be used for fragmentation analysis using a Bioanalyzer using RNA 6000 NanoLabChip Kit which will reveal the degree and uniformity of the fragmented products.

The fragmented samples can be stores at −20 °C until proceeding to the next step.

3.6 Labeling Fragmented Double-Stranded DNA

1. Set up the reaction by adding the following components [available in GeneChip WT Double stranded DNA terminal labeling Kit (P/N 900812)].

5× TdT buffer	12 μl
TdT	2 μl
DNA labeling reagent (5 mM)	1 μl
Total volume	15 μl

Add 15 μl of the labeling mix to the fragmented DNA samples. Flick mix the tube and spin down.

2. Incubate the labeling reaction at 37 °C for 1 h followed by 70 °C for 10 min and 4 °C for 3 min.
3. Use 2 μl of each sample for gel-shift analysis.

3.7 Hybridization and Array Processing

1. Prepare the hybridization mixture as follows.

Labeled DNA (5–7.5 μg)	60 μl
Control oligonucleotide B2 (50 pM)	3.3 μl
2× Hybridization mix	100 μl
DMSO	14 μl
Nuclease free water	To make up the total volume to 200 μl

The components for making the hybridization mixture are available in GeneChip hybridization, wash and stain kit (Affymetrix). For preparing buffers *see* Subheading 3.2, **step 2**.

Mix the components of the tube and give a brief spin.

2. Heat the mixture at 99 °C for 5 min, cool to 45 °C for 5 min and centrifuge at 17,000 × *g* for 1 min.
3. Inject 200 μl of the labeled sample into the array (GeneChip human promoter 1.0R array, Affymetrix) through one of the septa.

4. Keep the array in 45 °C hybridization oven, at 60 rpm, and incubate for 16 h.
5. After hybridization, remove the hybridization mix and store at −20 °C for future use.
6. For washing and staining Affymetrix arrays, the GeneChip Fluidics Station 450/250 or 400 is used and it is operated through GCOS. Fill the probe array completely with the appropriate volume of Non-stringent wash buffer (6× SSPE, 0.01 % Tween-20).
7. Select the experiment name from the drop-down experiment list in the fluidics station.
8. In the protocol drop down list, select FS450_0001 or FS450_0002 to control the washing and staining of the array.
9. To begin the washing and staining, choose RUN from the dialog box. Follow the instructions in the LCD window on the fluidics station.
10. Insert the appropriate probe array into the designated module of the fluidics station while the cartridge lever is in the eject position. The lever returns to the engaged position when finished.
11. Place a microcentrifuge tube (1.5 ml) containing 600 μl of SAPE solution mix/stain cocktail 1 in sample holder 1, one vial containing 600 μl of Antibody solution Mix/stain cocktail 2 in sample holder 2 and another vial containing 800 μl of Array holding buffer in sample holder 3.
12. Press down on the needle lever to snap needles into position and to start the run.
13. When the run is completed, remove the tubes containing the solutions and replace with fresh microcentrifuge vials.
14. Remove the array from the fluidics station by pressing down the lever to eject position.
15. Scan the array using GeneChip Scanner 3000 7G. Probe arrays can be stored for a short while at 4 °C, in the dark.
16. Turn the scanner on 10 min prior to use for warming up the laser.

3.8 Protocol Check Points

The protocol was successfully used for hybridization with Affymetrix human promoter 1.0 array in our lab. Protocol conditions should be optimized by each user depending on array type, cell type, specificity of antibodies, assay conditions, etc. The following steps are recommended as checkpoints to assess the success of the Immunoprecipitation protocol prior to performing hybridizations.

1. Visualization of sheared chromatin in a 1 % agarose gel. Ideally, 100–1,000 bp fragments should be obtained after successful sonication. Alternatively a bioanalyzer can be used to determine

the accurate size of the sheared DNA. Care should be taken not to overshear or oversonicate DNA as it may result in damaged DNA–protein complex and unsuccessful immunoprecipitation. The optimum sonication should be determined for each cell type.

2. Gene specific PCR of a known target to check IP enrichment. Validate the antibody with RNA polII to see whether the immunoprecipitation is working.
3. Monitor DNA yield after DNA cleanup and amplification of immunoprecipitated DNA preferably using NanoDrop. Unfortunately it does not provide information on the quality of amplified DNA.

3.9 Support Protocols

Preclearing of ChIP lysates is an efficient method to eliminate non-specific background in ChIP assay ([25, 42]) (*see* **Note 10**).

3.9.1 Preclearing of ChIP Lysate

1. Add 120 μl of 1:1 protein G slurry (or 400 μl of protein A) to the ChIP lysate (mentioned in Subheading 3.1) in a microcentrifuge tube.
2. Add 15 μl of 10 mg/ml salmon sperm DNA (Sigma-Aldrich, cat. no. D7696) and 10 μl of BSA (10 mg/ml).
3. Rotate on a nutator for 30 min at 4 °C. Spin the tubes at 17,000 × *g* for 30 min in a tabletop centrifuge at 4 °C.
4. Collect the supernatant and discard the beads. This supernatant fraction is used for the immunoprecipitation reaction described in Subheading 3.1, **step 2**.

3.10 Alternate Protocols

3.10.1 Amplifying Immunoprecipitated DNA by Ligation Mediated PCR

Blunting of DNA Ends Using T4 Polymerase

1. Take approximately 200 ng of immunoprecipitated DNA in a microcentrifuge tube. Set up separate reactions for sample and control DNA. Make up the volume to 55 μl using nuclease free water.
2. Make the following master mix in a 0.5 ml tube.

Final Conc.	Stock	1× Mix
1×	10× T4 DNA polymerase buffer	11.0 μl
5 μg	10 μg/μl BSA (NEB)	0.5 μl
40 nM	10 mM each dNTP	1.0 μl
1.5 U	3 U/μl T4 DNA polymerase (NEB)	0.5 μl
ddH_2O		42.0 μl
		55.0 μl

3. Add 55 μl of the above master mix to all samples. Flick-mix the tubes and briefly spin down the contents in the tube.
4. Incubate for 20 min at 12 °C in thermal cycler.

5. Transfer reaction to pre–chilled 1.5 ml tubes and extract the DNA by adding Add 120 μl phenol–chloroform–isoamyl alcohol (Fisher Biotech, cat. no. BP1752-100). Vortex well to mix the layers. Spin the tubes at 14,000 rpm (17,000×*g*) for 15 min at 4 °C to separate the layers. Transfer aqueous layer to new centrifuge tube containing 250 μl ethanol. Incubate for 30 min at −80 °C. Pellet the DNA by centrifuging at 15,000 rpm (17,000×*g*) for 20 min at 4 °C.
6. Wash the pellet with 70 % ethanol and centrifuge again at 15,000 rpm (17,000×*g*) for 20 min at 4 °C. Dry the pellet and resuspend DNA in 25 μl of water.
7. Atternatively, use a QiaQuick spin column (Qiagen corporation) to extract DNA and elute the DNA in 25 μl of TE.

