

Analysis of Nonsense-Mediated mRNA Decay in Mammalian Cells

Pamela Nicholson,¹ Raphael Joncourt,¹ and Oliver Mühlemann¹

¹Department for Chemistry and Biochemistry, University of Bern, Bern, Switzerland

ABSTRACT

The nonsense-mediated mRNA decay (NMD) pathway acts to selectively identify and degrade mRNAs that contain a premature translation termination codon (PTC), and hence reduce the accumulation of potentially toxic truncated proteins. NMD is one of the best studied mRNA quality-control mechanisms in eukaryotes, and it has become clear during recent years that many physiological mRNAs are also NMD substrates, signifying a role for NMD beyond mRNA quality control as a translation-dependent post-transcriptional regulator of gene expression. Despite a great deal of scientific research for over twenty years, the process of NMD is far from being fully understood with regard to its physiological relevance to the cell, the molecular mechanisms that underpin this pathway, all of the factors that are involved, and the exact cellular locations of NMD. This unit details some of the fundamental RNA based approaches taken to examine aspects of NMD, such as creating PTC+ reporter genes, knocking down key NMD factors via RNAi, elucidating the important functions of NMD factors by complementation assays or Tethered Function Assays, and measuring RNA levels by reverse-transcription quantitative PCR. *Curr. Protoc. Cell Biol.* 55:27.4.1-27.4.61. © 2012 by John Wiley & Sons, Inc.

Keywords: NMD • site-directed mutagenesis • RNAi • complementation assay • Tethered Function Assay • RNA • RT-qPCR

INTRODUCTION

The cascade of events during expression of protein-coding genes involves a series of complex and tightly linked steps from transcription of the respective DNA region to the eventual protein synthesis. While the intricacy of gene expression allows for fine-tuned regulation at many different levels, it also makes each step susceptible to errors. Therefore, to ensure the accuracy of gene expression, cells have evolved elaborate quality-control mechanisms both in the nucleus and in the cytoplasm at multiple stages during gene expression (Doma and Parker, 2007). At the level of messenger RNA (mRNA), two important features are monitored: (i) if the correct set of proteins has bound to a particular mRNA and (ii) if the coding potential of the mRNA is intact. The nonsense-mediated mRNA decay (NMD) pathway deals with the latter; it acts to selectively identify and degrade mRNAs that contain a premature translation termination codon (PTC), and hence reduce the accumulation of potentially toxic truncated proteins.

NMD was first recognized in 1979, when studies in yeast showed that a nonsense mutation in the *URA3* gene (coding for orotidine-5'-phosphate decarboxylase) destabilized the encoded mRNA without affecting its synthesis rate (Losson and Lacroute, 1979). In the same year, a nonsense mutation in the β -globin gene was discovered in patients suffering from the homozygous form of β -thalassemia (Chang and Kan, 1979). The low levels of PTC-containing (PTC+) β -globin mRNA indicated a rapid mRNA turnover rate because transcription of the gene and processing of the mRNA were indistinguishable from the wild-type (PTC-) counterpart (Maquat et al., 1981). At the time, it was proposed that

associated ribosomes protect mRNAs from nuclease attack, and that the ribosome-free regions downstream of PTCs render PTC+ mRNAs vulnerable to nucleases. However, with the discovery of stable untranslated mRNAs in mammalian cells and many reduced mRNA levels of PTC-containing genes in different species, it became apparent that nonsense mutations can activate a specific pathway leading to the specific degradation of the PTC+ mRNA (reviewed in Maquat, 1995).

A wealth of investigation has been done since 1979 to characterize and understand this degradation pathway in various model systems as well as in mammalian cells. Many of the proteins that play a role in NMD have been identified and characterized. To investigate the process of NMD, two fundamental questions need to be addressed: what are the exact molecular mechanisms involved in the recognition of PTC+ mRNA, and after its identification, how is it degraded. A great deal of effort has gone into answering these questions, and various models have been proposed (Chang et al., 2007; Isken and Maquat, 2007; Brogna and Wen, 2009; Nicholson et al., 2009; Rebbapragada and Lykke-Andersen, 2009). In addition to studies trying to elucidate its mechanism, NMD has also been extensively addressed from evolutionary, physiological, developmental, and genome-wide perspectives.

It is vital to understand the molecular mechanisms that underpin NMD because a third of all known disease-causing mutations are predicted to generate a PTC, and consequently NMD is a significant modulator of genetic disease phenotypes in humans (Frischmeyer and Dietz, 1999). In this sense, NMD can act either in a beneficial or in a detrimental way: the latter if it prevents the production of proteins with some residual function and the former if it prevents the synthesis of toxic truncated proteins (Holbrook et al., 2004; Khajavi et al., 2006; Kuzmiak and Maquat, 2006).

Many of the advances in dissecting the NMD process in mammalian cells have arisen from experiments involving the methods stipulated in this unit. Basic Protocol 1 in this unit details the use of site-directed mutagenesis to create PTC+ reporter genes or genes for expression of mutant NMD proteins. Basic Protocol 2 explains how to knock down various NMD factors using vector-based RNA interference (RNAi) technology. Basic Protocol 3 combines the first two methods and describes how to carry out NMD complementation assays by knocking down an endogenous NMD factor and simultaneously expressing a recombinant RNA interference (RNAi)-resistant variant of this factor. Basic Protocols 4-6 will discuss how to measure RNA levels by reverse transcription quantitative PCR (RT-qPCR), and finally, Basic Protocol 7 will aid in the establishment of a Tethered Function system, which has been used extensively in the analysis of NMD. The method or experimental setup will be explained in detail and often with the aid of NMD-specific examples. In the Commentary section at the end of the unit, background information for the techniques, anticipated results, and time considerations are provided for each experimental approach, as well as several recommended online resources. The critical parameters section details what needs to be thoroughly considered in setting up the experiment, and also provides guidance for some of the well-known problems of a given method.

STRATEGIC PLANNING

Creation of PTC+ mRNAs and Mutant NMD Proteins by Site-Directed Mutagenesis (Basic Protocol 1)

Designing synthetic primers for your site-directed mutagenesis reaction

The following considerations should be made for designing mutagenic primers:

1. For the QuikChange II and II XL kits, both mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid. Importantly, the primers should lack strong self-dimerization potential. Primers complement each other's strand on the 5' end, but do not completely overlap (as suggested in the manual of the kit manufacturer), which enlarges the melting temperature (T_m) difference between desired primer-to-template annealing and undesired primer-pair self-annealing. For the QuikChange Multi-Site Kits, only one mutagenic primer is required; a corresponding primer with the same mutation binding to the opposite strand is not necessary.
2. Primers should be between 25 and 45 bases in length.
3. Primer T_m should be at least 78°C.
4. The desired mutation should be in the center of the primer with at least 10 bases of correct sequence on both sides. When this is difficult (perhaps when several mutations are incorporated into one primer), the mutation(s) should be placed at least 4 bases away from the 5'-terminus and 6 to 8 bases from the 3'-terminus.
5. The primers should be as close to having a GC content of 40% as possible.
6. The primers should terminate in one or more C or G bases.
7. The mutagenesis efficiency is improved by using highly purified primers, prepared either by fast polynucleotide liquid chromatography (FPLC) or by preparative polyacrylamide gel electrophoresis (PAGE). However, many researchers have used cartridge-purified or even unpurified primers without any issues.
8. When making amino-acid changes, consider the codon usage frequency when designing the primers. For instance, when creating a mutation in a codon that will now direct the insertion of a leucine amino acid in a construct that will be expressed in a human cell line, consider using CTG (40% human usage frequency) instead of CTA (7% human usage frequency).
9. For further consultation on constructing primers containing several mutations, see (Zheng et al., 2004).

Preparation of the plasmid DNA

While plasmid DNA isolated from almost all of the commonly used *E. coli* strains (*dam*⁺), such as XL1-Blue, is methylated and is a suitable template for mutagenesis, plasmid DNA isolated from the *dam*⁻ *E. coli* strains, such as JM110 and SCS110, are not suitable. Also, the plasmid DNA to be used in the PCR reaction should be of good quality and purity. Importantly, very large plasmids (longer than 10 kb) may be difficult to mutagenize, which is why the protocol for QuikChange II XL has been included below. This kit is optimized for mutagenizing long templates. Alternatively, consider subcloning the region to be mutated into a smaller plasmid for mutagenesis and then subcloning the region back into the desired plasmid.

pSUPuro-Based RNAi to Knock Down NMD Factors (Basic Protocol 2)

Creating pSUPER vector system for expression of short interfering RNA to knock down NMD genes

This vector system for RNAi uses the RNA polymerase III H1-RNA gene promoter, as it produces a small RNA transcript lacking a polyadenosine tail, has a well defined start of transcription, and has a termination signal consisting of five thymidines in a row (T5). Importantly, transcription terminates after the second uridine when RNA polymerase III reaches the T5 stretch, yielding a transcript resembling the ends of synthetic siRNAs,

which also contain two 3' overhanging T or U nt's. This protocol is based on inserting annealed oligonucleotides encoding an shRNA to silence a specific NMD gene into a vector called pSUPuro (Paillusson et al., 2005), which was created from the pSUPER vector (Brummelkamp et al., 2002), and is explained in Figure 27.4.1A-B, along with an example (Fig. 27.4.1C). It should be noted that the creators of the pSUPER RNAi system also sell a pSUPER vector containing the puromycin gene. A similar RNAi vector system can also be purchased from BD Biosciences Clontech (RNAi-Ready pSIREN Vectors). The plan for generating a vector for the expression of shRNA for the knockdown of a desired NMD related mRNA can be carried out as follows:

1. Identify a target sequence within the NMD gene of interest.
2. Design the corresponding oligonucleotides to generate the shRNA.
3. Anneal the forward and reverse strands of the oligonucleotides that contain the shRNA sequence.
4. Linearize pSUPuro vector with *Bgl*III and *Hind*III.
5. Ligate the annealed oligonucleotides into the *Bgl*III and *Hind*III sites of the vector.
6. Transform the ligation reaction into *E.coli* cells.
7. Screen for positive clones by restriction digestion analysis and DNA sequencing.
8. Prepare plasmid DNA of a confirmed positive clone. Ensure the purity of the pSUPuro DNA for transfection by a method that will isolate high-quality, endotoxin-free plasmids.

Steps 1 to 2 are explained below; for step 3, see Support Protocol 1; for assistance with steps 4 to 8, please refer to *Molecular Cloning: A Laboratory Manual* (Sambrook and Russell, 2000) or a similar laboratory methods manual.

