RNA–Protein Interaction Protocols
RNA–Protein Interaction Protocols

Edited by

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Preface

The molecular characterization of RNA and its interactions with proteins is an important and exciting area of current research. Organisms utilize a variety of RNA–protein interactions to regulate the expression of their genes. This is particularly true for eukaryotes, since newly synthesized messenger RNA must be extensively modified and transported to the cytoplasm before it can be used for protein synthesis. The realization that posttranscriptional processes are critical components of gene regulation has sparked an explosion of interest in both stable ribonucleoprotein (RNP) complexes and transient RNA–protein interactions.

RNA is conformationally flexible and can adopt complex structures that provide diverse surfaces for interactions with proteins. The fact that short RNA molecules (aptamers; see Chapter 16) can be selected to bind many different types of molecules is evidence of the structural variability of RNA. RNA molecules are rarely entirely single- or double-stranded, but usually contain multiple short duplexes interrupted by single-stranded loops and bulges; in some RNAs, such as tRNAs, the short duplexes stack on each other. Further variability is generated by the presence of non-Watson-Crick base pairs, modified nucleotides, and more complex structures, such as pseudoknots and triple-strand interactions.

The techniques described in RNA–Protein Interaction Protocols cover a wide range of approaches to studying RNA–protein interactions. They address several broad methodological questions: How do I analyze the structural details of an RNA–protein interaction—what residues contact each other and how strongly do the components interact? If I know one component of a suspected RNA–protein interaction, how do I identify the other(s)? How do I purify RNP complexes from cells? How do I assay the effects of proteins and RNP complexes on mRNA metabolism?

Many of the techniques in this book require in vitro synthesized RNA, either unlabeled, uniformly labeled, or labeled at specific sites. The first chapter describes methods for generating and purifying large quantities of labeled or unlabeled RNAs by in vitro transcription from bacteriophage promoters. Chapter 2 covers the use of ligase to introduce a labeled phosphate at a specific internal location within a long RNA for label-transfer studies.

An important aspect of understanding the structural details of RNA–protein interactions is determining what portions of the two molecules are in
close contact. A variety of crosslinking techniques, described in the next four chapters, have been developed for this purpose. Each has advantages and disadvantages, and multiple approaches must often be used to analyze an RNP complex. Chapter 3 describes the use of photoactivatable nucleotide analogs, which are incorporated into RNA during transcription and form covalent crosslinks with adjacent amino acids after irradiation. This method is optimal for crosslinking proteins to single-stranded regions of RNA, but crosslinking protein to double-stranded (ds) RNA is much less efficient. Chapter 4 details a procedure using the intercalating dye methylene blue to generate efficient crosslinks to dsRNA. Although the dye facilitates the reaction, it does not itself form part of the crosslink. Crosslinking methods rely on the close apposition of reactive species, but not all residues form adducts efficiently. The use of photoactivatable nucleotide analogs addresses this problem for the RNA. A similar approach for protein, described in Chapter 5, uses site-directed mutagenesis to introduce cysteine at a specific location in the protein, which is subsequently derivatized with psoralen. Upon irradiation, the psoralen will crosslink with adjacent pyrimidines. The procedures in these chapters have generally been applied to the interaction of a single protein with a single RNA. Obviously, the analysis is considerably more complicated for multi-component RNP complexes. However, mass spectrometry techniques offer new tools for analyzing crosslinking in such RNP complexes. Chapter 6 describes the use of N-terminal sequencing and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) as applied to analyzing RNA–protein crosslinks in the native 30 S ribosomal subunit.

The absence of a crosslink does not imply the lack of close contact between RNA and protein in a particular region, and it is necessary to use additional methods for a complete understanding of the structure of an RNP complex. RNA footprinting and modification interference techniques (Chapter 7) provide a way of assessing all residues in an RNA for their association with protein. RNA footprinting yields information on how the binding of a protein affects the accessibility of an RNA to enzymatic and chemical probes. Modification interference studies allow one to identify bases that are essential for protein binding. In an approach that is conceptually similar to footprinting, short DNA oligonucleotides can be used to compare the regions of an RNA that are available for hybridization and cleavage by RNase H in the presence and absence of protein binding (Chapter 8).