Blunt-End Ligation

1. Prepare 15 μM Linker stock by mixing the following:

   250 μl Tris–HCl (1 M) pH 7.9.

   375 μl oligo A (5′-GCGGTGACCCGGGAGATCTGAATTC) (40 μM stock).

   375 μl oligo B (5′-GAATTCAGATC) (40 μM stock).

   Aliquot 50 μl into PCR tubes, place the tubes in a thermocycler and run the following program.

   95 °C for 5 min.

   70 °C for 1 min.

   Ramp down to 4 °C (0.4 °C/min).

   4 °C hold.

   Store the linkers at −20 °C.
2. Make master mix for ligation on ice (25 μl per reaction):

Final Conc.	Stock	1× Mix
1×	5× ligase buffer	10.0 μl
2 μM	15 μM linkers	6.7 μl
200 U	400 U/μl T4 DNA ligase (NEB)	0.5 μl
ddH_2O		7.8 μl
		55.0 μl

3. Add 25 μl of ligation mix to 25 μl of sample.
4. Incubate 16 h in 16 °C water bath.
5. Add 6 μl of 3 M sodium acetate and 130 μl ethanol. Incubate 30 min at −80 °C.
6. Spin at 15,000 rpm (17,000×*g*) for 20 min at 4 °C to pellet DNA. Wash pellets with 500 μl of 80 % ethanol.
7. Dry pellets and resuspend each in 25 μl H_2O.

Ligation-Mediated PCR

1. Transfer 25 µl of IP and control sample in PCR tubes.
2. Make two buffer mixes per rxn:

   Buffer mix A.

10× ThermoPol buffer (NEB)	4.00 µl
dNTP mix (2.5 mM each)	5.00 µl
oligo A (40 µM)	1.25 µl
ddH_2O	4.75 µl
Total	15.00 µl

   Buffer Mix B.

10× ThermoPol buffer (NEB)	1.0 µl
Taq polymerase (5 U/µl)	0.5 µl
ddH_2O	8.5 µl
Total	10.0 µl

3. Add 15 µl of Mix A to each sample and run the following program.

Step 1	55 °C	4 min
Step 2	72 °C	3 min
Step 3	95 °C	2 min
Step 4	95 °C	30 s
Step 5	60 °C	30 s
Step 6	72 °C	1 min
Step 7	GOTO Step 4	24 times
Step 8	72 °C	5 min
Step 9	4 °C	HOLD

4. Midway through Step 1, add 10 µl Mix B to each tube to hot start reactions. If necessary, pause program in Step 1 so tubes remain at 55 °C while adding Mix B.
5. After PCR is completed, precipitate the DNA by adding 25 µl of 7.5 M Ammonium acetate and 225 µl of 100 % ethanol.
6. Pool samples where appropriate. Add 250 µl precipitation mix per 50 µl of PCR reaction. Incubate 30 min at −80 °C.
7. Spin at 20,000 × *g* for 10 min at 4 °C to pellet DNA. Wash pellets with 500 µl of 80 % ethanol.
8. Dry pellets and resuspend each in 50 µl H_2O.
9. Measure DNA concentration with NanoDrop (use tenfold dilutions, if necessary) and normalize all samples to 500 ng/µl.

3.10.2 Whole-Genome Amplification PCR for Amplifying Immunoprecipitated DNA

This protocol is adapted from the protocol provided with Sigma GenomePlex kit (Product No. WGA1-50RXN) [43]. GenomePlex Whole Genome Amplification (WGA) Kit utilizes a proprietary technology based on random fragmentation of genomic DNA and conversion of the resulting small fragments to PCR-amplifiable library molecules flanked by universal priming sites. WGA is achieved by PCR amplification of the library molecules using universal oligonucleotide primers.

Library Preparation

1. Add 2 μl of 1× Library preparation buffer to 10 μl of immunoprecipitated DNA and transfer to PCR tubes (0.2 ml).

   Usually, the concentration of immunoprecipitated DNA is too low to get an accurate quantitation. The entire volume of reverse cross-linked, purified DNA (either by QiaQick or Affymetrix cDNA cleanup kit) is lyophilized and resuspended in 10 μl of DNA. If the concentration of DNA from a single IP is very low, a couple of IPs can be individually performed and the DNA can be subsequently pooled and concentrated as mentioned above.
2. Add 1 μl Library Stabilization solution, mix by pipetting and give a brief spin. Place the tubes in a thermocycler at 95 °C for 2 min.
3. Immediately cool on ice.
4. Add 1 μl of Library Preparation Enzyme, mix by pipetting and quick spin.
5. Incubate in thermocycler as follows.

   16 °C for 20 min.

   24 °C for 20 min.

   37 °C for 20 min.

   75 °C for 5 min.

   4 °C hold.
6. Proceed to first round of amplification or freeze at −20 °C.

Amplification

1. Prepare master mix for amplification for each sample as follows:

10× Amplification master mix	7.5 μl
Nuclease free water	47.5 μl
WGA DNA polymerase	5 μl

2. Add 60 μl master mix to each sample. Mix well and give a brief spin.
3. Incubate in thermocycler block as follows:

   95 °C for 3 min, then 14 cycles of

   94 °C for 15 s

   65 °C for 5 min, then 4 °C hold

Amplified material can be stored at −20 °C indefinitely, if needed.

4. Purify samples using QiaQuick PCR cleanup columns and elute the samples in water (Subsequent labeling reactions are efficient if eluted in water).
5. Quantitate DNA by NanoDrop or spectrophotometer. Usually, the DNA amount will be 1–4 μg which is sufficient for downstream labeling reactions. If the yield of DNA is low, reamplify the sample using Sigma GenomePlex WGA Reamplification Kit.

Reamplification

Transfer 15 μg purified amplification product in 10 μl volume to 0.2 ml PCR tubes. Use this as the starting material for second round amplification. Prepare master mix for each sample following the same method as in first round amplification. Follow the same thermocycler conditions as described earlier. Purify the samples using QiaQuick PCR cleanup columns.

3.10.3 Fragmentation of Chromatin Using Monococcal Nuclease

Monococcal nuclease treatment can be employed to cleave chromatin into oligonucleosomes [12, 37]. This method is known as native chromatin immunoprecipitation. However, this technique is incompatible with formaldehyde cross-linking since cross-linked chromatin is inefficiently cleaved by nucleases.

