Chapter 14

Generation of Cell Lines Stably Expressing a Fluorescent Reporter of Nonsense-Mediated mRNA Decay Activity

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Abstract

Nonsense-mediated mRNA decay (NMD) is a mechanism of mRNA surveillance ubiquitous among eukaryotes. Importantly, NMD not only removes aberrant transcripts with premature stop codons, but also regulates expression of many normal genes. A recently introduced dual-color fluorescent protein-based reporter enables analysis of NMD activity in live cells. In this chapter we describe the method to generate stable transgenic cell lines expressing the splicing-dependent NMD reporter using consecutive steps of lentivirus transduction and Tol2 transposition.

Key words Nonsense-mediated mRNA decay, Splicing, Fluorescent proteins, Genetically encoded sensor, Fluorescence-activated cell sorting, Mammalian cell lines, Tol2 transposition

1 Introduction

All eukaryotic cells contain a complex pathway called nonsense-mediated mRNA decay (NMD), which recognizes and degrades mRNA species with a premature termination codon (PTC) [1]. The main sources of such aberrant transcripts with truncated coding regions are gene mutations, alternative splicing, and DNA rearrangement in immune cells.

PTC recognition is based on positioning of the terminating ribosome in improper contexts of two main types: (1) a long 3′ untranslated region (3′UTR) and (2) an intron more than 50 nucleotides downstream of the terminal codon [2]. The long 3′UTR-based recognition mechanism functions in all organisms studied, whereas splicing-dependent NMD is characteristic for vertebrates and plants [3, 4]. Such unusual convergence of very distant taxa implies that both NMD pathways originated at the earliest stages of evolution of eukaryotes [3].

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For decades, NMD was thought to be a housekeeping mechanism of mRNA quality control that prevents accumulation of potentially toxic C-terminally truncated proteins. Recent data, however, strongly suggest additional functions of NMD in global regulation of gene expression during key biological processes such as cell differentiation, embryonic development, and stress response [5]. This new understanding of biological NMD significance is based on two groups of facts. First, it was demonstrated that many normal wild type transcripts are degraded by NMD machinery because their terminal codons are recognized as PTC (e.g., due to an intron in the 3′UTR) [2, 5]. Second, NMD activity was found to undergo specific regulation, for example, by some microRNAs [6, 7], Ca^{2+} [8], or expression level of NMD factors [9, 10]. Thus, modulation of NMD activity can result in up- or down-regulation of hundreds of mRNA species [11–13] providing a way of orchestrated regulation of gene expression.

The proposed regulatory function of NMD calls for new methods to measure detailed spatio-temporal activity patterns of this cascade. Indeed, the classical techniques for evaluating mRNA abundance and stability, namely northern blot analysis and quantitative real-time PCR, work with large populations of cells and provide a very restricted resolution in space and time. This problem can be solved by bioluminescence and fluorescence reporters of NMD activity based on NMD-dependent expression of a luciferase or a fluorescent protein [8, 14, 15]. It enables evaluation of live cells down to single cell sensitivity, although precautions should be taken when interpreting the data, as the measurements are performed at the protein not mRNA level.

Recently, we designed a dual-color reporter of NMD activity based on ratiometric signal from two fluorescent proteins [16]. This reporter consists of two vectors. The first vector (pNMD+) expresses NMD-targeted (due to the presence of an intron in its 3′UTR) mRNA for the green fluorescent protein TagGFP2, as well as NMD-insensitive mRNA for the red fluorescent protein Katushka (*see* Fig. 1). The second vector (pNMD−) is identical to the first one except the distance between the TagGFP2 stop codon and the exon junction site has been shortened to 35 nucleotides, which makes this transcript insensitive to NMD. Red-to-green ratio in pNMD+-expressing cells is proportional to NMD activity, while control cells expressing pNMD− provide a reference green-to-red ratio characteristic for particular detection parameters (intensity and wavelengths of excitation beams, detection wavelengths and detector sensitivity, or exposure time).

As this NMD reporter gives estimates of NMD activity in single live cells, it potentially can be used in a variety of models to visualize changes in NMD activity in space and time using fluorescence microscopy or flow cytometry [16–18]. However, to date only