Choosing target sequences

1. Search for AA dimers within the coding sequence of your NMD gene and identify 19 nt immediately downstream thereof.

Figure 27.4.1 (*appears on next page*) pSUPER vector system for expression of short interfering RNA to knockdown NMD genes. **(A)** pSUPuro is derived from pSUPER, a plasmid for stable expression of shRNAs in mammalian cells from the polymerase III H1-RNA gene promoter (Brummelkamp et al., 2002). It was created by insertion of a puromycin-resistance cassette lacking the *Hind*III site into the *Xho*I site of pSUPER (Paillusson et al., 2005). The legend outlines the other features of the plasmid. **(B)** Top shows the general design of the shRNA forward and reverse oligonucleotides, which are inserted as double-stranded oligonucleotides into pSUPuro between the *Bgl*II and *Hind*III sites. After this, these vectors contain dsDNA sequences that encode the shRNA cloned between the H1 promoter (a Pol III promoter) and a transcription termination site (gray sequence) comprising 5 thymidine residues. The transcript is terminated at position 2 of the termination site, and then folds into a stem-loop (gray sequence) structure with 3'-UU overhangs (gray). The ends of the shRNAs are processed *in vivo*, transforming the shRNA into a 21-nt siRNA-like molecule, which in turn initiates RNAi (see text for details and references). **(C)** Shows the forward oligonucleotide which, annealed with the reverse oligonucleotide (not shown) and ligated into pSUPuro, was used to generate pSUPuro-SMG6 (see Basic Protocol 2 and Fig. 27.4.7). The 19-nt target sequence and its complementary antisense sequence have been underlined (Redon et al., 2007). **(D)** Depicts a primer used in QuikChange Multi-Site Directed mutagenesis to mutate the sequence of the SMG6 open reading frame targeted by the shRNA shown in (B) in the pcDNA3-HA-SMG6 expression plasmid. The inclusion of these two silent mutations (shown in gray) means that the specific shRNA will no longer be able to bind to this region in SMG6 mRNA (see Basic Protocol 3 and Fig. 27.4.10).

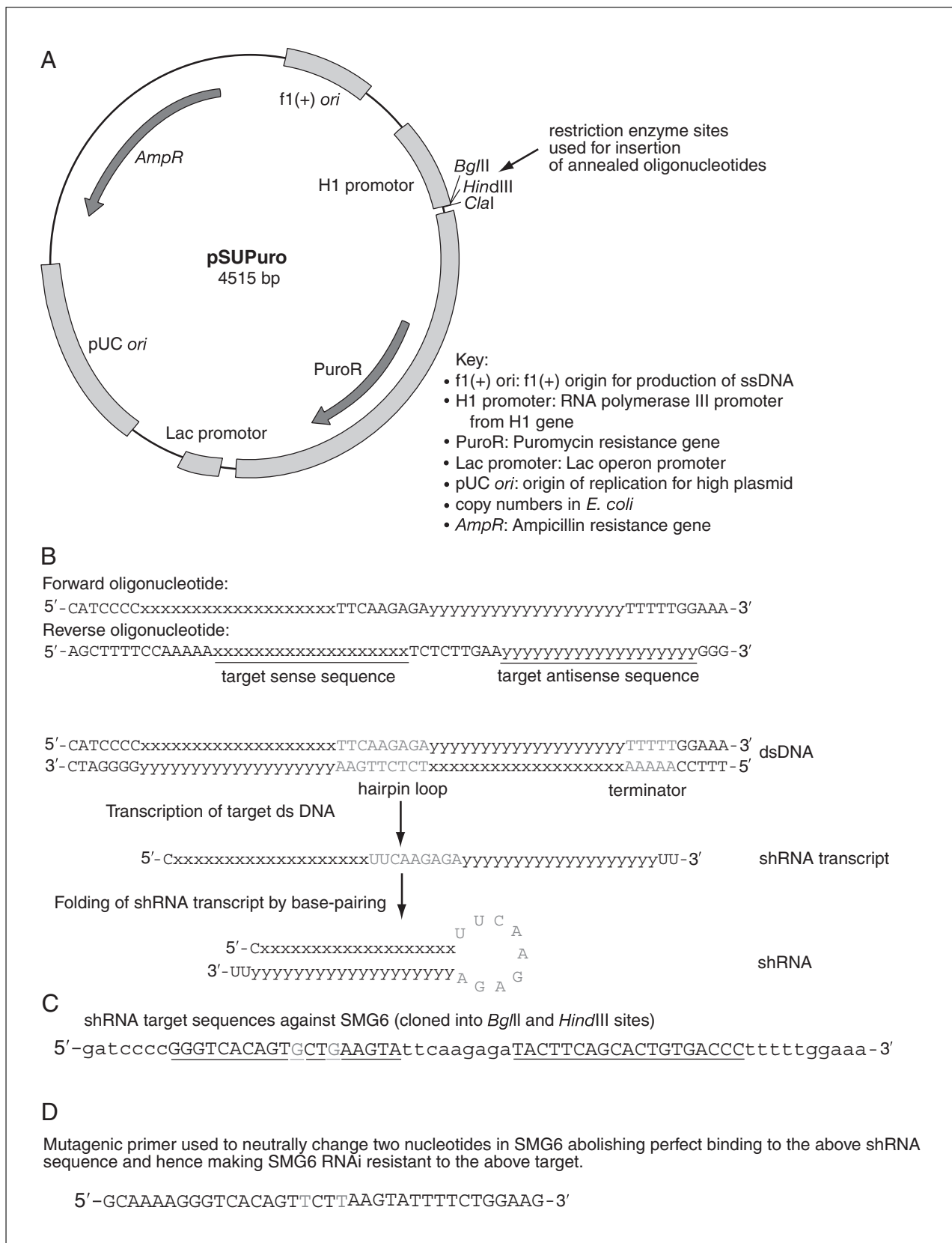


Figure 27.4.1 (legend appears on previous page)

2. Do not select sequences in the 5' or 3' untranslated regions (UTR) or within 75 bases of the start codon, as such sequences are highly likely to be regulatory protein binding sites which might interfere with the binding of RISC.
3. Long stretches of ≥ 4 T's or 4 A's in the target sequence should be avoided since the resulting ≥ 4 T stretch in the shRNA coding region may act as a termination signal for RNA pol III.
4. GC content of the selected 19-nt oligonucleotide sequence should be between 30% and 70%; approximately 50% is optimal for siRNA activity.
5. Including the AA dimer in the candidate sequence, check the 21-base oligonucleotide for secondary structure and long base runs, which can interfere with proper annealing. Eliminate candidate sequences with these features.
6. Compare the candidate sequence to an appropriate genome database to identify sequences that are specific for the NMD gene of interest and show no significant homology to other genes.
7. To optimize gene silencing, it is beneficial to identify and test multiple target sequences that are specific for the NMD gene.

Designing the oligonucleotides

To effect the silencing of a specific NMD factor, the pSUPuro vector is used in concert with a pair of custom-designed oligonucleotides that contain, among other features, a unique 19-nt sequence derived from the mRNA transcript of the gene targeted for suppression (see above and Fig. 27.4.1A,B). A single-nt mismatch in the 19-nt targeting sequence can abrogate the ability to suppress gene expression. Therefore, the sequence of the oligonucleotides can be designed using an online Web resource—in brief they should include the following features:

1. A 5'-*Bgl*III restriction site overhang on the top strand and a 5'-*Hind*III restriction site overhang on the bottom strand. These restriction sites will enable directional cloning of the annealed oligonucleotides into the pSUPuro vector. Note that the double-stranded oligonucleotide is designed to fit into the *Bgl*III site of the vector, but that the *Bgl*III site is destroyed upon ligation to enable more efficient screening of positive clones.
2. The 19-base oligonucleotide sense sequence (target sense sequence) of the shRNA target site.
3. A 7- to 9-nt hairpin loop sequence (see Fig. 27.4.1B, 5'-TTCAAGAGA- 3').
4. The 19-base oligonucleotide antisense sequence (target antisense sequence) of the shRNA target site; ensure proper orientation for correct formation of the hairpin structure. The 5' end of the antisense strand should be less stable than its 3' end in order to favor the incorporation of the antisense strand into the RISC complex.
5. RNA Pol III terminator sequence consisting of a 5 to 6 nucleotide poly(T) tract.
6. PAGE purification of the two oligonucleotides is highly recommended.

Working in tissue culture

A micro-flow advanced biosafety cabinet or similar cabinet should be used to carry out all tissue culture techniques, including making all tissue culture reagents. Aseptic techniques should be followed at all times and routine checks should be carried out to test for contamination or the existence of mycoplasma in cells. For further information read, *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications*, sixth edition, by R. Ian Freshney (Freshney, 2010).

NMD Complementation Assays (Basic Protocol 3)

Creating plasmids expressing NMD factors that are RNAi resistant

First, it is important to have generated and validated a pSUPuro vector that expresses a short interfering RNA to knock down a specific NMD gene, as explained below. Next, it is important to have constructed a plasmid that is able to exogenously express the same NMD gene (or a specific mutant of the gene, see Table 27.4.1). The strategic planning for this method involves performing site-directed mutagenesis on this plasmid to create one or more silent point mutations in the third base of codons within the region targeted by the shRNA sequence to make the NMD gene expressed from the plasmid RNAi resistant, as shown in Figure 27.4.1D for SMG6. If more than one siRNA target is used to create an effective knockdown, then all the target sequences within the ORF of the NMD gene in the expression plasmid will have to be slightly altered to abolish the ability of all the shRNA targets to bind and elicit silencing. Importantly, only a few nt changes in the target region have to be made and, unless specifically desired, they should be silent mutations, meaning that they should not change the amino acid sequence of the exogenously expressed NMD protein.

Extraction of RNA and Analysis of RNA Levels by Reverse-Transcription Quantitative PCR (Basic Protocols 4-6)

Working with RNA

The biggest challenge in any aspect of RNA research is preparing intact RNA molecules and keeping them undamaged throughout any subsequent manipulations. The RNA backbone is inherently more sensitive to damage than DNA because of the 2' hydroxyl group attached to the pentose ring. The 2' hydroxyl group adjacent to phosphodiester linkages can act as an intramolecular nucleophile attacking the adjacent phosphodiester bond, creating a 2', 3' cyclic phosphate and cleaving the RNA backbone. Alkaline pH, divalent metal ions, and ribonucleases (RNases) are the three major causes of RNA degradation. RNase A is a very abundant and very stable enzyme that hydrolyzes RNA, and is probably the most problematic source of RNA degradation. Extraction and isolation of undegraded RNA from cells requires careful laboratory technique; take all precautions to prevent RNase contamination by wearing gloves, using RNase-free water, reagents, equipment, plasticware, glassware, and workspace, and by using aerosol-barrier filter tips for pipetting, especially if your pipets are also used for DNA cloning work etc.