1. Prepare nuclear pellet from cells as described in refs. 12, 37.
2. Resuspend the nuclear pellet in 1 ml MNase digestion buffer and place on ice.
3. Aliquot into 1.5 ml microcentrifuge tubes with 500 μl of resuspended nuclei.
4. Add 1 μl of Mnase enzyme (Amersham Bioscience, cat. no. E70196Y) to each tube and mix gently.
5. Incubate the tubes at 37 °C in water bath for 10 min. The incubation times as well as MNase concentrations have to be optimized to generate mainly tri-, di-, and mono-nucleosomes.
6. Add 20 μl of stop solution. Chill on ice.
7. Centrifuge the suspension at 13,000 × *g* (12,000 rpm) for 20 s at 4 °C in a refrigerated microcentrifuge. The supernatant is made up of the second soluble fraction S2 containing larger fragments of chromatin, whereas the pellet contains the smaller fragment S1 fraction. Resolubilize the pellet in lysis buffer and pool with the S2 fraction in a fresh tube.
8. Continue Chip assay as described in Subheading 3.1, **step 1** from **step 6** onwards.

3.10.4 Preparation of Magnetic Beads

The use of magnetic beads for immunoprecipitation has been widely used in recent years [44, 45]. The beads are available in different forms, either precoulped with Protein G, Protein A, or secondary

antibodies (Dynabeads Protein G) (Invitrogen, cat. no. 100-03D), Dynabeads Protein A (cat. no. 100-01D). For each IP 100 μl of Dyna beads is recommended.

1. Transfer 100 μl of Dyna beads to 1.5 ml microfuge tubes.
2. Add 1 ml blocking buffer (0.5 % BSA in 1× PBS).
3. Collect the beads using a magnetic stand. Remove the supernatant.
4. Repeat washing with blocking buffer for two more times.
5. Resuspend beads in 250 μl blocking buffer and add antibody (10 g) to the tube.
6. Incubate overnight in a nutator at 4 °C.
7. Next day, wash the beads three times with blocking buffer and resuspend the beads in 100 μl of blocking buffer.
8. For immunoprecipitation, add 100 μl magnetic bead–antibody mix to the cell lysate (*See* 3.1 Preparation of ChIP lysate from cells) and incubate overnight in a nutator at 4 °C.

For detailed description of ChIP using Dynabeads refer the protocols from Young lab [43].

3.11 Conclusions

ChIP-chip or chromatin immunoprecipitation followed by DNA microarray analysis, has proven to be an efficient means of mapping protein–genome interactions on a genome-wide scale increasing our understanding of diverse cellular processes. Much of the work so far has focused on binding of transcription factors and many computational methods have been developed to identify the bound regions. Recently this technology has been extended to the genomic mappings of the other features such as histone modifications, transcriptionally active regions and binding sites for other protein complexes. In addition, coupling of ChIP with next generation DNA sequencing provides a platform for identifying genome scale DNA-protein interactions in an unbiased fashion (*see* **Note 11**). This chapter outlines the general strategies used to carry out ChIP-chip assays to study the differential recruitment of regulatory molecules based on the studies conducted in our lab as well as other published protocols.

4 Notes

1. GeneChip human promoter 1.0R array (Affymetrix, cat. no. 900775) is available as a single array comprising of over 4.6 million probes tiled through over 25,500 human promoter regions. Sequences used in the design of this array are based on the sequences available from NCBI genome assembly (Build 34). Oligonucleotide probes are synthesized in situ complementary to each corresponding sequence. Probes are tiled at an average

resolution of 35 bp, as measured from the central position of adjacent 25mer oligos, leaving a gap of approximately 10 bp between probes. Each promoter region covers approximately 7.5 kb upstream through 2.45 kb downstream of 5′ transcription start sites. For more than 1,300 cancer associated genes additional promoter region is included in the array, covering 10 kb upstream of transcription start site.

2. In addition to Affymetrix arrays, ChIP-chip microarrays are available from Agilent and NimbleGen. Available formats include whole-genome tiling arrays for model organisms like human and mouse as well as focused arrays for promoter regions and custom array designs. Each Agilent ChIP-on-Chip microarray features a total of ~244,000 60-mer oligonucleotide probes. Probes are spaced every ~100–300 bp across regions of interest in both coding and noncoding DNA sequence. Such focused microarrays include Proximal Promoter (−0.8 KB upstream to +0.2 KB downstream of identified transcriptional start sites) and Expanded Promoter (−8.0 KB upstream to +2.0 KB downstream) designs.
   NimbleGen's current high-density microarrays with long oligonucleotide probes (50–85-mer) contain 385,000 probes on a single glass slide. The entire nonrepetitive human and mouse genomes can be surveyed at 100 bp intervals, each with a set of 38 arrays. Agilent and NimbleGen microarray platforms utilize two color labeling as opposed to single color labeling of Affymetrix arrays. Two color labeling using Cy3/Cy5 can be done using CGH kit (Invitrogen) which utilizes a random primed, Klenow based extension protocol.

3. In conventional ChIP protocol, cells or tissues are treated with formaldehyde, a cell-permeable small molecule that can mediate protein–protein and protein–DNA cross-linking through Schiff base formation. To increase the degree of protein–protein cross-linking, treatment with dimethyl adipimidate can be done first, followed by formaldehyde treatment [46]. This is an efficient method to observe indirect protein–DNA interactions.

4. Antibodies used for ChIP-on-chip can be an important limiting factor. Not all antibodies can effectively immunoprecipitate protein–DNA complexes. An antibody that gives a specific signal in western blot need not be ideal for binding to a DNA bound, cross-linked protein. ChIP-on-chip requires highly specific antibodies that must recognize its epitope in free solution and also under fixed conditions. If the antibody is demonstrated to successfully immunoprecipitate cross-linked chromatin, it is termed "ChIP-Grade." Validated ChIP-grade antibodies are available from Abcam Inc., Santa Cruz Biotechnology Inc., and Upstate (Millipore Corporation). Since the affinity and avidity of antibodies can vary, the amount of antibody in

each ChIP-chip reaction needs to be empirically determined. To demonstrate that the antibody was successful in immunoprecipitating the protein–DNA complex, PCR amplification on a known binding-site region for the protein of interest should be performed using either conventional PCR methods followed by agarose gel electrophoresis or by quantitative PCR. The antibody used for immunoprecipitation should be a high affinity, high specificity antibody to avoid nonspecific background in ChIP assay. It is important to incorporate a positive control as well as an irrelevant antibody in the ChIP assay to facilitate critical interpretation of data.

5. It is necessary to have appropriate experimental controls in ChIP-chip experiments. Listed below are common controls that researchers are using in their experiments:
   (a) Nonspecific IgG antibodies—The most common type of negative control involves adding antibodies that do not recognize a specific epitope, for example pre-immune serum or IgG. A potential pitfall is that since the antibodies do not immunoprecipitate effectively, the nonspecific DNA yield is often extremely low. Hence, the hybridization tends to be much noisier and can result in many false positives due to amplification of trace amounts of nonspecific DNA. Alternatively, the primary antibody can be omitted (i.e., no antibody control).
   (b) Protein deficient cell line—Several upstream applications, such as target deletion or siRNA, can be used to perturb the expression of the protein of interest, hence decreasing the amount of immunoprecipitated material. Alternatively, a cell line that does not express the protein of interest could be used as a negative control.
   (c) Tag specific antibodies—If your protein of interest has a tag, such as GST or GFP, you may want to consider using antibodies against them in cells that do and do not express this tagged protein of interest.