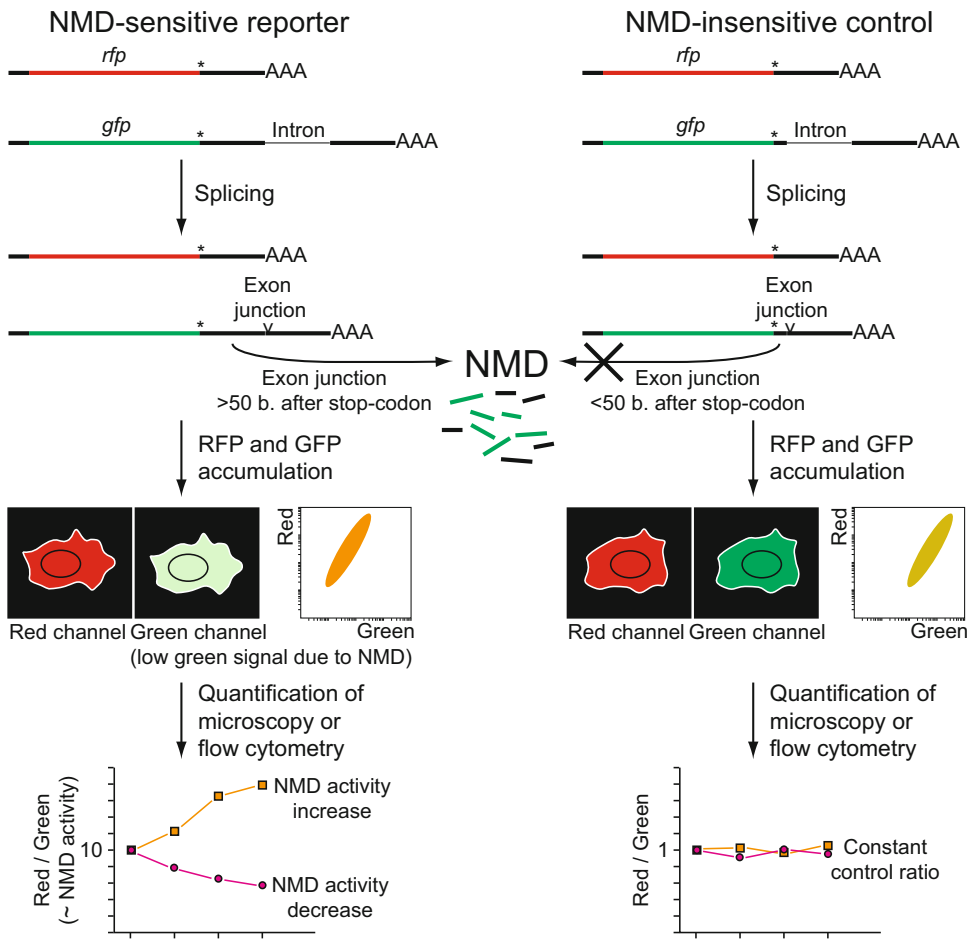

Fig. 1 Schematic outline of the NMD analysis method using dual-color fluorescence reporter. The splicing-dependent NMD pathway is evaluated by GFP-encoded transcript with an intron-containing 3′UTR (*left*). Control transcript contains the intron 35 nucleotides downstream of the stop codon and thus does not undergo NMD (*right*). RFP-encoding transcript acts as a reference for expression level. RFP and GFP fluorescence is detected by microscopy or flow cytometry, enabling quantification of NMD activity. For cells stably expressing the reporter, NMD activity can be followed in space and time during biological processes or treatments (*sketchy graphs at the bottom*)

transient expression of the NMD reporter has been described, which is inappropriate for long-term experiments.

In this chapter, we describe procedures to generate mammalian cell lines stably expressing the NMD reporter.

2 Materials

2.1 Genetic Vectors

1. Packaging lentivirus plasmids pR8.91 and pMD.G.
2. pLVT-Katushka transfer vector.

3. pCMVTol2 (AddGene) [19].
4. pTol2-NMD+ and pTol2-NMD− for Tol2-based stable integration (available upon request from the authors).
5. pNMD+ and pNMD− for transient transfections (available upon request from the authors).
6. pTurboFP635-N, pTagGFP2-N (Evrogen).
7. Standard *E. coli* strains, equipment and reagents for plasmid preparation.

2.2 Mammalian Cell Culture

Use standard protocols, equipment and reagents for cultivating mammalian cell lines. Use the HEK293T cell line for production of vector particles. The strongly adherent HeLa Kyoto cell line, adherent and suspension mouse Lewis Lung Carcinoma cells (LLC), adherent mouse Colon Cancer (CT26) can be used for both transient and stable expression experiments. Grow HeLa Kyoto and CT26 cells at 37 °C and 5% CO_2 in DMEM (Dulbecco's modified Eagle's medium) with 10% FBS (fetal bovine serum), 4 mM L-glutamine, 10 U/mL penicillin, 10 μg/mL streptomycin. For LLC cells use a mixture of DMEM and Ham's F12 medium (1:1).

For cell transfection, use appropriate common procedures using a transfection reagent, e.g., FuGENE HD (Promega) or electroporation, e.g., with Nucleofector 2b Device, (Lonza).

2.3 Cell Analysis

1. A Fluorescence-Activated Cell Sorter (FACS) machine.
2. A fluorescence microscope equipped with filter sets for detection in green and red channels.
3. A real-time PCR (qPCR) thermocycler.
4. Chemical inhibitors of NMD: caffeine, wortmannin, and/or NMDI14 (Sigma-Aldrich).

3 Methods

3.1 General Consideration on Choosing the Method of Transgenesis

The most convenient and efficient way to introduce an exogenous gene into genome of mammalian cells is recombinant lentiviral vectors [20]. However, this way is not well-suited for our NMD reporter as it contains an intron as well as two independent mRNAs. The life cycle of lentiviruses includes an intermediate mRNA-like form in the nucleus. As a result, spliceable introns are removed from the viral RNA. Also, the presence of transcription termination sites precludes formation of a full-length viral RNA genome. To solve this problem, antisense orientation of the insert can potentially be used, although often at expense of low virus titer [21, 22]. In our work, we failed to generate stable cell lines with NMD reporter using lentiviral vectors, possibly because there were

cryptic splice sites or transcription terminators in the antisense orientation as well.