Choosing the detection chemistries for your qPCR assays

The simplest and least costly approach to RT-qPCR uses DNA binding fluorophores for nonspecific detection of DNA targets (such as BEBO, BOXTO, Eva Green, ethidium bromide, or SYBR Green). With nonspecific detection chemistries, the sensitivity and specificity of the assay is determined only by the forward and reverse primers. The fluorophores used in these assays exhibit low fluorescence when unbound in solution, but start to fluoresce brightly when associated with dsDNA and exposed to a suitable wavelength of light. The fluorescence data are collected after each extension step to monitor the progress of the reaction. A drawback to using dsDNA-binding fluorophores is that they emit light in the presence of any dsDNA, even unwanted primer-dimer products or nonspecifically amplified DNA fragments, but this can be easily monitored by performing a post-PCR melting curve analysis, as explained below. The protocol outlines the use of SYBR Green, which is the most commonly used DNA-binding fluorophore; its chemistry is illustrated in Figure 27.4.2B. The resulting DNA-SYBR Green complex absorbs blue light ($\lambda_{\max} = 497$ nm) and emits green light ($\lambda_{\max} = 520$ nm). Actually, the stain preferentially binds to dsDNA, but will stain ssDNA with lower performance. SYBR Green can also stain RNA, albeit with a lower performance than with DNA (Zipper et al., 2004).

Table 27.4.1 List of the Main NMD Factors and the Most Important Mutants That Have Been Published So Far^a

Name	Accession number	Description	Localization	Isoforms	Size	References
UPF1 RENT1 (human, mouse) SMG2 (<i>C.elegans</i>)	Gene ID: 5976 NM_002911.3	RNA helicase, nucleic acid dependent ATPase, and RNA-binding protein. Phosphorylated at serine residues in C-terminal SQ motifs	Shuttling protein; at steady-state predominantly in the cytoplasm	1: canonical sequence 2: AA 353-363 missing	1: 1129 AA / 124,345 kDa 2: 1118 AA / 123,036 kDa	
DE636AA	Highly conserved Asp and Glu in motif II of the NTP-binding and hydrolysis motifs changed to Ala. ATPase and helicase activities are lost.					Bhattacharya et al. (2000)
C126S	Phosphorylation state is greatly reduced. Coprecipitates more SMG1, eRF1 and eRF3, modestly more SMG7. Coprecipitates no UPF2, Y14 and eIF4A3.					Kashima et al. (2006)
K498Q	Putative ATP-binding residue is substituted. ATPase activity is abolished, binding to ATP is lost. Hyperphosphorylated. Modest decrease in eRF3 coprecipitation. Increase in UPF2 and SMG7 coprecipitation. Preferentially associates with UPF3A isoform 2.					Kashima et al. (2006)
4SA	Four AA substitutions preferentially phosphorylated by SMG1 (S1073A, S1078A, S1096A, and S1116A). Modest decrease in eRF3 coprecipitation. Greatly reduced coprecipitation of SMG7. Preferentially associates with UPF3A isoform 1.					Kashima et al., (2006)
dNT	N-terminal 63 AA deleted. Reduced ability to associate with SMG5. Modest decrease in eRF3 coprecipitation. Increase in UPF2 coprecipitation. Hyperphosphorylated. Greatly reduced coprecipitation of SMG7. Preferentially associates with UPF3A isoform 1.					Ohnishi et al. (2003); Kashima et al. (2006)
ΔCHR	AA 130–250 including CH-rich region deleted. Reduced binding to eRF3.					Ivanov et al. (2008)
R844C	Mutation in the helicase domain. Inhibits NMD.					Sun et al. (1998)
G495R/ G497E	G495R and G497E in the ATPase/RNA helicase domain. Hyperphosphorylated. Reduced co-immunoprecipitation of SMG1 and eRF3. Slightly increased co-immunoprecipitation of eIF4AIII. Increased co-precipitation with Dcp1a, Xrn1 and Rrp4.					Isken et al. (2008)

continued

Table 27.4.1 List of the Main NMD Factors and the Most Important Mutants That Have Been Published So Far^a, *continued*

Name	Accession number	Description	Localization	Isoforms	Size	References
UPF2 RENT2 (human, mouse)	Gene ID: 26019 NM_080599.1	Phosphoprotein, phosphorylated at serine residues in N-terminal part.	Cytoplasmic; mainly perinuclear	1: canonical sequence 2: AA 54-83 missing. AA 588-609: AMDFCMNM-NTKANRRKKLVRALF->EKAFCN	1: 1272 AA / 147,810 kDa 2: 579 AA / 66,842 kDa	
Nmd2p (S. cerevisiae) SMG3 (<i>C. elegans</i>)	NM_015542.2	Promotes phosphorylation of UPF1		GNLARVNLRLR-MAVKK AA 610-1272 missing		
Δ (94-133)	AA 94-133 deleted.	Deletes N-terminal UPF1 binding site. Weakens the interaction to UPF1.				Serin et al. (2001)
Δ (94-133, 1095-1272)	AA 94-133 and AA 1095-1272 deleted.	Deletes UPF1 binding sites. Eliminates interaction to UPF1.				Serin et al. (2001)
Δ (526-722)	AA 526-722 deleted.	Eliminates interaction to UPF3B only.				Serin et al. (2001)
Δ (711-928)	AA 711-928 deleted.	Eliminates interaction to UPF3B.				Serin et al. (2001)
Δ (710-1272)	AA 710-1272 deleted.	Eliminates interaction to UPF3A and UPF3B.				Serin et al. (2001)
Δ (711-928, 1095-1272)	AA 711-928 and AA 1095-1272 deleted.	Eliminates interaction to UPF3A and UPF3B.				Serin et al. (2001)
Δ (788-1272)	AA 788-1272 deleted.	Eliminates interaction to UPF3A and UPF3B.				Serin et al. (2001)
Δ (1095-1272)	AA 1095-1272 deleted.	Eliminates the interaction to UPF3B only. Deletes C-terminal UPF1 binding site. Weakens interaction to UPF1.				Serin et al. (2001)
E858R	Eliminates UPF3B interaction in vitro.					Kadlec et al. (2004)
F1113E	Almost completely abolishes UPF1 binding in vitro.					Clerici et al. (2009)
F1171E	Abolishes UPF1 binding in vitro.					Clerici et al. (2009)
M1173E	Abolishes UPF1 binding in vitro.					Clerici et al. (2009)
L1174E	Abolishes UPF1 binding in vitro.					Clerici et al. (2009)
R1176E	Reduces UPF1 binding in vitro.					Clerici et al. (2009)

continued

Table 27.4.1 List of the Main NMD Factors and the Most Important Mutants That Have Been Published So Far^a, *continued*

Name	Accession number	Description	Localization	Isoforms	Size	References
UPF3A (=UPF3B) (<i>C. elegans</i>) UPF3B	Gene ID: 65110 NM_023011.3 NM_080687.2 Gene ID: 65109 NM_080632.2 NM_023010.3	Associates with the EJC. Promotes phosphorylation of UPF1.	Shuttling protein; at steady-state primarily in the nucleus (UPF3B)	UPF3A: 1 (hUpf3p; hUPF3AL): canonical sequence 2 (hUpf3pdelta; hUPF3AS): AA 141-173 missing UPF3B: 1: canonical sequence 2: AA 270-282 missing	UPF3A 1: 476 AA / 54,696 kDa 2: 443 AA / 51,008 kDa UPF3B 1: 483 AA / 57,762 kDa 2: 470 AA / 56,213 kDa	
NES 3A	AA 54-58	VVIRRL -> AVARRA in UPF3B. Mutation in the putative nuclear export signal (NES). Eliminates interaction to UPF2.				Serin et al., 2001
YVVF->DVD	AA 117-119	YVVF->DVD in UPF3B. Eliminates the interaction to UPF2.				Serin et al., 2001
Δ(30-255)	AA 30-255	deleted in UPF3B. Eliminates the interaction to UPF2.				Serin et al., 2001
K52E	In UPF3B.	Eliminates interaction to UPF2 in vitro.				Kadlec et al., 2004
Upf3AΔY14	Conserved C-terminal stretch of 14 AA (434-447)	in UPF3A deleted. Weak increase of reporter levels when tethered compared to WT.				Kunz et al. (2006)
Upf3BΔY14	Conserved C-terminal stretch of 14 AA (421-434)	in UPF3B deleted. Increase of NMD reporter mRNA levels when tethered compared to WT. Complete loss of function. Eliminates the interaction to Y14/Magoh, eIF4AIII and BTZ.				Gehring et al. (2003); Kunz et al. (2006)
R417A, R419A, K421A, R423A, P424A, and Y429F	Point mutations within the conserved C-terminal 14 AA stretch of UPF3B that result in weaker NMD, particularly R423A. R423A eliminates the interaction to Y14.					Gehring et al. (2003)
Upf3aΔUpf2	AA 66-140	deleted in UPF3A. Eliminates interaction to UPF2.				Kunz et al. (2006)
Upf3bΔUpf2	AA 49-143	in UPF3B deleted. Eliminates interaction to UPF2.				Kunz et al. (2006)
Upf3aL-432R	A432R	in UPF3AL. Increases NMD activity.				Kunz et al. (2006)
Upf3a+3b Cterm	C-terminal 150 AAs of UPF3AL	are exchanged for the corresponding sequence of UPF3B. Increases NMD activity.				Kunz et al. (2006)
R434E/ R436E/ R438E	In UPF3B.	Increase of NMD reporter mRNA levels when tethered compared to WT.				Buchwald et al. (2010)

continued

Table 27.4.1 List of the Main NMD Factors and the Most Important Mutants That Have Been Published So Far^a, *continued*

Name	Accession number	Description	Localization	Isoforms	Size	References
SMG1 ATX	Gene ID: 23049 NM_015092.4	Ser/Thr-kinase of PIKK family, phosphorylates UPF1	Cytoplasm and nucleus		3661 AA / 410,501 kDa	
2068–3657	AA 2068-3657.	Does not bind SMG8 and SMG9 anymore.				Arias-Palomo et al. (2011)
618–3657	AA 618-3657.	Interaction to SMG8 and SMG9 is significantly reduced.				Arias-Palomo et al. (2011)
KD1	D1705A.	Abolishes kinase activity.				Denning et al. (2001)
KD2	D1724A.	Abolishes kinase activity				Denning et al. (2001)
KD3	D1724E.	Abolishes kinase activity.				Denning et al. (2001)
SMG-1-DA	D2331A.	Abolishes kinase activity.				Yamashita et al. (2001)
SMG5 EST1B	Gene ID: 23381 NM_015327.2	Required for UPF1 dephosphorylation, directs PP2A to phosphorylated UPF1	Cytoplasm; co-localizes with SMG7 to P-bodies		1016 AA / 113,928 kDa	
dCT	C-terminal 23 AA deleted.	Leads to accumulation of phosphorylated UPF1. Increased coprecipitation of UPF1.				Ohnishi et al. (2003)
SMG-5-DA	D860A.	Leads to accumulation of phosphorylated UPF1. Increased coprecipitation of UPF1.				Ohnishi et al. (2003)
SMG6 EST1A	Gene ID: 23293 NM_017575.4	Ribonuclease with endonucleolytic activity. Required for UPF1 dephosphorylation	Cytoplasm		1: 1419 AA / AA 1-1089 missing AA 1090-1119: ILEEDRLL-SGFVPLLAAPQDPCY-VEKTS DK-> MR-FRLCHQRGCCPHER-ENTCTCKMIISSLQ	
R656E-R737E	R656E and R737E	in 14-3-3-like domain. Binding to UPF1 is reduced in vitro.				Fukuhara et al. (2005)
D1251A	One of the three catalytic AAs	in the PIN-domain mutated. Nonfunctional in NMD.				Huntzinger et al. (2008)
D1353A	One catalytic AA	in the PIN-domain mutated. Nonfunctional in NMD. Endonuclease activity is lost in vitro.				Glavan et al. (2006); Eberle et al. (2009)
D1251A, D1392A	Two of the three catalytic AAs	in the PIN-domain mutated. Nonfunctional in NMD.				Huntzinger et al. (2008)