   Generally genomic DNA is used as an input control and samples from no antibody or immunoglobulin G groups are used as negative controls. Other control designs such as transformed cell lines versus empty vector cell lines, wild type target versus mutation target and drug treated versus untreated can all be used as control options [47].

6. As mentioned previously, the amount of starting material required for successful microarray hybridization is very critical in a ChIP-chip experiment. The amount is highly variable depending on the quality of the antibody, binding frequency of protein to DNA and abundance of the protein. It is recommended that pooling of immunoprecipitated DNA samples

to acquire enough DNA for subsequent amplification is a feasible strategy. Unfortunately, pooling ChIP samples is not always possible, especially if specialized cell types or tumor tissues are used. The amplification of immunoprecipitated DNA using T7 polymerase is widely used to linearly amplify ChIP DNA. However, a PCR bias may occur, especially in mammalian systems where large amounts of repeat sequence may skew data.

7. The statistical analysis of the huge amount of data generated from arrays is a challenge and normalization procedures should aim to minimize artifacts and determine what is really biologically significant. So far, application to mammalian genomes has been a major limitation, for example, due to a significant percentage of the genome that is occupied by repeats. However, as ChIP-on-chip technology advances, high resolution whole mammalian genome maps are achievable.
8. Although ChIP-on-chip can be a powerful technique in the area of genomics, it is very expensive. Most published studies using ChIP-on-chip repeat their experiments at least three times in order to obtain biologically meaningful maps. The cost of the DNA microarrays is often a limiting factor to whether a laboratory should proceed with a ChIP-on-chip experiment.
9. Another limitation is the size of DNA fragments that can be achieved. Most ChIP-on-chip protocols utilize sonication as a method of breaking up DNA into small pieces. However, sonication is limited to a minimal fragment size of 200 bp. In order for higher resolution maps, this limitation should be overcome to achieve smaller fragments, preferably to single nucleosome resolution.
10. Although ChIP-on-chip is a powerful technique for genome-wide analysis of protein binding, it can result in high background signals leading to false positive target site detection. Recent studies present alternate approaches and modified protocols to reduce such artifacts [8, 48, 49].
11. With the availability of next-generation sequencing (NGS), global mapping of DNA–protein interactions can be achieved with higher resolution and greater accuracy [50]. For ChIP-sequencing (ChIP-seq), ChIP-isolated DNA fragments are sequenced directly instead of hybridizing on an array [51–53]. ChIP-seq offers greater coverage of the genome with a larger dynamic range than ChIP-on-chip and provides substantially improved data with fewer artifacts [54–56]. Several variations of ChIP-seq such as MeDIP-seq has been developed to map methylated DNA by sequencing DNA isolated using 5-methylcytosine-specific antibody [57].

Direct sequencing of methylated DNA after bisulfite conversion, known as BS-seq, has been successfully employed to generate high resolution maps of methylome [58]. In addition, a variation of ChIP-seq, referred to as RIP-seq or CLIP-seq has been used to identify regions in mRNA that bind to specific RNA-binding proteins [59]

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Chapter 27

ChIP-on-Chip Analysis Methods for Affymetrix Tiling Arrays

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Abstract

Although the ChIP-sequencing has gained significant attraction recently, ChIP analysis using microarrays is still an attractive option due to the low cost, ease of analysis, and access to legacy and public data sets. The analysis of ChIP-Chip data entails a multistep approach that requires several different applications to progress from the initial stages of raw data analysis to the identification and characterization of ChIP binding sites. There are multiple approaches to data analysis and there are several applications available for each stage of the analysis pipeline. Each application must be evaluated for its suitability for the particular experiment as well as the investigator's background with computational tools. This chapter is a review of the commonly available applications for Affymetrix ChIP-Chip data analysis, as well as the general workflow of a ChIP-Chip analysis approach. The purpose of the chapter is to allow the researcher to better select the appropriate applications and provide them with the direction necessary to proceed with a ChIP-Chip analysis.

Key words ChIP–chip, ChIP-on-chip, Affymetrix, Tiling, Data, Analysis, Normalization, Algorithm, Application, Chromatin, Binding

1 Introduction

Chromatin Immuno-precipitation followed by microarray hybridization (ChIP-Chip) allows for a genome-wide survey of in vivo transcription factor to DNA binding events, as well as the identification of histone modifications. With a range of a 7–35 base pair resolution, Affymetrix high-density oligonucleotide tiling arrays can interrogate both coding and noncoding regions, generating an enormous amount of data. This presents multiple challenges for data normalization and analysis. Multiple studies have been published describing novel algorithms that intend to address the statistical problems associated with ChIP-Chip data, but many of these may not be appropriate for every researcher's experiment or level of computer expertise and in some cases have yet to be implemented into an accessible software package.

The analysis of ChIP-Chip data is a process that requires the identification ChIP-enriched binding regions, the mapping of

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those hits to the genes or the genome, and possibly the sequence analysis and binding motif discovery of those hits. There are various approaches to each stage of ChIP-Chip data analysis, and each application will employ one or more of these techniques to arrive at a conclusion. Each of these approaches is explained here, hopefully allowing the reader to better understand the implementation of each application as it is reviewed.

Each of the stages of ChIP-Chip data analysis requires a specific treatment of the data and often requires a different application to produce the output for the next stage. It should be stressed that a complete treatment of ChIP-Chip data will most likely include the use of at least one of each of the applications available for each stage of data analysis. Fortunately, there are standard file formats that may be exported at one stage and subsequently imported into one or more of the applications at the next stage. As long as the user is cognizant of the file types used for input and output, as well as the genomic annotation version that was employed when generating the output, ChIP-Chip data can be processed through the analysis pipeline in a straightforward manner.

This chapter provides a survey of the algorithms and associated software applications that are currently available for Affymetrix ChIP-Chip analysis. Many algorithms were developed to address specific concerns or shortcomings of previous analysis solutions, but each will inherently include its own set of statistical assumptions and biases. Therefore, the researcher will ultimately need to decide which approach is the most appropriate for their experiment. The intent of this chapter is to provide enough information to allow the researcher to make an informed decision about ChIP-Chip data analysis, as well as downstream visualization and annotation. It also addresses the hardware and software requirements of each application as well as the level of computer expertise that is necessary to install and use the software effectively. Finally, the chapter describes a workflow of the ChIP-Chip data analysis process from raw data to binding site detection to visualization and biological characterization of the binding sites.

2 Methods

2.1 Array Normalization and Probe Analysis

Affymetrix tiling arrays are designed using the typical perfect match (PM) and mismatch (MM) probe pair approach where the PM is a 25-mer oligonucleotide probe that is identical to the genomic sequence data at a specific locus, and the MM is a 25-mer that is identical to the PM except for the central nucleotide. The purpose of the MM probe is to estimate the contribution of the signal intensity that is derived from nonspecific hybridization. Typically, the algorithms in Affymetrix analysis packages are designed to subtract the MM from the PM intensity, but many applications allow

the user to ignore the MM data and estimate signal from the PM values alone. Currently, Affymetrix offers a variety of tiling arrays that includes a set that interrogates all the nonrepetitive sequences of the human genome, a set that focuses on chromosomes 21 and 22, as well as an array that focuses only on the promoter regions of each gene. There are also arrays available for several other species that are commonly used in research. The analysis of any of these arrays may be carried out in the same manner.