An alternative way to make transgenes is based on transposons. For example, the Tol2 transposon derived from *Oryzias latipes* (medaka fish) is autonomously active [23]. DNA of Tol2 consists of the transposase gene flanked by inverted terminal repeats required for transposition. Application of the Tol2 transposon system to generate stable expression in vertebrates including mammals has been reported [19, 24–26]. The smallest active variant *miniTol2* containing the combination of deletions has been developed [27]. The Tol2-mediated system was considered to have several advantages: (1) it has proved to be rather tolerant to increasing cargo size, which allows more complex transgenes to be incorporated without reducing transposition efficiency; (2) *Tol2* can transpose efficiently in a different cell types (either differentiating somatic cells or germ cells); (3) *Tol2* can integrate almost everywhere in the host genome, showing no preferences with respect to position on a chromosome; in contrast to other transposons, no consensus DNA sequence has been observed in the 8 bp target duplications created after integration of *Tol2;* (4) *Tol2* integration does not produce DNA rearrangements around its sites of integration [28].

As Tol2-based transgenesis is compatible with intron-containing and bicistronic inserts, we have chosen this system for our work. We have tested two alternative protocols to generate cell lines stably expressed NMD reporter. The first one used transgenesis by Tol2 vector with a long bicistronic insert carrying both intron-containing GFP- and intronless RFP-encoding genes. The potential advantage of this protocol is that it is based on a single-step transgenesis and ensures one-to-one copy numbers for GFP- and RFP-encoding genes. However, we encountered a significant problem of low stability of the integrated inserts. Even after careful selection of target dual-color transfectants by antibiotic selection and FACS, further cell passages resulted in a rather frequent appearance of green-only cells. We concluded that direct repeats close to each other in the bicistronic insert led to high probability of recombination events and elimination of the RFP-encoding part.

The second protocol used two consecutive steps (Fig. 2): (1) lentiviral transgenesis with the intronless RFP-encoding gene, and (2) Tol2-based transgenesis with intron-containing GFP-encoding genes (Fig. 3). This protocol includes more steps but provides efficient and stable transgenesis; it also provides flexibility in choosing a desirable green-to-red signal ratio. We have successfully used the suggested procedure for CT26 and LLC cells.

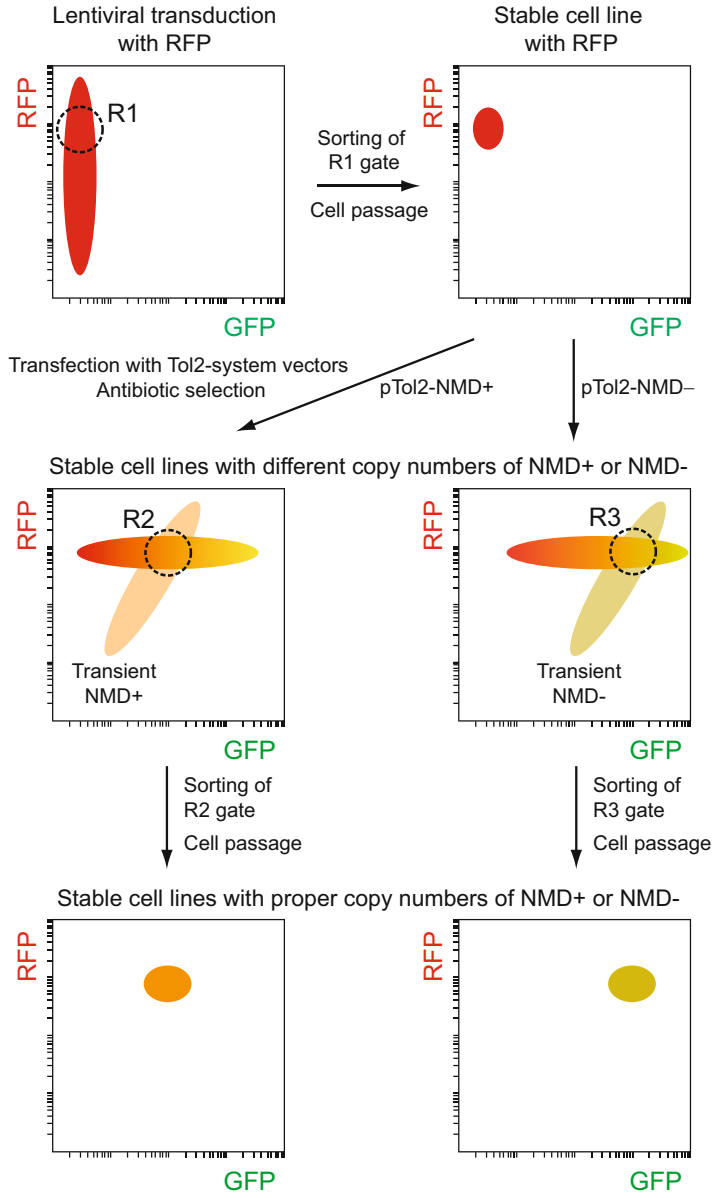

Fig. 2 Main steps to generate cell lines stably expressing the NMD reporter. Schematic flow cytometry bivariate plots in the green and red channels are shown to illustrate generation of RFP-expressing stable cell line by lentiviral transduction and FACS sorting (*upper panels*); subsequent integration of NMD-reporter using Tol2-based system (*middle panels*); and final FACS sorting of stably expressing cells (*bottom panels*) with proper green-to-red ratios chosen by analysis of cells transiently transfected with pNMD+ or pNMD− (regions R2 and R3, respectively)