*continued***27.4.11**

Table 27.4.1 List of the Main NMD Factors and the Most Important Mutants That Have Been Published So Far^a, *continued*

Name	Accession number	Description	Localization	Isoforms	Size	References
D1251A, D1353A, D1392A		The three catalytic AAs in the PIN-domain mutated. Nonfunctional in NMD.				Eberle et al. (2009)
D1251N, D1353N, D1392N		The three catalytic AAs in the PIN-domain mutated. Nonfunctional in NMD.				Eberle et al. (2009)
ΔN	AA 1-576 deleted.	Interaction to NMD factors, Y14 and Magoh is eliminated. Interaction to PABP (via RNA) is eliminated.				Kashima et al. (2010)
ΔPIN	Deletion of the PIN-domain, AA 1239-1419.	Interaction with SMG5 is eliminated.				Kashima et al. (2010)
ΔEBM1	AA 42-58 (EJC binding motif 1, EBM1) deleted.	Strong reduction of the interaction with Y14 and Magoh. Some residual Y14 co-immunoprecipitation.				Kashima et al. (2010)
ΔEBM2	AA 136-152 (EJC binding motif 2, EBM2) deleted.	Strong reduction of the interaction with Y14 and Magoh.				Kashima et al. (2010)
ΔEBM1+2	EBM1 and 2 deleted (AA 42-58, 136-152).	Interaction to Y14 and Magoh is eliminated.				Kashima et al. (2010)
EBM1-Mut	R46E and Y52E in EBM1.	Strong reduction of the interaction with Y14 and Magoh. Some residual Y14 co-immunoprecipitation.				Kashima et al. (2010)
EBM2-Mut	K140E & Y146E in EBM2.	Strong reduction of the interaction with Y14 and Magoh.				Kashima et al. (2010)
EBM1+2 Mut	R46E and Y52E and K140E & Y146E in EBM1 and 2.	Interaction to Y14 and Magoh is eliminated.				Kashima et al. (2010)
SMG7 EST1C	Gene ID: 9887	Required for UPFI	Cytoplasm;	1: canonical sequence 2: 1: 1137 AA /		
Ebs1p (<i>S. cerevisiae</i>)	NM_173156.2	dephosphorylation	co-localizes with SMG5 to P-bodies	AA 569-614 missing AA 569-614 missing AA 914-914: E->EDPKSSPL- 1091 AA / 122,020 kDa 4: 1178 AA /		
	NM_201568.2			LPPDLLKSLAAALEE- 131,654 kDa 5: 1145 AA / 127,854 kDa		
	NM_201569.2			EEELIFS NTPDLY- 1145 AA / 127,854 kDa		
	NM_001174061.1			PALLGPLASLPGRSLF AA 1101-1137: SIWSSMMHP...RGQG-TMNPPH-> KQQH-GVQQLG...PFWKRRKKKGK		
				5: Lacks a portion of the 5' coding region, initiates translation at downstream start codon. Contains alternate in-frame exon in the 3' coding region.		

continued

Table 27.4.1 List of the Main NMD Factors and the Most Important Mutants That Have Been Published So Far^a, *continued*

Name	Accession number	Description	Localization	Isoforms	Size	References
K66E-R163E	K66E and R163E	in the 14-3-3-like domain. Binding to UPF1 is strongly reduced in vitro.				Fukuhara et al. (2005)
SMG8	Gene ID: 55181 NM_018149.6	Forms a complex with SMG1 Inhibits SMG1 kinase activity			991 AA / 109,684 kDa	
SMG9	Gene ID: 56006 NM_019108.2	Forms a complex with SMG1			520 AA / 57,651 kDa	
2-181	AA 2-181.	Interaction to SMG1 and SMG8 is lost.				Fernández et al. (2011)
185-520	AA 185-520.	Interaction to SMG1 and SMG8 is lost.				Fernández et al. (2011)
175-520	AA 175-520.	Interaction to SMG1 is heavily impaired.				Fernández et al. (2011)
NAG SMGL1 <i>(C. elegans)</i>	Gene ID: 51594 NM_015909.2	Essential for NMD in humans and C. elegans			2371 AA / 268,571 kDa	
DHX34 SMGL2 <i>(C. elegans)</i>	Gene ID: 9704 NM_014681.5	RNA helicase activity Essential for NMD in humans and C. elegans			1143 AA / 128,120 kDa	

^aThe wild-type factors and the corresponding information are in bold font; the specific references can be found in Nicholson et al. (2009). Name, alternative names (name shown underneath), and NCBI accession numbers are provided for identification. A short description, cellular localization, isoforms, and molecular masses are also provided. Mutants of each factor are described below the wild-type information.

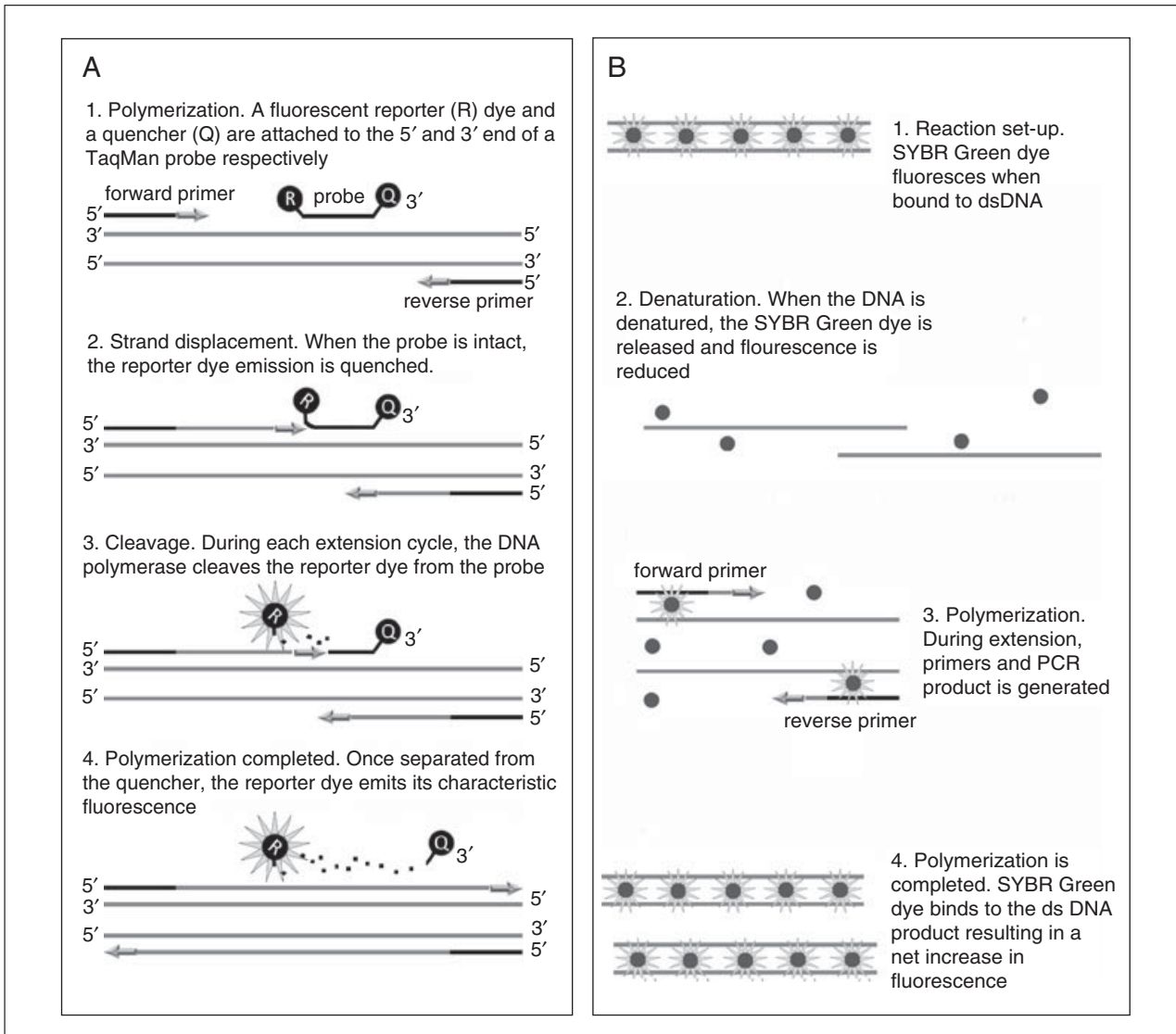


Figure 27.4.2 An illustration of TaqMan probe and SYBR Green chemistries. **(A)** A fluorophore (R) and a quencher (Q) dye are attached to opposite ends of a sequence-specific oligonucleotide probe (Holland et al., 1991). These two dyes form a donor-acceptor pair, with the fluorophore usually attached to the 5' end of the probe and the quencher to the 3' end. When a fluorophore is excited by light, it passes its energy to the quencher via FRET. As long as the donor and acceptor are in close proximity, very little fluorescence is generated. During each cycle of a qPCR protocol the dual-labeled probe hybridizes to its complementary sequence just downstream from one of the PCR primers, encounters the probe, and hydrolyzes it from the 5' end, releasing the fluorophore from the probe. As a result, the emission of the fluorophore is no longer quenched and the increase in the fluorescence can be measured with a real-time qPCR device (reviewed in Bustin, 2000; Kubista et al., 2006). **(B)** The fluorophores used in nonspecific qPCR assays exhibit low fluorescence when unbound in solution (depicted by circles). Then, in the reaction, primers anneal and SYBR Green molecules bind to the dsDNA. The DNA polymerase elongates the template and more SYBR Green molecules bind to the product formed, resulting in an exponential increase in the fluorescence level. The fluorescence data are collected after each extension step to monitor the progress of the reaction. SYBR Green is the most commonly used fluorophore for these assays; see text for further details and references.