Several techniques have been developed to provide quantitative measures of the affinity or kinetics of RNA–protein interactions, as well as allowing one to assay for the presence of an RNA-binding protein in a
complex mixture. The most commonly used are nitrocellulose filter binding (Chapter 9) and gel retardation assays (Chapter 10). These assays are most effective for relatively strong, stable interactions. The polyacrylamide coelectrophoresis (PACE; Chapter 11) procedure has been developed to study weaker interactions that may not be kinetically stable under the conditions of gel retardation assays. More sophisticated technology is also available: Biosensors employing surface plasmon resonance measurements can provide data on real-time interactions, their stoichiometry, and the effects of other molecules on those interactions. The applications of these instruments to RNA–protein interactions are discussed in Chapter 12.

The analysis of variant protein or RNA sequences, either naturally occurring or experimentally-induced, provides important information on the contributions of different residues to an interaction. Methods that allow screening of populations of variant molecules are described in the next few chapters. Two of the methods utilize bacterial genetic assays, in which the binding of a protein to its RNA target results in either repression of translation (Chapter 13) or transcription antitermination (Chapter 14). In both systems, the level of β-galactosidase expression provides a readout of the extent of interaction. Other procedures utilize in vitro selection of populations of variant molecules. Chapter 15 describes the use of phage display technology to generate a pool of mutated proteins that can be assayed for binding to a specific RNA target. The opposite approach, involving preparation of combinatorial RNA libraries and selection of molecules that bind a particular protein, is discussed in Chapter 16.

Identifying an unknown, biologically relevant partner in an RNA–protein interaction can be a major technical challenge. For example, although selection of a target RNA sequence from a combinatorial library of short RNAs may provide a consensus binding sequence, that information alone may not be sufficient to identify an in vivo target mRNA. Chapter 17 describes the use of libraries of natural RNA sequences to identify such RNAs. Two approaches for identifying unknown proteins are described: screening of expression libraries for binding to an RNA target by a Northwestern assay (Chapter 18), and by a solution-based assay (Chapter 19). The methods in these three chapters assume that the interaction is bimolecular, but that is not always true. Purification of RNP complexes by immunoprecipitation and the identification of component RNAs by PCR-based approaches (Chapter 20) should be particularly useful in situations in which the RNA binding specificity requires multiple proteins, only one of which is known.

Studies of RNP complex composition and function depend critically on the ability to purify the complex from contaminating cellular components.
The remainder of the book provides methods for purification and analysis of RNP complexes, and assays for some of the major RNA–protein interactions that occur in the cell. Chapter 21 describes the use of biotinylated antisense oligoribonucleotides to either purify or deplete specific RNP complexes from cell extracts by affinity chromatography. Immunoaffinity chromatography, either alone or in combination with other separation methods, has also proved to be a useful technique for purification of RNP complexes. A protocol describing its use in purification of the small nuclear RNP (snRNP) complexes required for splicing is given in Chapter 22; immunoaffinity purification of heterogeneous nuclear RNP complexes, which are composed of nuclear proteins and newly synthesized mRNA, is described in Chapter 23. Analyses of the mechanism and regulation of constitutive and alternative splicing are major topics of current research in the RNA field. The next eight chapters provide protocols for such studies in mammalian tissue culture cells and yeast. Chapters 24 and 25 cover the preparation and use of HeLa cell extracts for in vitro splicing assays, and Chapter 26 provides similar protocols for studying splicing in yeast. In vivo, splicing occurs in large multicomponent complexes termed spliceosomes, and Chapters 27 and 28 describe methods for their purification and analysis from mammalian cells and yeast, respectively. The regulation of splicing of mRNAs transcribed from complex transcription units depends on a combination of cis elements and trans factors. Chapter 29, while not a protocol chapter in the strict sense, discusses strategies and pitfalls in defining the cis elements that regulate alternative mRNA splicing. A protocol for selecting functional cis elements in vivo from a randomized pool is given in Chapter 30. The SR protein family constitutes a major class of non-snRNP splicing factors that regulate splicing in trans, and Chapter 31 describes their purification from tissue culture cells or organs.