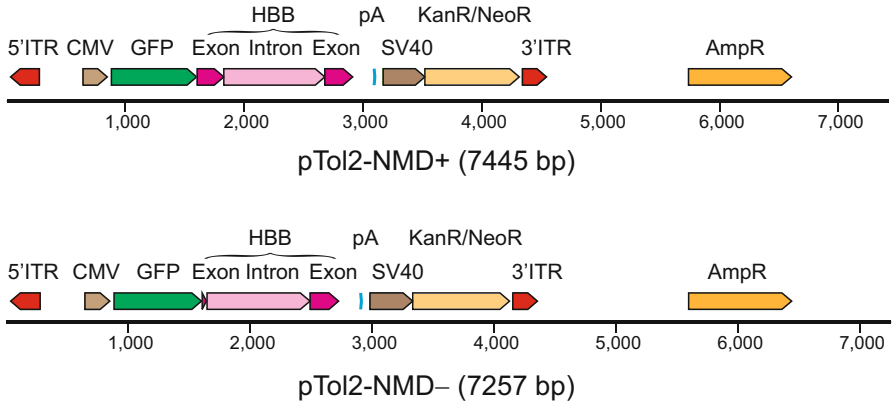

Fig. 3 Main elements of the reporter vectors pTol2-NMD+ and pTol2-pNMD−. Between inverted terminal repeats (5′ITR and 3′ITR) required for Tol2-based transposition, these vectors carry TagGFP2-encoding transcripts under control of CMV IE promoter. In their 3′UTR, these transcripts contain a fragment of human beta-globin (HBB) gene with a spliceable intron. In pTol2-NMD+, the intron is 230 bases downstream of the TagGFP2 stop-codon making this mRNA a classical target of splicing-dependent NMD. In pTol2-NMD−, the intron is only 35 bases downstream of the TagGFP2 stop-codon; thus this mRNA is not targeted to NMD

3.2 Generation of Katushka-Expressing Stable Cell Line by Lentiviral Transduction

In our method, Katushka provides a reference red signal for normalization. In fact, any RFP can be used for this purpose. Thus, if you already have a stable RFP-expressing cell line of interest (self-made or from a commercial source, e.g., from Anticancer Inc., www.anticancer.com), skip this section and go directly to the steps of Tol2-based integration of NMD reporter (Subheading 3.3).

3.2.1 Lentiviral Transfer Vector

The pLVT transfer vector was derived from the pRRLsin.cPPT.PGK.GFP.WPRE vector kindly provided by Prof. Didier Trono by replacing the PGK promoter with the intron-less version of the human elongation factor 1-α (EF1-α) promoter. The coding region of Katushka was excised from pTurboRFP-N and inserted into pLVT between BamHI and SalI restriction sites, replacing the GFP-encoding fragment, to produce the pLVT-Katushka transfer vector.

3.2.2 Production of Lentiviral Particles

Lentivirus particles can be produced by any convenient method. In our work we use the protocol according to [22, 29] with some modifications as described below.

1. Culture HEK293 T cells in DMEM, supplemented with 10% FBS and antibiotics (50 U/mL penicillin G and 50 μg/mL streptomycin).
2. Seed out 1.5×10^6 HEK293T cells into a 60 mm culture dish 24 h before transfection.
3. Use manufacturer's protocol for transfecting HEK293T cells using FuGENE 6. We recommend using 4 μg and 1.2 μg of the

two packaging plasmids, pR8.91 and pMD.G, respectively, and 5 μg of transfer vector plasmid (pLVT-Katushka) for this transfection.

4. Replace the medium with 4 mL of fresh DMEM/10% FCS after 17 h.
5. After 24 h of incubation collect the medium with lentiviral particles and filter it through 0.45-μm filter.
6. Freeze the virus sample in 100–200 μL aliquots at −80 °C (without addition of DMSO). Use it for generating further stable cell lines of interest (*see* Subheading 3.2.3, **step 2**).
7. To determine the lentivirus titer, thaw one aliquot and prepare 2–3 serial tenfold dilutions of the virus in DMEM. Add each virus sample to 1×10^5 HeLa or HEK293T cells grown in 35-mm plastic dishes for 24 h in advance. Forty eight hours after transduction, count Katushka-positive (red fluorescent) cells by flow cytometry (*see* Subheading 3.2.4). To estimate the titer, use cell sample with 1–30% infection rate according to a standard protocol [30].

3.2.3 Lentiviral Transduction for Stable Expression of Katushka

The lentivirus sample should be used to generate cell line(s) of interest stably expressing Katushka. The protocol for CT26 and LLC cells given below should be adapted for a particular cell line (according to its size, growth rate, medium requirements, etc.).

1. Seed about $1–3 \times 10^5$ CT26 or $3–5 \times 10^4$ LLC cells into 35-mm plastic dishes the day before transduction.
2. For transduction, incubate the cells with virus-containing medium (*see* Subheading 3.2.2, **step 6**) using about 30 virus particles per cell for 24 h, then replace the medium.
3. Propagate the cells for 4–6 passages to reach a constant level of expression.