The first stage of ChIP-Chip data analysis ultimately results in the initial identification of ChIP-enriched segments of the genome. This stage of data analysis is crucial as all downstream binding site analysis is based on these results. Initially, the raw data files, Affymetrix .cel files, are imported into an application and normalized or globally scaled to a specific target intensity. Next, the application must estimate the background-corrected signal of each probe on the array. This may include the estimation of signal, *p*-value, or other statistical scoring mechanisms which will be carried into the next step. Finally, the application must employ one or more statistical algorithms that will detect the regions of DNA that are potentially bound by the protein of interest by evaluating the signal or statistical measurements generated in the previous stage. Each probe along the interrogated site is reported and compared to neighboring probes with the intent of identifying a statistically significant region of binding activity. Typically, these regions are reported as lists of genomic coordinates, but other metrics such as ChIP fold-enrichment score and false discovery rate (FDR) may be reported by the software. The output files from this stage of analysis are commonly of the .bed file format which may be visualized in the genome browsers that are discussed later. Next, several of the most commonly used and some recently reported algorithms and analysis applications are discussed.

2.1.1 Affymetrix Tiling Analysis Software (TAS)

An early Chip-ChIP study using Affymetrix arrays was performed in collaboration with Affymetrix and employed the approach used in the Affymetrix GTRANS, or the later named, Tiling Analysis Software (TAS) [1]. TAS is a freely available standalone Windows application that is not tied into the Affymetrix GCOS system. Because it is flat file based, the user only needs to point to the array .cel files to initiate an analysis. The application requires several library files, including BPMAP and GRC files that are supplied by Affymetrix.

After downloading the appropriate library files for the subject arrays and creating the tiling array group (TAG file) within the software, the TAS workflow starts with the .cel file normalization. The software can perform a global median scaling to specified target intensity or optionally perform quantile normalization of the probe intensity data [2]. Quantile normalization assumes that the data have the same underlying distribution, so it is most likely

inappropriate to normalize all control and enriched samples together. The software allows the user to indicate the preference of a one-sample or two-sample analysis (control and enriched IP sample) or the corresponding quantile normalization treatment. It is important to note that although TAS will perform analysis on a one-sample experiment, the array must be of the PM/MM type or the results from the analysis will not be accurate.

After normalization and scaling, TAS Probe Analysis is performed which will generate reports of absolute signal intensity and *p*-values. The first step of Probe Analysis employs a sliding window approach where bandwidth is set to half the expected IP DNA fragment length, which is typically near 500 bp. A larger bandwidth may increase statistical power, but it may also increase background noise. Next, TAS performs a nonparametric Wilcoxon signed-rank test (also knows as a Mann–Whitney *U*-test) [3] which is a nonparametric test for samples lacking a normal distribution. The test ranks all probe pairs in the window and confirms that the sum of the ranks in the ChIP-enriched sample is higher than that of the control sample. The *p*-values generated from this test may be reported in a $-10 \log_{10}$ format which allows convenient viewing along with sequence, interval, or intensity data in a genome browser. TAS Probe Analysis also results in a file with signal intensity estimates at each probe, as generated by the Hodges–Lehmann estimator [4], which are the median of the pairwise averages of the PM–MM scores in the sliding window. Additionally, in a two-sample analysis, fold enrichment is also reported as a $\log_2$-fold change between the treatment and control group signals.

The final stage of Probe Analysis is an interval analysis which uses the output files from the previous steps to report discrete regions of potential target protein binding along the sequence of interest. The interval analysis is dictated by three parameters including a *p*-value or signal intensity threshold (depending on which is used for input), a *max_gap* parameter which results in a higher level of stringency with a smaller value, and *min_run* which is more permissive with a smaller threshold value. The final set of the detection regions, or intervals, is output in a .bed file format and can be used as an input for downstream analysis or data visualization.

Affymetrix Tiling Analysis Software could be considered a good starting point for the analysis of ChIP-Chip tiling data since the software is free, easy to install, and relatively straightforward to use. However, there is a learning curve to the software for those starting into ChIP-Chip analysis, so it is recommended to thoroughly review the documentation as well as the Affymetrix Web talks that specifically address TAS with ChIP-Chip data.

There are some caveats of the TAS package that should be considered. First, the software does not allow one-sample analysis for the tiling arrays that are not designed with MM probes.

This may present challenges for those who would like to perform a small experiment without a control sample or for those who are checking QC of one array at a time. In addition, and perhaps more importantly, TAS does not account for probe affinity which is the variability in probe behavior based on sequence and GC content as well as high copy number probes that are specific to multiple sites of the genome. Some applications that address these concerns are examined below.

2.1.2 HMMTiling

In order to address some of the potential shortcomings of the Affymetrix Tiling Analysis Software, Li et al. [5] developed a ChIP-Chip algorithm based on a hidden Markov model (HMM) [6] called HMMTiling. The software is offered as source code and runs in a Python command line environment on a Linux operating system only and is available for download at http://liulab.dfci.harvard.edu/Software/HMMTiling/HMMTiling_Readme.htm. The authors state that a compiled binary version with a graphical user environment (GUI) will be released for both Windows and Linux, but at the time of this writing these alternatives are not yet available.

HMMTiling was designed with the approach of developing a repeat masked probe model that is defined using multiple samples from several experimental data sets of the user's choice. The result is a model that estimates the behavior of each individual probe with the intention of minimizing the effects of poorly performing probes. In addition, the authors assert that the use of a HMM addresses the notion that the Wilcoxon rank test used in TAS requires additional replicates and a larger window size (which will consequently include more noisy probes or miss short binding regions) to evaluate the ChIP-enriched regions with a sufficiently small *p*-value

The strategy of the HMMTiling software starts with a quantile normalization of the .cel files. Next, repetitive probes, those that are represented multiple times on the array as well as those that map multiple times to the genome, are filtered out before further analysis. Essentially, probes must have only one measurement within 1 kb of the genome, otherwise latter occurrences of the sequence are removed. Probes must also occur only once for each genomic position. Baseline probe behavior is then estimated by evaluating each probe across every sample in the dataset. An HMM is then applied to estimate the probability of ChIP enrichment at each probe location, taking all replicates into consideration. A summary enrichment score is reported for the discovered ChIP-enriched regions, and the data may be exported as a .bed file for downstream analysis and data visualization.