The final chapters cover protocols for analyzing mRNA polyadenylation, translation, and turnover. These processes can be crucial control points for regulating the expression of specific mRNAs during cellular metabolism and development. The preparation and use of extracts for studying mRNA 3' end cleavage and polyadenylation reactions are described in Chapter 32. The length of the poly(A) tail on a particular mRNA plays an important role in its stability and translatability; Chapter 33 describes a PCR-based assay for measuring poly(A) tail lengths. The ability to prepare translation extracts (Chapter 34) from different cell types has been important for studying the regulation of translation of specific mRNAs, particularly the cap-independent translation of certain viral and cellular mRNAs. Finally, regulation of mRNA stability
can be a crucial aspect of gene regulation, and Chapter 35 provides assays for studying mRNA turnover in cell-free extracts.

The protocols in RNA–Protein Interaction Protocols were written with the novice RNA researcher in mind, although a knowledge of basic molecular biology, biochemistry, and cell culture is assumed. They should be particularly useful for the scientist who discovers that the expression of his or her favorite gene is posttranscriptionally regulated, and who wants to know how to proceed in analyzing that regulation. Although every effort has been made to provide a comprehensive protocol book, it is impossible to include all the variations and applications that have been developed for studying RNA-protein interactions. However, a broad range of methods is covered in this book, and many of the protocols can be readily adapted to other systems. These protocols distill a great deal of wisdom and experience, and provide an excellent starting point for investigating many types of RNA–protein interactions.

Susan R. Haynes
## Contents

Preface ................................................................................................................................. v
Contributors ......................................................................................................................... xv

1  Labeling and Purification of RNA Synthesized by In Vitro Transcription .......................... 1
   **Paul A. Clarke**

2  Joining RNA Molecules with T4 DNA Ligase ............................................................... 11
   **Melissa J. Moore**

3  RNA–Protein Crosslinking with Photoreactive Nucleotide Analogs ............................ 21
   **Michelle M. Hanna, Lori Bentsen, Michael Lucido, and Archana Sapre**

4  The Methylene Blue Mediated Photocrosslinking Method for Detection of Proteins that Interact with Double-Stranded RNA ...................................................... 35
   **Zhi-Ren Liu and Christopher W. J. Smith**

5  Probing RNA–Protein Interactions by Psoralen Photocrosslinking .............................. 49
   **Zhuying Wang and Tariq M. Rana**

6  Identification and Sequence Analysis of RNA–Protein Contact Sites by N-Terminal Sequencing and MALDI-MS ................................................................. 63
   **Bernd Thiede, Henning Urlaub, Helga Neubauer, and Brigitte Wittmann-Liebold**

7  RNA Footprinting and Modification Interference Analysis ........................................... 73
   **Paul A. Clarke**

8  Oligonucleotide-Targeted RNase H Protection Analysis of RNA–Protein Complexes ............................................................. 93
   **Arthur Günzl and Albrecht Bindereif**

9  Nitrocellulose Filter Binding for Determination of Dissociation Constants .................. 105
   **Kathleen B. Hall and James K. Kranz**