Unlike DNA-binding fluorophores, target-specific chemistries that utilize fluorescent probes and/or primers only detect the DNA containing the probe sequence; therefore, use of the reporter probe significantly increases specificity, and enables quantification even in the presence of nonspecific DNA amplification. Several types of specific detection chemistries are available commercially, such as hairpin probes (molecular beacons, scorpions, sunrise primers, or LUX fluorogenic primers), hybridization probes, and hydrolysis probes. They all utilize fluorescently tagged oligonucleotide probes that are specifically designed to detect the target DNA sequence. Hydrolysis probes, typified by the TaqMan chemistry and also known as 5' nuclease assays, are the most commonly used and are advocated in Basic Protocol 6. Here, a fluorophore and a quencher dye are attached to opposite ends of a sequence-specific oligonucleotide TaqMan probe. These two dyes form a donor-acceptor pair, with the fluorophore usually attached to the 5' end of the probe and the quencher to the 3' end. When a fluorophore is excited by light, it passes its energy to the quencher via fluorescence resonance energy transfer (FRET). As long as the donor and acceptor are in close proximity, i.e., linked to the same oligonucleotide, quenching is efficient and very little fluorescence is generated. During each cycle of a qPCR reaction, the dual-labeled probe hybridizes to its complementary sequence just downstream from one of the PCR primers. When the elongating DNA polymerase encounters the probe, its 5'-3' exonuclease activity hydrolyzes the TaqMan probe, releasing the fluorophore from the quencher. As a result, the emission of the fluorophore is no longer quenched, and the increase in the fluorescence can be measured with a real-time qPCR-instrument (Fig. 27.4.2A; Bustin, 2000; Wong and Medrano, 2005; Kubista et al., 2006).

Designing specific qPCR primers for SYBR Green I or TaqMan chemistry

1. The optimal primer length is 15 to 25 bp.
2. The primers can have a G+C content between 20% to 70%.
3. The forward and reverse primer should have a similar T_m of approximately 60°C (−/+ 1°C).
4. No more than two G/C bases should be located in the last five bases at the 3' end of the primer, because these base interactions are stronger than A/T interactions and may lead to mispriming.
5. Minimize the formation of primer-dimers by avoiding complementary sequences between the forward and reverse primers, especially near their 3' ends.
6. Where possible, try to design the primers in a way such that one of the primers is spanning an exon-exon boundary. This reduces the risk of amplification of any contaminating genomic or plasmid DNA, since these intron-containing DNA sequences cannot be amplified by such primer pairs.
7. If the primers have to anneal to a very AT-rich sequence (hence making the T_m very low), it might be advisable to substitute one or more bases with modified nucleic acid analogs such as locked nucleic acids (LNA).
8. The optimal amplicon length for SYBR Green assays is a minimum of 100 bp, which is needed to bind sufficient dye. Typical amplicons are between 100 and 200 bp long. Smaller amplicon sizes (60 to 100 bp) can be used in TaqMan assays (see below). Generally, shorter amplicon sizes are more favorable because they can be amplified more efficiently.
9. Check that the target amplicon is not predicted to form any unfavorable secondary structures.

Dye	Excitation max- Emission max (nm)	BHQ Quenching ranges (nm)
FAM	495-520	
TET	521-540	
VIC	530-552	
JOE	526-552	
HEX	540-557	
Cy3	552-570	
TAMRA	552-578	
ROX	585-605	
Texas Red	595-615	
Cy5	643-667	
Cy 5.5	675-694	BHQ-3 620-730
Quasar 705	644-705	

Figure 27.4.3 Fluorophores and quenchers for TaqMan probes. The figure shows the approximate excitation spectra for many of the commercially available fluorophores. It also details the quenching ranges of the commercially available BHQ quenchers. For further details on TaqMan assays, refer to the text.

- If possible, the target amplicon should have a G+C content of no more than 60%. High GC content can reduce the amplification efficiency.

Designing fluorescently labeled probes for TaqMan assays

- Design the probe with a fluorophore at the 5' end and a suitable "black hole quencher" (BHQ) at the 3' end. The emission spectrum of the fluorophore has to overlap with the excitation spectrum of the quencher to achieve efficient quenching. The approximate excitation and emission spectra of the most frequently used fluorophores and quenchers are detailed in Figure 27.4.3. Use this to select an optimal commercially available fluorophore/quencher pair.
- Avoid using G near the 5' end as it can quench fluorescence.
- Avoid long stretches of the same base, especially G.
- The probe length should be no more than 30 bp in length to maximize the quenching of the fluorophore.
- The TaqMan probe T_m should be in the region of 68° to 70°C (which is 10°C higher than the T_m of the primers to ensure that the TaqMan probe will always bind the template before the primer).
- Modifications such as minor groove binders (MGB) can be incorporated into probes to increase T_m , allowing the use of shorter probes or more AT-rich sequences.

Finally, for designing both primers and probes, a BLAST search should be carried out to identify any significant matches to any other sequences in the database that could result in false-positive results. Likewise, analyze the probe sequence for sources of variability such as SNP sites and splice variants. It is highly beneficial to use software tools that are available for primer and probe design.

CREATION OF PTC+ mRNAs AND MUTANT NMD PROTEINS BY SITE-DIRECTED MUTAGENESIS

BASIC PROTOCOL 1

PTCs can appear in mRNAs for a variety of reasons. At the DNA level, PTCs arise from erroneous bases in the gene sequence of the genome (mutations) or due to insertions or deletions leading to frame-shift mutations in genes. At the RNA level, PTCs can be produced by alternative splicing (or splicing errors in general), or less frequently by transcriptional errors. It is estimated that in mammals about one-third of alternatively spliced transcripts contain PTCs and are substrates for NMD (Lewis et al., 2003). In addition to having a damage-control function, NMD is a critical process in normal cellular development. For example, the immunoglobulin (Ig) superfamily and T cell-receptor genes in mammalian lymphocytes undergo dramatic rearrangement during maturation of the immune system. This somatic recombination results in a high frequency (~66%) of frame-shifted genes containing PTCs. To cope with this, the PTC+ mRNAs are down-regulated by 90% to 99% by NMD (Carter et al., 1995). For this reason, researchers investigating NMD often utilize these genes, where they compare the wild-type and PTC+ versions. For experiments relying on NMD reporter genes, the PTC is specifically introduced into the open reading frame of these genes by site-directed mutagenesis, as shown in Figure 27.4.4A for the Ig mini μ gene.

Messenger RNAs are associated with a host of protein factors throughout their lifetime, and mRNAs targeted for NMD are associated with many proteins that will direct the mRNA to degradation. Therefore, to investigate the intricacies of degradation of nonsense mRNAs, it is important to know all of the factors involved in NMD. In particular, it is vital to structurally understand these factors and try to elucidate which parts of these factors might be essential for the degradation of nonsense mRNAs. A comprehensive summary and discussion of all known NMD factors and factors associated with NMD are provided by recent reviews (Chang et al., 2007; Nicholson et al., 2009). A great deal of what is known about the different functions of the NMD proteins has been generated by mutational studies. To serve as a useful reference for your NMD experiments, an extensive collection of existing NMD factor mutants is presented in Table 27.4.1. Examples of altering one or a few specific nucleotides (nt) in plasmid DNA by site-directed mutagenesis to generate a PTC in a NMD reporter gene or to inactivate a functional domain of a NMD factor are shown in Figure 27.4.4.

Site-directed mutagenesis is a method in which a mutation is created at a precise site in a DNA molecule. The protocols below use the QuikChange II kit from Agilent Technologies to make point mutations, insertions, or deletions in plasmid DNA, and the QuikChange Multi-Site kit from Agilent Technologies to make up to five amino acid changes in the plasmid DNA, but the necessary reagents can also be purchased separately. The concepts behind these protocols are explained below and illustrated in Figure 27.4.5. These procedures are based on a combination of the Agilent Technologies QuikChange protocols and experience from our own laboratory. For further information about the method of site-directed mutagenesis see (Fisher and Pei, 1997; Wang and Malcolm, 1999; Zheng et al., 2004).

NOTE: Essential details to this protocol are included in the Commentary (see sections entitled “Basic Protocol 1: Creation of PTC+ mRNAs and mutant NMD proteins by site-directed mutagenesis” in Background Information, Critical Parameters and Troubleshooting, Anticipated Results, and Time Considerations) and must be read in conjunction with this protocol before proceeding with any experiment.