HMMTiling is a good alternative to Affymetrix TAS since the algorithm takes baseline probe performance into consideration, further filters out repetitive probes that will potentially confound

the data, and it obviates the need for the fixed-size sliding window of the Wilcoxon rank test. Additionally, the user has the option to pool together data from multiple independent data sets to estimate probe behavior. The authors claim that reasonable results may be attained from a single ChIP experiment so this application may be very useful to those who are testing multiple ChIP conditions and would rather not make use of multiple arrays during the optimization stages of their experiment. However, since this application is only available as a command line application for Linux operating systems with limited documentation, it may be out of reach for the majority of biologists at this time. Perhaps because of this, at this time, other applications described below may be a good alternative to researchers.

2.1.3 MAT

An algorithm titled, Model-based Analysis of Tiling-arrays (MAT) [7], was developed by the same group as the previously described HMMTiling application. This particular application is endorsed by Affymetrix as another alternative to their TAS application. MAT is open-source and is available for download for Linux operating systems at http://liulab.dfci.harvard.edu/MAT/.

The approach of MAT has some similarities to HMMTiling, but probe behavior modeling and ChIP-enriched region detection are performed very differently. The authors first remapped all of the 25-mer probe sequences in the BPMAP file of each tiling array type to the latest genome assembly while filtering out probes that map to more than one location in any 1 kb window, as well as those that map to the same genomic location. Probe behavior is estimated by assessing signal intensity, sequence, and copy number of all probes on each array, unlike HMMTiling where it requires multiple arrays to build the probe affinity model. Next, a probe affinity standardization method is employed to background correct and, in effect, normalize the arrays so that they may be directly compared. The authors report that quantile normalization is not necessary, and that it offers no further benefits after probe affinity standardization is performed.

Once the arrays have been examined for probe affinity, ChIP region detection may be performed using a single ChIP-enriched sample only, multiple ChIP sample replicates, or multiple ChIP samples with input controls including those with unbalanced designs (i.e., one control to multiple ChIP-enriched samples).

MAT uses a sliding window approach to identify enriched regions. The size of the window is user-adjustable and dependent on ChIP DNA fragment size. A trimmed mean of MAT-calculated *t*-values (where the upper and lower 10 % of *t*-values from the window are removed) and a MATscore are calculated and reported. Computed *t*-values are standardized probe intensities that have been adjusted for the baseline predicted by the probe behavior model (sequence, copy number, etc.) and standard deviation of a predetermined probe affinity bin. ChIP-enriched regions are then

identified based on p-values, false discovery rate (FDR) or MATscores, depending on the user preference. Resulting MATscores for each probe and the genomic coordinates for enriched binding regions are exported as .bar files and .bed files, respectively, for downstream analysis and visualization.

MAT is a powerful alternative to TAS that is reported to work sufficiently with as little as one ChIP-Chip sample, with no replicates or controls, similar to HMMTiling. Currently, the application is only available for Linux platforms running Python and it has a command line interface. This limitation will exclude many biologists, but those who have some experience with Linux may find the software tractable. The program expects a user-created text file called a .tag file which describes the experiment and a few library files that are downloadable from the MAT Web site. Launching MAT then requires a single line of code.

2.1.4 TileMap

TileMap is a free application developed by Ji and Wong [8] that also steps away from the sliding window approach of Affymetrix TAS. TileMap features user-selectable analysis options including a moving average (MA) or an HMM, a pseudo false discovery rate (FDR) calculation, and repeated probe filtering. TileMap is available in a command line interface for Windows and Unix operating systems http://jilab.biostat.jhsph.edu/software/tilemap/index.htm, but recently the software has been released as a graphical user interface along with the authors' CisGenome project, available at http://www.biostat.jhsph.edu/~hji/cisgenome/.

TileMap employs a hierarchical empirical Bayes model to pool information from all probes on the array, followed by the generation of test-statistics that are used to identify protein-binding patterns. The Bayes method allows one to estimate the probabilities of an individual probe by using information from empirical measurements of that probe and on the entire population [9]. Neighboring probes are then combined using either a moving average (MA) or an HMM. The application is intended to accurately handle experiments with few replicates or complex multiple sample comparisons, and the authors claim that the algorithm runs >10 times faster than that of TAS.

The implementation of TileMap involves a workflow that includes functions that normalize the raw data followed by log transformation of the PM or PM–MM values. In order to reduce noise, the application includes a repeat masking step that filters out any probe that occurs more than once in a 2 kb window. The algorithm then calculates an unbalanced mixture subtraction (UMS) which is similar to a local false discovery rate (FDR) for the MA or HMM model parameters. The output of TileMAP includes summaries for each probe with a .bed file containing the identified genomic binding regions. As usual, this file may be further processed by downstream applications.

TileMap is navigated via command line interface for Windows and Unix, and requires the user to set up two parameter files, but the documentation and examples may be adequate for many biologists to get started with analysis. However, an attractive alternative may be the CisGenome application since the features of TileMap have been incorporated into the interface along with a graphical user environment.

2.1.5 TiMAT

The TiMAT application essentially serves to address the issues of noisy probe data, calculate FDR, and identify and evaluate ChIP-enriched intervals. TiMAT, freely available at http://bdtnp.lbl.gov/TiMAT/, is an open source Java-based command line application that was specifically designed for ChIP-Chip data. It will run on Windows, Mac OS X, and Linux operating systems with the Java Runtime Environment (JRE) installed.

TiMAT consists of a series of small programs that perform specific functions in the processing pipeline. The process involves multiple steps starting with the creation of a Tpmap file from an Affymetrix BPMAP file (this file must only be created once per array type). Next the .cel files are loaded and converted to a TiMAT-specific format, followed by normalization and scaling where replicates are averaged, log intensities are calculated, and subtraction of control values from ChIP-enriched values is performed. Next, window scores and optionally FDR are calculated using a sliding trimmed mean window. Then, enriched intervals are identified by employing a number of user selectable filters including FDR, minimum interval length, and maximum gap. Finally, significant interval peaks are selected and the multiple reports are generated and exported from the application, however not of the typical .bed file format. The files will most likely have to be modified for downstream visualization in genome browsers.

TiMAT uses a sliding window approach similar to that of Affymetrix TAS, but the content of the data files that are exported is much more robust in terms of scoring of intervals, FDR filtering, and peak finding. However, the software does require control samples and does not take into account the individual variation of each probe (probe affinity), nor does it address the repetitive probes on the array. In addition, the application must be installed and configured as a Java command line interface. The user will need to be comfortable working in DOS and addressing memory allocation by using the Java –Xmx flag, or memory errors will likely occur. Additionally, the fact that each process of the pipeline requires the user to launch a different program, specifying multiple input files, may pose as a significant burden to the user. For these reasons, many biologists may not easily traverse the complete analysis pipeline using TiMAT, and may be advised to work with one of the applications that include a graphical interface (TAS or CisGenome).

2.1.6 Tilescope

Tilescope is a graphical, Web-based application developed by Zhang et al. [10] which is intended to be an integrated pipeline featuring an automated workflow. The application is freely downloadable at http://tilescope.gersteinlab.org, and will run on any platform with a Java-enabled Web browser.