3.2.4 Analysis and Sorting of Cells with Stable Lentiviral Expression of Katushka by FACS (See Note 1)

1. To prepare cells for flow cytometry analysis, wash the cells twice with 1 mL of versene solution and trypsinize in 0.5–1 mL of trypsin/EDTA solution for 5 min at 37 °C. Centrifuge the required number of cells (10^6–10^7 cells per sample) at $300 \times g$ for 5 min at room temperature. Remove supernatant and carefully resuspend the cell pellet in 0.5–1 mL of PBS/0.5% BSA.
2. To remove cell aggregates, pass the sample through a 70-μm cell strainer immediately before sorting.
3. Analyse the cells by FACS. Apply commonly used flow cytometry gates in the forward/side scattering plots to select single live cells only.
4. Analyse cell fluorescence in green and red channels (e.g., 488 nm excitation and 510–530 nm detection for the green

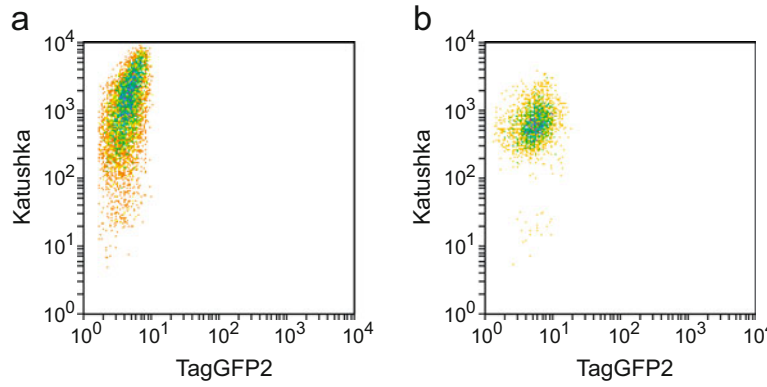

Fig. 4 Generation of a cell line stably expressing Katushka by lentiviral transduction. Shown are results of flow cytometry of CT26 cells after transduction with pLVT-Katushka (**a**) and further FACS sorting (**b**)

signal, and 561 nm excitation and 660–680 nm detection for the red signal; *see* Subheading 3.3.2 and **Note 1**). In a green-red bivariate plot (green versus red signal), the cells should give a vertical oval-shaped cloud (*see* Fig. 2, top left panel and Fig. 4a).

5. Sort a sufficiently bright (but not the brightest—*see* Fig. 2, gate R1) red cells under sterile conditions into a tube with 0.5 mL DMEM. Collect 1–5 × 10^4 cells (*see* **Note 2**). Seed the sorted cells into 35-mm dish or in a well of a 6-, 12-, or 24-well plate and grow for 96 h in DMEM with 20% FBS. If you collected less than 3 × 10^3 cells, seed in a 96-well plate and grow for 7 days.
6. Propagate the cells for 2–3 passages.
7. Repeat flow cytometry analysis at the same settings as above to prove that cells form a compact cloud within the previously selected region with no RFP-negative or dim cells in the sample (*see* Fig. 2, top right panel and Fig. 4b).
8. Freeze a portion of the obtained cell line stably expressing Katushka with DMSO in liquid nitrogen for future use.

3.3 Tol2-Based Integration of NMD Reporter

3.3.1 Tol2 Transposon Vectors

NMD reporter plasmids were constructed on the base of pminiTol2 vector [19] as follows: (1) neomycin-resistance (kanR/neoR) cassette under SV40 promoter was added; (2) fragment encoding either TagGFP2-HBB+ (NMD-sensitive) or TagGFP2-HBB− (NMD-insensitive) under control of CMV promoter was inserted to generate the final pTol2-NMD+ and pTol2-NMD− plasmids, respectively (*see* Fig. 3 and **Note 3**). Prepare sufficient amounts (20–50 μg) of pTol2-NMD+ and pTol2-NMD− plasmids using standard bacterial transformation, growth and ampicillin selection, and plasmid isolation protocols (*see* **Note 4**).

3.3.2 Cell Transfection and Antibiotic Selection

1. In preliminary experiments, determine the concentration of G418 that kills your cells of interest (usually 400–1000 μg/mL) (*see* **Note 5**).
2. Perform two separate transfections of the Katushka-expressing cells obtained at Subheading 3.2.4, **step 7** with a mixture of (1) pTol2-NMD+ and pCMVTol2, (2) pTol2-NMD− and pCMVTol2. Use a molar ratio of 1.5:1 for pTol2-NMD +(−)/pCMVTol2 for cotransfection (i.e., taking into account their lengths, 500 ng of pTol2-NMD+(−) and 250 ng of pCMVTol2); higher or lower ratios result in decreased percentage of cells with stable integration. Use any common method of transfection (*see* **Note 6**).
3. Twenty four hours after transfection, trypsinize the cells and plate about 1/10 of the volume of cells onto 3.5-cm plates with complete medium (DMEM) supplemented with 100 μg/mL G418 (*see* **Note 5** and 7).
4. After 8–10 days of selection evaluate cells for the formation of foci (antibiotic-resistant cell clones). Foci may require an additional week or more to develop depending on the cell line and transfection/selection efficiency.
5. Trypsinize the cells and transfer about 1/5 of cells into new flask with fresh medium containing G418. Seed the cells at no more than 25% confluency, otherwise the rate of cell division decreases and antibiotic selection will not be efficient.
6. Replace antibiotic-containing medium every 4 days.
7. Collect cells by trypsinization and seed to additional culture flasks to obtain approximately 1×10^7 cells for further FACS sorting (Subheading 3.3.4). Gradually increase G418 concentration when replating cells in fresh media.