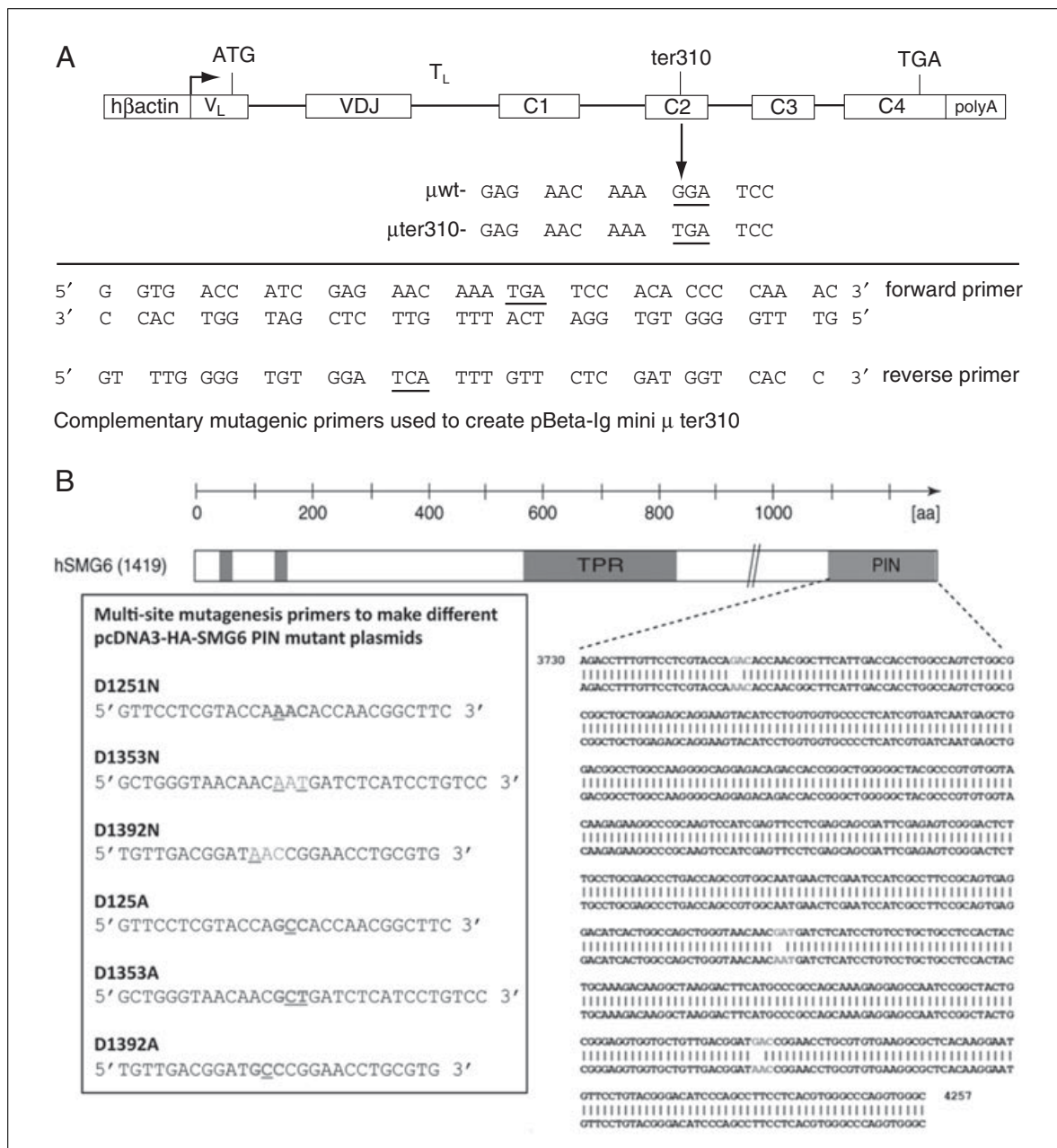


Figure 27.4.4 Using site-directed mutagenesis to introduce premature stop codons in Ig mini μ gene and abolish endonuclease activity in SMG6. **(A)** Top, a schematic of the Ig-μ mini gene constructs. The Ig-μ mini gene (Bühler et al., 2004) is derived from the plasmid pR-Sp6 (Ochi et al., 1983). The nonsense mutation in the Ig-μ mini gene was generated by PCR-mediated site-directed mutagenesis using QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies). The PTC at amino acid position 310 was generated by mutations of GGA to TGA using the forward and reverse primers shown below the diagram. **(B)** Top, an illustration of SMG6 protein organization with the scale above representing the amino acids and various different domains highlighted, in particular the C-terminal PIN domain. Most PIN domains, including SMG6, contain four acidic residues which are highly conserved and coordinate a metal ion that activates water for a nucleophilic attack on the phosphodiester bond of the RNA. In human SMG6, these four key residues are D1251, E1282, D1353, and D1392; they are crucial for endonuclease activity (Wilusz, 2009). Therefore, to make SMG6 endonucleolytically inactive, one or several of these amino acids have to be mutated. The box on the bottom left shows six mutagenic primers to change the three aspartic acid (D) residues to asparagine (N) or to alanine (A) using the QuikChange Multi-Site Directed Mutagenesis Kit (Agilent Technologies) (Eberle et al., 2009). The nt sequence of the PIN domain before (upper strand) and after (lower strand) generating mutations D1251N, D1353N and D1392N is represented on the bottom right (SMG6 PIN mutant sequence P. Nicholson, unpub. observ.).

Materials

Mutagenic primers (see Strategic Planning)
Template dsDNA of interest (see Strategic Planning)
Kits containing PCR reaction components (Table 27.4.2)
Necessary controls (see Table 27.4.2)
Restriction endonuclease *DpnI*
Ultracompetent cells (e.g., from XL10-Gold Ultracompetent cells from Agilent Technologies)
2-mercaptoethanol (optional)
0.1 ng/μl pUC18 transformation control plasmid
Luria-Bertani (LB) liquid medium (APPENDIX 2A) with and without appropriate antibiotic (usually 100 μg/ml ampicillin or 50 μg/ml kanamycin; see recipe for antibiotic stocks) for the plasmid vector
LB agar plates (APPENDIX 2A) containing 100 μg/ml ampicillin (see recipe for antibiotic stocks), 80 μg/ml Xgal (prepare from 50 mg/ml stock; see recipe), and 0.5 mM IPTG (prepare from 0.1 M stock; see recipe)
Plasmid DNA alkaline lysis miniprep kit
0.2-ml thin-walled PCR tubes
Thermal cycler
42°C water bath
14-ml polypropylene round-bottom tubes (e.g., BD Falcon)
37°C shaking incubator

NOTE: In theory, for simple alterations, it is possible to follow the protocol described below (excluding the control reactions) for the QuikChange II kit and provide the following reagents from elsewhere: dNTP mix, high-fidelity DNA polymerase, and reaction buffer containing Mg²⁺, *DpnI* restriction endonuclease, and competent bacteria.

Amplify mutant DNA by PCR

1. In a 0.2-ml thin-walled PCR tube, mix the components shown in Table 27.4.2 and program the thermal cycler for the cycling parameters shown in Table 27.4.3 for the appropriate kit. Perform PCR.

Digest methylated (parental) DNA using the restriction endonuclease *DpnI*

2. Add 1 μl of 10 U/μl *DpnI* to the PCR reaction product from step 1. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 min, and immediately incubate for 1 hr at 37°C.

In the multi-site Lightning kit, the DpnI enzyme has been specifically enhanced so that a 5-min incubation time is sufficient to digest the parental DNA, hence making the whole procedure faster.

IMPORTANT NOTE: *If low mutagenesis efficiency is observed, the volume of DpnI used and/or the incubation time of the reaction can be increased.*

Transform bacteria using XL-10 Gold Ultracompetent *E. coli* cells

3. Gently thaw the XL-10-Gold Ultracompetent cells on ice and aliquot 45 μl of the cells into pre-chilled 14-ml polypropylene round-bottom tubes.

Escherichia coli strain, XL10-Gold Ultracompetent Cells (Agilent Technologies):

Genotype: *Tet^r Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hie [F' proAB lacIqZΔM15 Tn10 (Tet^r) Amy Cam^r].*

Resistance: tetracycline and chloramphenicol.

Efficiency: $\geq 5 \cdot 10^9$ cfu/μg pUC18 DNA.

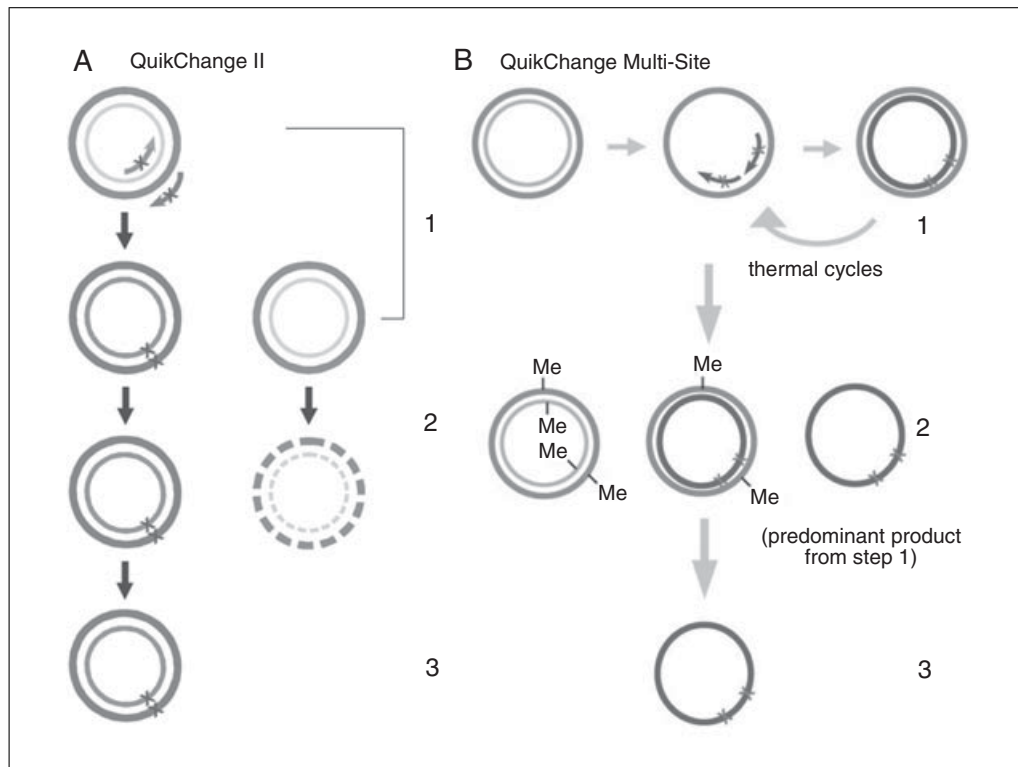


Figure 27.4.5 Overview of various QuikChange Kits (Agilent Technologies). **(A)** The method employs a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers, both containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during PCR, without primer displacement. Extension of the oligonucleotide primers generates a mutated plasmid containing staggered nicks (step 1 in this figure). Then, the product is treated with *DpnI*, which is specific for methylated and hemi-methylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA, because DNA isolated from almost all *E. coli* strains is *dam* methylated and therefore susceptible to *DpnI* digestion (step 2 in the figure). The nicked vector DNA containing the desired mutations is then transformed into *E. coli* cells (step 3 in the figure). The QuikChange II XL is a more specialized version of the method shown in (A), whereby the kit is tailored to the efficient mutagenesis of large (or difficult to mutagenize) plasmid templates. Here, QuikSolution is added to the PCR to aid in the replication of large templates, and the bacterial cells used in this kit have much higher transformation efficiency. **(B)** For the QuikChange Multi Site-Directed mutagenesis, PCR is carried out with supercoiled dsDNA template, two or more synthetic phosphorylated oligonucleotide primers containing the desired mutations all binding to the same strand, and the kit-provided enzyme blend, which can seal the nicks in the one strand bearing multiple mutations and containing nicks. This ssDNA PCR product is treated with *DpnI* as in (A). Then, the reaction mixture, enriched for multiply mutated ssDNA, is transformed into *E. coli* cells, where the mutant closed circle ssDNA is converted into duplex form in vivo. The QuikChange Lightning Multi Site-Directed mutagenesis method is the same as outlined in (B). However, it is faster due to speedier cycling parameters, and the *DpnI* enzyme has been enhanced so that a reaction time of 5 min rather than 60 min is sufficient. The diagrams have been adapted from the Agilent Technologies QuikChange manuals.

4. *Optional:* Add 2 μl of 2-mercaptoethanol to the cells and incubate the tubes on ice for 10 min, gently swirling the contents of the tube every 2 min.
5. Transfer 2 μl of the *DpnI*-treated DNA into the 45 μl of XL-10-Gold ultracompetent cells. Keep the remainder of the *DpnI*-treated DNA at -20°C in case the reaction does not work optimally.

As a transformation control, 1 μl of pUC18 control plasmid (0.1 ng/ μl) can also be transformed into 45 μl of cells.