The processing pipeline for Tilescope involves three stages: data input, tile scoring, and feature identification. First, Affymetrix .cel and BPMAP files are uploaded through the use of a Web form, and quantile normalization is then performed by default. Tile scoring is performed using a TAS-like sliding window approach with an added nonparametric statistical test to further assess the differences between the control and ChIP-enriched samples at each probe. Both fold-enrichment and *p*-value scores at each genomic location are reported. Binding sites are identified by one of three methods: the max-gap and min-run TAS-like metric, a novel iterative peak method, or an HMM method. Upon completion of data processing, a Web page is displayed that includes a summarization of parameters, scatter plots, and histograms. Downloadable feature lists include genomic coordinates (including those for updated genome builds) along with the calculated metrics, as well as links to custom tracks on the UCSC genome browser.

Because Tilescope is integrated and Web-based, it may be one of the easiest-to-use applications available to researchers who are just getting involved in ChIP-Chip analysis. It offers three choices for binding site identification as well as a seamless transition to data visualization in a genome browser. Although probe affinity and repetitive probes are not addressed here, the application may still offer a solid starting point for non-programmers who would like an alternative to TAS.

2.1.7 CisGenome

CisGenome is a publicly available integrated application that was developed by the authors of TileMap [8]. The application is available with a graphical user interface (GUI) for Windows operating systems, as well as a command line interface for Linux and Mac OS. Both source code and binary executables for CisGenome are available at http://www.biostat.jhsph.edu/~hji/cisgenome/index.htm.

The CisGenome GUI enables a quick and easy approach to data analysis which starts with the loading of the appropriate BPMAP files and .cel files of interest. Next, the .cel files may be quantile normalized or analyzed using the MAT algorithm [7], and then binding site peak detection is performed using the TileMap algorithm. Once the analysis is complete, CisGenome can visualize the binding regions in the resulting output file from within the application. Tracks of interval data or gene annotations may be added to the genome browser and the user may traverse through each chromosome narrowing in on sites of interest.

At the time of this writing, CisGenome was newly released and is currently in beta stages, but this application may be the most integrated, easy-to-use freely available package. The fact that it installs and runs as a graphical Windows application including a genome browser within the interface makes CisGenome an excellent application for the average biologist to use. In addition, the inclusion of the MAT and TileMap algorithms along with user-selectable parameters gives users the opportunity to easily step away from the Affymetrix TAS software. One caveat is that at this time the documentation for the graphical version of the software is limited to a basic tutorial, but the authors do offer a comprehensive manual for the command line version of the software which describes the implementation of the algorithms in detail.

2.1.8 Partek Genomics Suite

For researchers who are processing larger volumes of ChIP-Chip as well as other types of microarray samples, or for those who need the support and ease-of-use of a commercial analysis application, Partek Genomics Suite (Partek GS) may serve as an alternative to the freely available applications. Information as well as a free trial of the software for Windows, Mac, and Linux operating systems may be found at http://www.partek.com.

Partek GS is designed to handle Affymetrix data for studies including gene expression, exon tiling, copy number analysis, SNP association, as well as tiling data. The application workflow starts with .cel file import and carries through to normalization, binding region detection, and visualization including mapping of binding regions to probe sets that are represented on the HG-U133 Plus 2.0 human expression array. In addition, the software automatically provides links to external databases including NetAffx, UCSC Genome Browser, and the Affymetrix Integrated Genome Browser.

Normalization and signal intensity estimation of the .cel files is achieved using the Robust Multiarray Average (RMA) algorithm [2] without a median polishing step, including quantile normalization and $\log_2$ conversion. Detection of potential ChIP binding sites is then performed using a user-adjustable sliding window approach similar to that used in Affymetrix TAS.

The benefits of Partek GS include its straightforward workflow and easily navigable interface, as well as its ability to handle multiple types of Affymetrix data, including the ability to merge data sets from these different array types within the same workflow. However, it should be noted that the software does not implement a probe affinity model or repetitive probe masking such as those included with MAT. If a commercial application that includes these features is preferred, Biotique Systems' XRAY may be of interest.

2.1.9 Biotique Systems XRAY for ChIP-Chip

XRAY is an Excel add-in available commercially for Windows operating systems at https://www.biotiquesystems.com/Products-Solutions/XRAY. The graphical interface offers a wizard-driven

workflow for the identification and scoring of binding sites. XRAY is targeted towards researchers on a budget who are comfortable with the Excel working environment. According to the Biotique Systems, XRAY is priced similar to the cost of a "couple of arrays." XRAY may be a good choice for those who have limited funds and are do not have the need for an application that supports multiple types of Affymetrix microarrays.

XRAY includes built-in .cel file quality and normalization metrics, and users may select from the TAS [1], HMM [6] or MAT [7] algorithms for array processing and binding site identification. The resulting output may be viewed on the UCSC genome browser from within the application without the need to link externally to the site. Additionally, the software will export a document outlining the analysis methods employed in the array processing, and users may export the Excel charts to publications.

2.1.10 Genomatix ChipMiner

ChipMiner is a newly available application from Genomatix (http://www.genomatix.de) that is designed to handle any of the Affymetrix ChIP tiling arrays including promoter and whole genome sets. The graphical user interface of ChipMiner allows the user to import Affymetrix .cel files for enriched region detection analysis. The results are visualized against the overlapping genomic annotation including upstream and downstream loci. In addition to finding transcription factor binding sites, the software can identify phylogenetically conserved transcription factor binding motifs including a mechanism to compare these motifs to ten vertebrate species. The final stage of the ChipMiner pipeline involves the display of all results as a pathway mining component.

2.1.11 R/Bioconductor Analysis Packages

Several ChIP-Chip analysis packages are freely available in the R statistical programming environment [11]. Two of these packages support Affymetrix tiling arrays, and some commonly used packages are be summarized here. An updated list of current packages is available at http://master.bioconductor.org/packages/release/bioc/.

rMAT is a commonly used R package developed by Droit et al. [12], which implements the popular MAT algorithm into the R environment, while extending the software with an additional normalization model that is based on nucleotide pairs In addition to implementing MAT functionality, rMAT can interact with other R modules such as GenomeGraphs and biomaRt in order to visualize and annotate the enriched binding sites.

Starr [13] is another package available in R which implements a strategy for the evaluation of how ChIP signals align along annotated gene features. In addition, the software will update BPMAP library files with current genomic annotations, allows for quality control reporting and visualization, and can incorporate gene expression data to allow for an integrated analysis of the two platforms.

For users accustomed to working in the R environment, normalization and post-processing options are vast. In addition, an R module that focuses on tertiary ChIP analysis (ChIPpeakAnno) is described in a later section.