3.3.3 Fluorescence Microscopy of Cells

Fluorescence microscopy is a useful method to check progress in stable cell line generation. During antibiotic selection, analyze cell samples every 3 days to make sure that the cells fluoresce in both red and green channels (*see* **Note 8**). Use any microscope equipped with standard filter sets for GFP and RFP (*see* Spectra Viewer at http://evrogen.com/spectra-viewer/viewer.shtml to check the appropriateness of filters) (*see* **Note 9**). During first 10–14 days of selection, a rapid decrease in green fluorescent signal is expected because of gradual elimination of transiently introduced plasmids. The nascent cells with stable integration possess lower fluorescence than transiently transfected cells.

3.3.4 Flow Cytometry Analysis and Sorting of Cells with Stable Tol2-Based Integration of NMD Reporter

1. Two days before cell sorting, prepare 4 control cell samples that are transiently transfected with the following vectors: (a) pTagGFP2-N, (b) pTurboFP635-N, (c) pNMD+, (d) pNMD−. Transfect cells using the same protocol as in Subheading 3.3.2, **step 2** and incubate cells for 2 days.

2. Prepare cell suspensions (four control samples and two target cell samples with stable integration of pTol2-NMD+ and pTol2-NMD−) for flow cytometry analysis (*see* Subheading 3.2.4, **steps 1** and **2**).
3. Use control samples (a) and (b) to set up appropriate detection parameters in the green and red channels of the FACS machine that ensure well-detectable signals without cross talk of the green cells into red channel and vice versa. Apply commonly used flow cytometry gates in the forward/side scattering plots to select single live cells only. Keep these settings unchanged for all further cytometry analyses and sorting.
4. Analyze control samples (c) and (d). It should give diagonal clouds in the bivariate plot (green versus red signal); pNMD+ cell diagonal distribution is shifted to the region of lower green signal compared to the pNMD− cells, proportionally to the NMD activity (*see* Fig. 2, middle panels and Fig. 5a, b). These control samples provide proper green-to-red signal ratios corresponding to equal copy numbers of TagGFP2 and Katushka-encoding genes in NMD-sensitive and NMD-insensitive constructs, respectively.
5. Analyse cell sample pTol2-NMD+, which should give a horizontal oval-shaped cloud in the bivariate plot (*see* Fig. 2, left middle panel and Fig. 5c). This distribution corresponds to the presence of different numbers of integrated TagGFP2-encoding genes into the genome of the cells obtained after antibiotic selection.
6. Draw a region at the virtual intersection of pNMD+ and pTol2-NMD+ cell clouds (R2 in Fig. 2) (*see* **Note 10**). Sort pTol2-NMD+ cells from this region under sterile conditions into a tube with 0.5 mL DMEM. Collect 1–5 $\times$ 10^4 cells (*see* **Note 2**). Analogously, analyze cell sample pTol2-NMD−, which should give a horizontal oval-shaped cloud in the bivariate plot somewhat right-shifted compared to the pTol2-NMD+ cells (*see* Fig. 2, right middle panel and Fig. 5d).
7. Draw a region at the virtual intersection of pNMD− and pTol2-NMD− cell clouds (*see* Fig. 2, gate R3).
8. Sort pTol2-NMD+ cells from this region under sterile conditions into a tube with 0.5 mL DMEM. Collect 1–5 $\times$ 10^4 cells (*see* **Note 2**).
9. Seed the sorted cells into 35-mm dishes or into wells of a 6-, 12-, or 24-well plate and grow for 96 h in DMEM with 20% FBS.
10. Propagate the cells for 2–3 passages.
11. Repeat flow cytometry analysis at the same settings as above to prove that cells form a compact cloud within the previously