Table 27.4.2 Reaction Components of Several Different QuikChange Kits (Agilent Technologies)^a

Component	QuikChange II	QuikChange II XL	QuikChange Multi	QuikChange Lightning
10× reaction buffer	5 µl	5 µl	2.5 µl	2.5 µl
ds DNA template	5-50 ng	10 ng	50 ng if template <5 kb; 100 ng if template >5 kb	50 ng if template <5 kb; 100 ng if template >5 kb
Primer # 1	125 ng	125 ng	100 ng each 1-3 primers; 50 ng 4-5 primers	100 ng each 1-3 primers; 50 ng 4-5 primers
Primer # 2	125 ng	125 ng		
dNTP mix (200 µM)	1 µl	1 µl	1 µl	1 µl
QuikSolution reagent	–	3 µl	Up to 0.75 µl if template >5 kb	Up to 0.75 µl if template >5 kb
Polymerase	Pfu Ultra HF DNA polymerase 2.5 U/µl	Pfu Ultra HF DNA polymerase 2.5 U/µl	QuikChange Multi enzyme blend	QuikChange Lightning Enzyme
	1 µl	1 µl	1 µl	1 µl
H ₂ O	to 50 µl	to 50 µl	to 25 µl	to 25 µl

^aIt is important to make sure that the 10× reaction buffers are fully dissolved and well mixed. The DNA polymerases should be kept on ice and the dNTPs should not be subjected to multiple freeze-thaw cycles. Titrate the volume of QuikSolution if low mutagenesis efficiency is observed in the multi-site reactions. It is also recommended to include mutagenesis controls for the QuikChange II and XL reactions: 10 ng of pWhitescript 4.5 kb control template (2 µl), and 125 ng (1.25 µl) of each control primer should be used. For the QuikChange Multi-Site control reactions, 1 µl of the pBluescript II SK (-) control plasmid and 1 µl of the control primer mix should be used. See Critical Parameters for Basic Protocol 1 for more information regarding controls.

6. Incubate the microcentrifuge tubes for 15 to 30 min on ice.
7. Heat-shock the cells for 45 sec in a 42°C water bath.
8. Immediately cool the tubes for 2 min on ice.
9. Add 500 µl of LB medium without antibiotics preheated to 42°C to the cells.
10. Incubate the tubes for 60 min in a 37°C in a shaking incubator at 200 rpm.
11. Spread 100 µl of cells onto LB agar plates containing the appropriate antibiotic for the plasmid vector. If controls have been prepared (see Table 27.4.2 and Critical Parameters for Basic Protocol 1), mix 10 µl of the pBluescript mutagenesis control transformation reaction with 100 µl LB medium and spread on to LB agar plates containing 100 µg/ml ampicillin, 80 µg/mL Xgal, and 0.5 mM IPTG. Finally, mix 5 µl of the pUC18 transformation control reaction with 100 µl LB medium and spread on to LB agar plates containing 100 µg/ml ampicillin.

LB plates should be made as described in APPENDIX 2A; let the medium cool to 50°C before adding antibiotic (see recipe for antibiotic stock solutions); normally ampicillin and kanamycin are used at final concentrations of 100 µg/ml and 50 µg/ml respectively. Supplement the medium with 0.5 mM IPTG and 80 µg/ml Xgal and pour the plates (see recipes for the respective stock solutions). Alternatively, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml Xgal can be spread over the surface of an LB ampicillin/kanamycin plate and allowed to absorb for 30 min at 37°C prior to use.

12. Incubate the transformation plates overnight (for at least 16 hr) at 37°C.

IMPORTANT NOTE: *If low mutagenesis efficiency is observed, if the plasmid DNA is large, or if the E. coli cells have a lower competency than the protocol details, then it will be beneficial to vastly increase the volume of DpnI-treated DNA to be transformed and*

Table 27.4.3 Thermal Cycling Programs for Different QuikChange Protocols^a

Step	QuikChange II		QuikChange II XL		QuikChange Multi-Site		QuikChange Lightning Multi-Site	
	Time/temp. Cycles		Time/temp. Cycles		Time/temp. Cycles		Time/temp. Cycles	
Initial denaturation	30 sec at 95°C	1×	60 sec at 95°C	1×	60 sec at 95°C	1×	120 sec at 95°C	1×
Denaturation	30 sec at 95°C	12-18×	50 sec at 95°C	18×	60 sec at 95°C	30×	20 sec at 95°C	30×
Primer annealing	60 sec at 55°C		50 sec at 60°C		60 sec at 55°C		30 sec at 60°C	
Elongation	60 sec/kb at 68°C		60 sec/kb at 68°C		120 sec/kb at 65°C		30 sec /kb at 65°C	
Final Elongation	—	—	420 sec at 68°C	1×			300 sec at 65°C	1×

^aThe reaction stringency can be increased, if many false positives are being observed using the QuikChange II and XL cycling parameters, by increasing the annealing temperature.

to also plate more of the transformed bacteria on to the agar plates. However, always ensure that the DNA comprises no more than 10% of the transformation reaction.

As an indication that the positive control supplied with the site-directed mutagenesis kit of choice has been successful, there should be predominantly blue-colored colonies on the LB/ampicillin/IPTG/Xgal plates. As an indication that the transformation procedure was successful, there should be as many white colonies on the pUC18 transformation control LB/ampicillin plate. For further information regarding these controls, please refer to Critical Parameters for Basic Protocol 1 (section on “Controls”).

13. Pick single colonies and inoculate each into 5 ml of LB medium containing the appropriate antibiotic.
14. Incubate the cultures overnight in a 37°C shaking incubator at 200 rpm.
15. Prepare small-scale high quality double-stranded DNA using a miniprep kit according to the alkaline lysis method.
16. Perform DNA sequencing to check that you have introduced your desired change(s) but that the PCR has not introduced any unintentional errors.

BASIC PROTOCOL 2

pSUPuro-BASED RNAi TO KNOCK DOWN NMD FACTORS

Four different types of RNA molecules are commonly used for knockdown experiments: synthetic siRNAs, small hairpin RNAs (shRNAs), small hairpin microRNAs (shmiRNAs), and long double-stranded RNAs (dsRNA) (Echeverri and Perrimon, 2006; Lee and Kumar, 2009). Synthetic siRNAs are small RNA duplexes composed of 19 complementary base pairs (bps) and 2-nt 3' overhangs. They are transfected into cells or injected into animals. On entering cells, the strand with thermodynamically less stably paired 5' end of the siRNA duplex is incorporated into the multi-subunit RNA-induced silencing complex (RISC) and directs RISC to the target mRNA by complementary base-pairing, resulting in mRNA degradation. The effects of the siRNAs are transient, especially in actively dividing cells. In contrast, shRNA and shmiRNA-synthesizing vectors allow for controlled or continuous expression of effector RNA molecules in the cell. The shRNA and shmiRNA transcripts consist of both the sense and antisense strand connected by a loop sequence. The DNA-encoding shRNA or shmiRNAs are either transfected into

cells as plasmids or delivered in the form of viral particles, and are maintained as extra-chromosomal copies or stably integrated in the genome as transgenes. The 50- to 70-bp single-stranded RNA (ssRNA) transcripts fold back to form stem-loop structures that are processed in the cytoplasm by Dicer to generate siRNAs, of which one strand then assembles into RISC as for synthetic siRNAs.

NOTE: Essential details to this protocol are included in the Commentary (see sections entitled “Basic Protocol 2: pSUPuro-based RNAi to knock down NMD factors” in Background Information, Critical Parameters and Troubleshooting, Anticipated Results, and Time Considerations) and must be read in conjunction with this protocol before proceeding with any experiment.

Materials

Human cervix epithelioid carcinoma cell line (HeLa cells) or human cell line of choice
pSUPuro plasmid with inserted oligonucleotides that contain the shRNA-expressing sequences targeting gene of choice (see Strategic Planning and Fig. 27.4.1)
pSUPuro plasmid with no inserted oligonucleotides (pSUPuro-empty)
DMEM+/+ medium (see recipe)
DMEM-/- medium (see recipe)
DreamFect transfection reagent (OZ Biosciences, <http://www.ozbiosciences.com/>)
DMEM+/- medium (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
0.05% trypsin/EDTA (e.g., Invitrogen, cat. no. 25300-062)
0.5 mg/ml puromycin stock solution (see recipe)

Hemocytometer
6-well culture plates
25-cm² culture flasks
Tissue culture inverted microscope

Additional reagents and equipment for basic cell culture techniques including counting cells (*UNIT 1.1*), harvesting cells (Support Protocol 2), protein blotting (*UNIT 6.2*), and RT-qPCR (Basic Protocols 5 and 6)

Day 1

1. Use a hemacytometer or automated cell counter to determine the quantity of cells present (*UNIT 1.1*) and to calculate the dilution factor necessary to seed 2×10^5 cells/well in 6-well plate(s). Seed the cells using DMEM+/+ medium.

Day 2

2. Ensure that the cells seeded the day before are at least 60% confluent.
3. Allow the transfection reagent to warm to room temperature and gently vortex the solution.
4. Dilute 400 ng of the pSUPuro containing the siRNA-expressing sequences or pSUPuro empty plasmid with DMEM-/- (without serum and antibiotics) to a final volume of 100 μ l.
5. Dilute 4 μ l of DreamFect transfection reagent per μ g of DNA to a final volume of 100 μ l. Ensure that the transfection reagent doesn't touch the side of the tube, dilute directly into the DMEM-/- solution, and move on to step 6 within 5 min.

6. Combine the diluted DNA and diluted DreamFect, gently mix (do not vortex or centrifuge) the solution and incubate the DNA-DreamFect mixture for at least 15 min at room temperature.
7. Carefully add the 200 μ l transfection mixture drop-wise to the cells growing in DMEM+/- medium and gently rock the plate from side to side to ensure a uniform distribution of the mixture.
8. Incubate the cells at 37°C in a CO₂ incubator under standard conditions until day 3.

IMPORTANT NOTE: Contamination of plasmid DNA samples with bacterial endotoxin can cause massive cell death and interfere with transfection efficiencies. Therefore, ethanol precipitation of the plasmid DNA after using a kit is recommended to obtain high-quality endotoxin-free DNA.

Day 3

9. Remove medium from the cells, being careful not to disturb the cells.
10. Wash monolayers twice with PBS.
11. Add 100 μ l of 0.05% trypsin/EDTA to the cells in each well of the 6-well plate, then gently rock the plate to ensure uniform coverage of the cells with TE buffer.
12. Return the plate to the incubator for 5 min to allow the cells to detach.
13. Meanwhile, calculate the volume of medium required for 5 ml, 25-cm² flasks and dilute the stock solution of puromycin into the calculated volume of DMEM+/+ to a final concentration of 1.5 μ g/ml puromycin.
14. Transfer the cells in 5 ml of puromycin-containing medium from each well in the 6-well plate to a 25-cm² flask to specifically select for the cells containing the pSUPuro plasmids.
15. For the next 48 hr incubate the cells at 37°C in a CO₂ incubator under standard conditions.

Day 4

Observe the cells under the microscope. The selection process is on-going and untransfected cells should be dying.

Day 5

16. Remove the puromycin-containing medium from the 25-cm² flasks.
17. Wash the transfected cells twice with PBS, being careful not to disturb the cells.
18. Add 5 ml fresh DMEM+/+ lacking puromycin.
19. Incubate the cells at 37°C in a CO₂ incubator under standard conditions until the next day.

Day 6

20. Remove the medium from the 25-cm² flasks.
21. Wash twice with PBS.
22. Harvest the cells for both protein (see Support Protocol 2) and RNA analyses (see Basic Protocols 4-6).
23. Check the knockdown efficiency at the protein level by SDS-PAGE and western blotting and at the mRNA level by RT-qPCR or northern blotting.