2.1.12 Cistrome Analysis Pipeline

The Galaxy framework [14] is an open-source Web-based graphical interface that has greatly enhanced the accessibility of applications that are typically only available in command line interfaces on Unix environments. Cistrome [15] is a Galaxy implementation that offers extensible support for ChIP-Chip, ChIP-seq, and expression array data. Cistrome enables a seamless flow from import of local expression or tiling CEL files (or public data from the NCBI GEO database) to downstream analysis using well-supported tools such as MAT for peak-calling and CEAS for peak annotation. The software, available at http://cistrome.org/ap/root, allows the user to identify differentially expressed genes that may be regulated through transcription factor binding or histone modification. Finally, the software uses commonly used databases to annotate the regions identified in the analysis, and allows for the export of images and plots. In addition to data sharing with collaborators, a primary benefit of Cistrome is that since it is Web-based, the software can be upgraded and extended in a manner that is transparent to the user. For users who prefer a graphical environment, and perhaps who are not using Windows, Cistrome may be an excellent alternative to CisGenome, since the GUI version of CisGenome is only available for Windows.

2.2 Binding Site Visualization

Accurate signal estimation and binding site identification are central to ChIP-Chip analysis, but to garner meaningful results from the data it is necessary to further visualize and report the data using other downstream tools. Genome browsers are powerful interfaces that allow the simultaneous visualization of ChIP-Chip experimental data as a track against an annotated genome. Users may add additional annotation tracks containing information such as SNP sites, known genes, predicted genes, ESTs, or even data merged from gene expression data sets.

The UCSC Genome Browser is a commonly used Web-based genome browser that is available at http://genome.ucsc.edu/index.html [16]. The Genome Browser includes annotation tracks that are computed at UCSC from publicly available data, as well as tracks that are provided by collaborators. The user can view the data along the chromosome by zooming in or providing genomic coordinates from genome BLAST searches, etc. The browser includes clickable areas that allow the user to explore external databases from the NCBI and others such as the BLAT sequence alignment tool which may be used for further downstream analysis.

Affymetrix also offers a freely available genome browser entitled Integrated Genome Browser (IGB) located at http://www.affymetrix.com/estore/partners_programs/programs/

developer/tools/download_igb.affx. Similar to the UCSC browser, IGB allows users to dynamically explore the genomic annotations by adding multiple tracks of user-generated data along with annotations from public databases. IGB is launched from within a browser as a Java application. Although similar in function to the UCSC browser, IGB has a completely different style of interface that allows rapid zooming and sliding across the chromosomal data. IGB is connected to multiple public databases, so the user may choose from multiple annotation tracks and genomes. In addition, IGB will open Affymetrix .chp files as generated from Tiling Analysis Software for a relatively seamless progression through the analysis pipeline. The software will also directly link to the NCBI Entrez nucleotide database and the UCSC Genome Browser.

2.3 Binding Site Analysis and Characterization

In addition to data exploration in genome browsers, it is extremely helpful to identify the genes that may be associated with the potential DNA binding regions as well as to additionally characterize these sites. The *cis*-regulatory element annotation system (CEAS) [17] is an integrated Web application that retrieves repeat masked genomic sequences, calculates GC content, maps nearby genes, reports evolutionary conservation at the site of interest, and identifies transcription factor binding motifs from ChIP-Chip data. CEAS is available at http://ceas.cbi.pku.edu.cn.

CEAS accepts input files with ChIP binding regions in either the UCSC .bed format or Sanger GFF format. Once the files are uploaded, the application will create conservation plots from a phylogenetic HMM which are then generated as phastCons thumbnails and exported to a .pdf file for review. In addition, CEAS will also search for the nearest RefSeq genes on both strands within 300 bp from the binding site of interest. This allows the researcher to potentially identify which particular genes may be affected by their protein of interest. CEAS reports the relative binding location within the gene (intron, exon, UTR, etc.), the length and GC content of the binding site, and it provides a link to the UCSC genome browser. In addition to the basic binding site characteristics, CEAS finds the enriched sequence motifs from within the binding sites using the precomputed motif-scanning methods TRANSFAC and JASPAR. Fold change, *p*-value, and other metrics are reported with each motif along with a helpful graphical sequence logo (covered below) which gives a quick visual indication of sequence conservation at each base in the binding motif. Additionally, CEAS can retrieve RepeatMasked genomic sequences at each binding site which allows the researcher to design qPCR primers or perform additional motif searching, which is covered in more detail.

Genome browsers typically allow the user to export the genomic sequence data associated with the potential binding sites. The next step of the ChIP-Chip analysis pipeline may include the processing of this sequence data in order to identify and report these potential

regulatory elements as conserved motifs. Several motif discovery tools exist which allow the user to verify or identify de novo binding sites from the exported sequence data. Some common de novo search applications include MEME, AlignAce, and GLAM among many others which were previously reviewed and evaluated [18]. Using a comprehensive scan of the sequence data exported from a genome browser or application such as CEAS, the user may quickly report a list of potential binding motifs from the ChIP-Chip data. Most of these applications report multiple potential motifs along with scoring metrics to allow the user to evaluate the search results.

Once the potential binding motifs are identified, the biologist may then report them as a graphical sequence logo by utilizing the WebLogo application [19]. WebLogo starts with an aligned set of potential binding site sequences and creates a graphic that represents each nucleotide by a color-coded stack of letters corresponding to each of the four bases. The height of each letter indicates the proportion of the sequence conservation of that site across the series of aligned DNA sequences. WebLogo can then export the graphic to one of many formats including GIF and PNG, as well as print-friendly vector-based formats such as EPS and PDF. WebLogo is a freely available Web-application that may be accessed at http://weblogo.berkeley.edu.

For users who are comfortable working in the R environment, ChIPpeakAnno [20] is a recently developed tool which offers significance reporting, batch annotation, and visualization options beyond other tools, and without the challenges of working with multiple tracks in genome browsers. The input of ChIPpeakAnno is a .bed file of enriched regions identified by an upstream analysis tool. ChIPpeakAnno differs from other such as CEAS or CisGenome in that it will compare peaks and other coordinates *between* samples or replicates, along with significance testing and Gene Ontology (GO) enrichment. In addition, the package can interact with other R libraries specific for peak calling, annotation and visualization. Due to the platform-agnostic nature of genomic coordinates, and the flexibility of tools implemented in R, ChIPpeakAnno may highly reward the investigator willing to invest the time to learn to use R packages.

3 Conclusions

ChIP-Chip analysis is a microarray technique that, along with ChIP-seq, is still evolving. Many applications and approaches to ChIP-Chip data analysis have been developed, and in recent years these have been extended in functionality or have been made accessible to a broader user group through a graphical user interface implementation. The methods covered here are among the most well known and well supported in the scientific community. As work progresses with ChIP data, the most fruitful techniques will undoubtedly be improved by

data management, simplification of the workflow, and by the introduction of more graphical user interfaces that facilitate the use by biologists with limited programming experience. ChIP-Chip data analysis may initially seem daunting, but if the user starts with applications that are appropriate for his or her level of computer expertise, budget, and particular experiment, then analysis efforts will be both rewarding and fruitful.

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