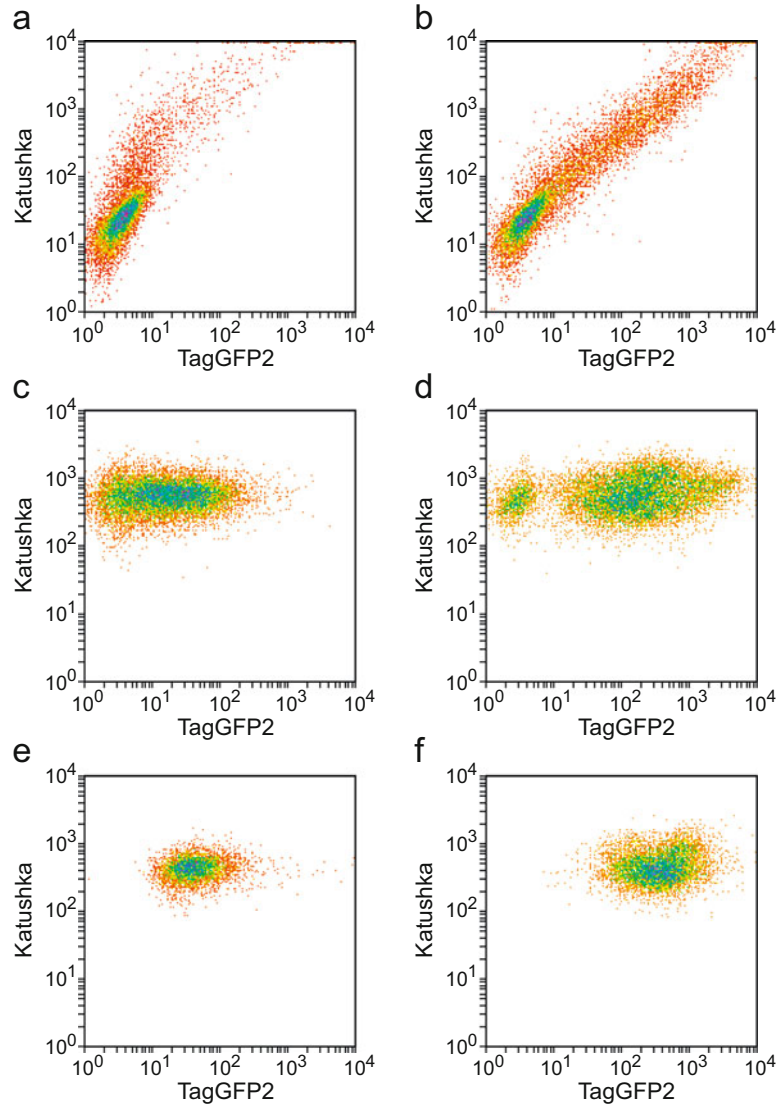

Fig. 5 Generation of cell lines stably expressing NMD reporter. Results of flow cytometry of CT26 cells are shown (the same settings for all panels). (**a**, **b**) Transient transfection with pNMD+ (**a**) or pNMD− (**b**) for estimation of green-to-red signal ratios corresponding to about 1:1 copy numbers of TagGFP2 and Katushka-encoding genes. (**c**, **d**) Tol2-based transposition of pTol2-NMD+ (**c**) or pTol2-NMD− (**d**) into Katushka-expressing stable cell line. (**e**, **f**) Katushka/pTol2-NMD+ (**e**) and Katushka/pTol2-NMD−(**f**) stable lines after FACS sorting (according to the chosen *green-to-red* ratios) and propagation

selected region with no RFP- or GFP-negative or very dim cells in the sample (*see* Fig. 2, bottom panels and Fig. 5e, f).

12. If required, repeat sorting of target cell populations (**steps 7–13**).
13. Freeze the obtained cell lines stably expressing Katushka and pTol2-NMD+ or pTol2-NMD− with DMSO in liquid nitrogen for future use.

3.4 Analysis of Stable Cell Lines

3.4.1 Inhibitory Analysis

To check whether the stably expressed NMD reporter correctly follows NMD activity, inhibitory analysis can be performed. Use treatments known to inhibit the NMD machinery such as:

1. Nonspecific (caffeine and wortmannin [31, 32]) or specific (NMDI14 [33]) NMD chemical inhibitors;
2. Inhibitory RNAs, e.g., shRNA against key NMD factor UPF1 [14, 15].

These treatments should result in significant suppression of NMD activity. Thus, an increase of green fluorescence in pTol2-NMD+-expressing cells is expected upon treatment. For each inhibition experiment use 4 samples of cells: NMD+ with inhibitor, NMD+ without inhibitor, NMD− with inhibitor, NMD− without inhibitor.

Treatment with Wortmannin

1. Seed out about 3–5 × 10^4 stably expressed cells (both Katushka/pTol2-NMD+ and Katushka/pTol2-NMD−) on 35-mm plastic dishes. Use two dishes for each cell culture.
2. Culture cells for 12–14 h in complete growth medium DMEM at 37 °C, 5% CO_2 in a humidified incubator.
3. Prepare 3 mL DMEM with 20 μM wortmannin by adding 30 μL of 2 mM wortmannin stock solution in DMSO. Also, prepare 3 mL DMEM with 30 μL DMSO (for vehicle controls).
4. Change cell medium to fresh prewarmed DMEM with wortmannin or DMSO in Katushka/pTol2-NMD+ and Katushka/pTol2-NMD− samples.
5. Culture cells for at least 24 h at 37 °C, 5% CO_2 in a humidified incubator.
6. Prepare 10^4–10^5 cells for flow cytometry analysis (*see* Subheading 3.2.4, **steps 1** and **2**).
7. Perform flow cytometry analysis (*see* Subheading 3.3.4, **steps 3** and **4**). Wortmannin treatment should result in a considerable increase of green signal (increase in green/red ratio) in Katushka/pTol2-NMD+ cells.
8. Estimate ratio of the green/red ratios in NMD+ and NMD− without wormannin (vehicle controls). Estimate the same value for wortmannin-treated samples. Difference between vehicle controls and wortmannin-treated samples provides a level of NMD inhibition.

Treatment with Caffeine

1. Seed out about 3–5 × 10^4 stably expressed cells (both Katushka/pTol2-NMD+ and Katushka/pTol2-NMD−) on 35-mm plastic dishes. Use two dishes for each cell culture.