10 Measuring Equilibrium and Kinetic Constants Using Gel Retardation Assays ................ 115
    **David R. Setzer**

11 PACE Analysis of RNA–Peptide Interactions ............................................................... 129
    **Christopher D. Cilley and James R. Williamson**
12 Detection of Nucleic Acid Interactions Using Surface Plasmon Resonance ................................................................. 143
Robert J. Crouch, Makoto Wakasa, and Mitsuru Haruki
13 An Escherichia coli-Based Genetic Strategy for Characterizing RNA Binding Proteins ...................................................... 161
Chaitanya Jain
14 Screening RNA-Binding Libraries Using a Bacterial Transcription Antitermination Assay .................................................. 177
Kazuo Harada and Alan D. Frankel
15 In Vitro Genetic Analysis of RNA-Binding Proteins Using Phage Display ................................................................. 189
Ite A. Laird-Offringa
16 In Vitro Selection of Aptamers from RNA Libraries ......................... 217
Daniel J. Kenan and Jack D. Keene
17 Identification of Specific Protein–RNA Target Sites 
Using Libraries of Natural Sequences ........................................ 233
Lucy G. Andrews and Jack D. Keene
18 Northwestern Screening of Expression Libraries ....................... 245
Paramjeet S. Bagga and Jeffrey Wilusz
19 Screening Expression Libraries with Solution-Based Assay ............ 257
Philippa J. Webster and Paul M. Macdonald
20 An Immunoprecipitation-RNA:RPCR Method for the In Vivo Isolation of Ribonucleoprotein Complexes ............... 265
Edward Chu, John C. Schmitz, Jingfang Ju, and Sitki M. Copur
21 Purification and Depletion of RNP Particles by Antisense Affinity Chromatography ................................................... 275
Benjamin J. Blencowe and Angus I. Lamond
22 Purification of U Small Nuclear Ribonucleoprotein Particles ........... 289
Berthold Kastner and Reinhard Lührmann
23 Preparation of Heterogeneous Nuclear Ribonucleoprotein Complexes ................................................................. 299
Maurice S. Swanson and Gideon Dreyfuss
24 Preparation of Hela Cell Nuclear and Cytosolic S100 Extracts for In Vitro Splicing .......................................................... 309
Akila Mayeda and Adrian R. Krainer
25 Mammalian In Vitro Splicing Assays ........................................... 315
Akila Mayeda and Adrian R. Krainer
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Author(s)</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>Yeast Pre-mRNA Splicing Extracts</td>
<td>Stephanie W. Ruby</td>
<td>323</td>
</tr>
<tr>
<td>27</td>
<td>Prespliceosome and Spliceosome Isolation and Analysis</td>
<td>Laura A. Lindsey and Mariano A. Garcia-Blanco</td>
<td>351</td>
</tr>
<tr>
<td>28</td>
<td>A Yeast Spliceosome Assay</td>
<td>Stephanie W. Ruby</td>
<td>365</td>
</tr>
<tr>
<td>29</td>
<td>Defining Pre-mRNA cis Elements that Regulate Cell-Specific Splicing</td>
<td>Thomas A. Cooper</td>
<td>391</td>
</tr>
<tr>
<td>30</td>
<td>In Vivo SELEX in Vertebrate Cells</td>
<td>Thomas A. Cooper</td>
<td>405</td>
</tr>
<tr>
<td>31</td>
<td>Purification of SR Protein Splicing Factors</td>
<td>Alan M. Zahler</td>
<td>419</td>
</tr>
<tr>
<td>32</td>
<td>Processing mRNA 3' Ends In Vitro</td>
<td>Michael J. Imperiale</td>
<td>433</td>
</tr>
<tr>
<td>33</td>
<td>Analysis of Poly(A) Tail Lengths by PCR: The PAT Assay</td>
<td>Fernando J. Sallés and Sidney Strickland</td>
<td>441</td>
</tr>
<tr>
<td>34</td>
<td>In Vitro Translation Extracts from Tissue Culture Cells</td>
<td>Kazuko Shiroki and Akio Nomoto</td>
<td>449</td>
</tr>
<tr>
<td>35</td>
<td>Messenger RNA Turnover in Cell-Free Extracts</td>
<td>Jeff Ross</td>
<td>459</td>
</tr>
<tr>
<td></td>
<td>from Higher Eukaryotes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Index</td>
<td></td>
<td>477</td>
</tr>
</tbody>
</table>
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