2. Culture cells for 12–14 h in complete growth medium DMEM at 37 °C, 5% CO_2 in a humidified incubator.
3. Prepare 10 mM solution of caffeine powder (Sigma) in DMEM, sterilize it by filtering via 0.22 μm filter. Store this solution at +4 °C for up to 2 months.
4. Change cell medium in both Katushka/pTol2-NMD+ and Katushka/pTol2-NMD− samples to fresh prewarmed DMEM with 10 mM caffeine every 12 h. Replace cell medium to fresh DMEM without caffeine in control pair of samples Katushka/pTol2-NMD+ and Katushka/pTol2-NMD−.
5. Culture cells for at least 24 h.
6. Prepare 10^4–10^5 cells for flow cytometry analysis (*see* Subheading 3.2.4, **steps 1** and **2**).
7. Perform flow cytometry analysis (*see* Subheading 3.3.4, **steps 3** and **4**). To estimate the value of NMD inhibition, compare the ratio of green/red signal values for the treated and untreated NMD reporter samples.

3.4.2 DNA and mRNA Analysis

DNA and mRNA analysis by quantitative PCR can be used to quantify integration copy number and expression levels of Katushka- and TagGFP2-encoding genes according to standard protocols [18, 34, 35].

4 Notes

1. Fluorescence-activated cell sorting is a key approach for this protocol. It is a highly sophisticated technique, which requires complex and expensive equipment (cell sorter) and an experienced operator, who is able to conduct calibration and set up proper detection and gating parameters to sort high-purity target cell populations. Various FACS machines can be used according to common practice of sorting of live mammalian cells [36, 37]. Ideally, to avoid spectral cross talk, it should be equipped with blue (488 nm) and green-yellow (e.g., 561 nm) lasers focused at spatially separated spots for independent excitation of TagGFP2 and Katushka. If there is no laser in the 520–600 nm region in a particular FACS machine, single excitation line at 488 nm can be used for both TagGFP2 and Katushka [16, 18]. In this case, however, it is especially important to use control cells transfected with TagGFP2 or Katushka alone to select proper detection and compensation settings and avoid spectral cross talk between green and red signals. See Spectra Viewer at the Evrogen website (http://evrogen.com/spectra-viewer/viewer.shtml) to select optimal lasers and detection filters for TagGFP2 and Katushka (TubroFP635).

In our work we used FACSAria III (Becton Dickinson). TagGFP2 was excited with 488 nm laser and detected at 530/30 nm; Katushka was excited with 561 nm laser and detected at 670/14 nm.

2. You can use an option to sort single cells into 96- or 384-well plates. Single cell-derived clones require more time to grow but provide much more homogeneous fluorescence signals.
3. We use the CMV promoter to drive expression of the GFP-HBB cassette. This viral promoter is highly active in many cell lines (such as HeLa), but it is not ubiquitously expressed in primary cells [38]. To solve this problem, human PGK and EF-1α promoters can be used [39].
4. Nearly all commonly used *E. coli* strains (e.g., XL1-Blue) can be used for plasmid propagation. The quality of purified plasmid DNA may greatly affect transfection efficiency. In particular, treatment with endotoxin-removal buffer is very important to increase transfection efficiency for some cell lines (e.g., LLC).
5. We recommend determining optimal concentrations of antibiotic required to kill your host cell line before transfection with pTol2-NMD plasmid by treating the cells with several concentrations, ranging from 100 μg/mL to 1 mg/mL. Prepare a sterile 1600 μg/mL stock solution of G418 in PBS or DMEM and store it at 4 °C for several months. To find the minimum effective G418 concentration, a kill curve should be measured. After treatment, cell death occurs rapidly, allowing the selection of transfected cells with plasmids carrying the *neo* gene in a few passages.
6. In our work, we used FuGENE HD or FuGENE 6 reagents for transfecting CT26 cells, and electroporation (nucleofection) with Nucleofector 2b for difficult-to-transfect cells such as LLC. These procedures are described in detail in ref. 18.
7. Do not seed more than 1/10 of transfected cells (no more than 5×10^4 onto a plate with antibiotic). If the cells are plated with too high density, efficiency of antibiotic selection gradually decreases.
8. Monitoring cell fluorescence during selection of stable cell lines allows any red-negative green-positive cells that may appear to be removed. When a population of cells with fluorescence in only one channel is observed, use analysis and sorting of live cells by FACS. We do not recommend using this analysis before 14 days of G418 selection.
9. In our work we imaged cells with BZ9000 fluorescence microscope (Keyence) using 20× objective and standard filter sets for green (excitation at 450–490, emission at 510–560 nm) and red (excitation at 540–580, emission at 600–660 nm)

channels. We also used ZOE Fluorescent Cell Imager (Bio-Rad) with LED light sources.

10. Katushka/pTol2-NMD+ stably expressed cells with green-to-red ratio similar to that of transiently transfected pNMD+ cells carry close to one-to-one ratio of TagGFP2 and Katushka-encoding genes into their genome. Optionally, you can also select Katushka/pTol2-NMD+ cells with a higher level of green signal, which have more copies of integrated pTol2-NMD+ (*see* Fig. 2). Bright green fluorescence of these cells would be helpful in some biological models, e.g., analysis of tumors in vivo (where dim green signal is hard to detect reliably), or when increased NMD activity (and thus decreased green fluorescence) is expected during the experiment.

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