Blotting techniques are described in UNIT 6.2; RT-qPCR is described in Basic Protocols 5 and 6.

NMD COMPLEMENTATION ASSAYS

The protocol in this section is based upon the previous two methodologies: creating specific mutations in genes by site-directed mutagenesis and knocking down genes by vector-based RNAi. Experiments investigating NMD show that a reporter mRNA containing a PTC will be rapidly degraded compared to its wild-type counterpart. Therefore, if an essential protein for NMD is depleted by RNAi, then the PTC reporter mRNA levels will be similar to the wild-type counterpart because it will no longer be subjected to NMD. The basis of the NMD complementation assay is to knock down an essential NMD protein, which will abolish NMD, and then rescue the phenotype by exogenously expressing the same gene that has been made resistant to the RNAi via silent mutations in the shRNA binding sites.

All of the same materials are required as listed in Basic Protocol 2 but the following additional materials are necessary.

NOTE: Essential details to this protocol are included in the Commentary (see sections entitled “Basic Protocol 3: NMD complementation assays” in Background Information, Critical Parameters and Troubleshooting, Anticipated Results, and Time Considerations) and must be read in conjunction with this protocol before proceeding with any experiment.

Materials

- pSUPuro empty (pSUPuro-E)
- pSUPuro Vector that expresses a short interfering RNA, to knockdown SMG6 gene (pSUPuro-SMG6, see Fig. 27.4.4C)
- Empty vector is from Invitrogen (pcDNA3-E) or pcDNA3 plasmid expressing another NMD factor
- Plasmid exogenously expressing SMG6 (pcDNA3-HA-SMG6)
- Plasmid exogenously expressing RNAi resistant version of SMG6 gene (pcDNA3-HA-SMG6-RNAi^R, see Fig. 27.4.4D)
- Plasmid exogenously expressing RNAi resistant version of SMG6 gene that has mutations to make it endonucleolytically inactive, as shown in Figure 27.4.4B (pcDNA3-HA-SMG6 PIN mut-RNAi^R)
- Plasmid expressing green fluorescence protein (GFP) to act as a transfection control (p-EGFP-C1 BD Biosciences, Clontech)
- pBeta-actin-Ig mini μ wild-type (Ig mu wt)
- pBeta-actin-Ig mini μ Ter 310 (Ig mu ter 310; see Fig. 27.4.4A)

Methodology

The only difference from Basic Protocol 2 is the transfection procedure. On Day 2, the plasmids exogenously expressing NMD factors must be included in the transfection. Table 27.4.4 shows an example protocol detailing a typical transfection scheme for a SMG6 complementation experiment.

EXTRACTION OF RNA AND ANALYSIS OF RNA LEVELS BY REVERSE TRANSCRIPTION–QUANTITATIVE PCR

For determination of relative RNA levels, reverse transcription (RT) followed by quantitative PCR (RT-qPCR) is a sensitive, accurate, and relatively quick—and hence popular—technique. The RNA is first isolated and purified from cells (Basic Protocol 4), then transcribed into complementary DNA (cDNA; Basic Protocol 5), which is used in turn as a template in the qPCR assay (Basic Protocol 6). Since cDNA is usually created from spliced messenger RNA (mRNA), it differs from genomic DNA in that it does not contain introns. The lack of introns in cDNA can be exploited for designing cDNA-specific primers over exon-exon junctions that will not amplify possible contaminants of the RNA

Table 27.4.4 An Example of a Co-transfection Plan for an SMG6 Complementation Assay^a

Transfection	PTC-/+	Transfection control	RNAi	Expression plasmid
TF 1	Ig-mu wt	p-EGFP-C1	pSUPuro-E	pcDNA3-E
TF 2	Ig-mu ter310	p-EGFP-C1	pSUPuro-E	pcDNA3-E
TF 3	Ig-mu wt	p-EGFP-C1	pSUPuro-E	pcDNA3-HA-SMG6
TF 4	Ig-mu ter310	p-EGFP-C1	pSUPuro-E	pcDNA3-HA-SMG6
TF 5	Ig-mu wt	p-EGFP-C1	pSUPuro-E	pcDNA3-HA-SMG6-RNAi ^R
TF 6	Ig-mu ter310	p-EGFP-C1	pSUPuro-E	pcDNA3-HA-SMG6-RNAi ^R
TF 7	Ig-mu wt	p-EGFP-C1	pSUPuro-E	pcDNA3-HA-SMG6-PIN-mut-RNAi ^R
TF 8	Ig-mu ter310	p-EGFP-C1	pSUPuro-E	pcDNA3-HA-SMG6-PIN-mut-RNAi ^R
TF 9	Ig-mu wt	p-EGFP-C1	pSUPuro-SMG6	pcDNA3-E
TF 10	Ig-mu ter310	p-EGFP-C1	pSUPuro-SMG6	pcDNA3-E
TF 11	Ig-mu wt	p-EGFP-C1	pSUPuro-SMG6	pcDNA3-HA-SMG6
TF 12	Ig-mu ter310	p-EGFP-C1	pSUPuro-SMG6	pcDNA3-HA-SMG6
TF 13	Ig-mu wt	p-EGFP-C1	pSUPuro-SMG6	pcDNA3-HA-SMG6-RNAi ^R
TF 14	Ig-mu ter310	p-EGFP-C1	pSUPuro-SMG6	pcDNA3-HA-SMG6-RNAi ^R
TF 15	Ig-mu wt	p-EGFP-C1	pSUPuro-SMG6	pcDNA3-HA-SMG6-PIN-mut-RNAi ^R
TF 16	Ig-mu ter310	p-EGFP-C1	pSUPuro-SMG6	pcDNA3-HA-SMG6-PIN-mut-RNAi ^R

^aMost probably, 100 ng of Ig mini μ expression plasmids, 100 ng of the EGFP expression plasmid, and 400 ng of the pSUPuro plasmids would be used in each transfection. However, it is difficult to state the exact amounts of the RNAi-resistant NMD factor expression plasmids that would be required to express these genes at similar levels to each other. Hence, this table simply describes the plasmids that should be co-transfected only.

samples with genomic or plasmid DNA. The RT phase (RNA to cDNA) is crucial for precise and sensitive quantification, because the amounts of cDNA produced must be proportional to the original amounts of RNA. Successful cDNA synthesis is dependent on the integrity and purity of the template RNA and on using well optimized conditions. RT-qPCR can be performed in a one-step or a two-step assay. This protocol uses the two-step RT-qPCR method. In two-step assays, the RT and qPCR steps are performed in separate tubes, with different optimized buffers and reaction conditions. By performing the RT step separately, a stable cDNA pool is generated that can be stored for long periods of time and used for multiple measurements. It has also been reported that one-step protocols are less sensitive than two-step protocols (Battaglia et al., 1998). However, the two-step method does mean more pipetting, different tubes, and more time. In Basic Protocol 6, the qPCR procedure follows the basic principle of PCR. Its key feature is that the amplified cDNA is detected as the reaction develops in real time, unlike in standard PCR, where the product of the reaction is detected at its endpoint. qPCR is based on the observation that there is a quantitative relationship between the amount of the target material present before PCR and the amount of product formed during the PCR process. Two common methods for detection of products in real-time PCR are: (1) nonspecific chemistries that usually detect fluorescence of a DNA-binding dye, and (2) target-specific

chemistries that utilize fluorescence probes and/or primers. The fluorophores used in RT-qPCR assays absorb light energy at one wavelength and almost immediately re-emit the light energy at another, longer wavelength. Each fluorophore has a distinctive range of wavelengths at which it absorbs light and another at which it emits light. This property enables their use for specific detection of PCR products by qPCR-instruments.

NOTE: Essential details to these protocols are included in the Commentary (see sections entitled “Basic Protocols 4-6: Extraction of RNA and analysis of RNA levels by reverse transcription–quantitative PCR” in Background Information, Critical Parameters and Troubleshooting, Anticipated Results, and Time Considerations) and must be read in conjunction with these protocols before proceeding with any experiment.

Guanidium Thiocyanate–Phenol–Chloroform Extraction of RNA

Methodology

Briefly, this method depends on phase separation by centrifugation of a mix of the aqueous sample and a solution containing water-saturated phenol, chloroform, and a chaotropic denaturing agent called guanidinium thiocyanate, resulting in an upper aqueous phase and a lower organic phase (mainly phenol/chloroform). RNA partitions in the aqueous phase and genomic DNA partitions in the interphase, while protein partitions in the organic phase. In a last step, RNA is recovered from the aqueous phase by precipitation with isopropanol. This RNA isolation method was originally devised by Piotr Chomczynski and Nicoletta Sacchi and published in 1987 (referred to as *Guanidinium thiocyanate-phenol-chloroform extraction*; Chomczynski and Sacchi, 1987, 2006). This type of reagent used for RNA extraction can be purchased from Sigma-Aldrich (TRI-reagent), from Invitrogen (TRIzol), and also from Bionline (Trisure). In our laboratory, we prepare TRI-mix ourselves for a fraction of the cost of the commercially available products (see Reagents and Solutions for the recipe). The TRI-mix protocol takes slightly longer than column-based methods such as the silica-based purification, but it has higher capacity and can yield more RNA. It is important to note that most column-based methods are typically unsuitable for purification of short (<200 nts) RNA species. For all applications involving cell pellets or tissues, 10 times more TRI-mix is used than biological material to be extracted (volume/volume).

Materials

- Cell samples for extraction of RNA (see protocols above)
- Phosphate-buffered saline (PBS; *APPENDIX 2A*)
- TRI-mix (see recipe; very toxic)
- Chloroform (potential narcotic)
- 20 mg/ml glycogen, molecular biology grade (e.g., Sigma, cat. no. G1767)
- Isopropanol (2-propanol)
- 75% ethanol in DEPC-treated water
- RNase-free water: filtered or DEPC treated as described in *APPENDIX 2A*
- Ambion TURBO DNA-*free* DNase Treatment and Removal Reagents (Invitrogen Applied Biosystems):
 - 10 × TURBO DNase Buffer
 - TURBO DNase, 2 U/μl
 - DNase inactivation solution and nuclease-free water
- Aerosol-barrier pipet tips
- Nuclease-free 1.5-ml microcentrifuge tubes
- Refrigerated microcentrifuge
- Thermomixer heat block (e.g., Eppendorf Thermomixer Compact)
- Additional reagents and equipment for spectrophotometric quantitation of RNA (Support Protocol 3)

BASIC PROTOCOL 4

RNA-Based Methods in Cell Biology

27.4.27

Separate phases

1. Remove the medium from the cells.
2. Wash monolayer twice with PBS.
3. Add 1 ml TRI-mix per 10-cm² of culture dish surface area directly to the cells in the dish.

For example, for a 6-well plate, each well would require 1 ml of TRI-mix.

4. Lyse the cells directly in the culture dish by pipetting up and down several times. If the sample is not solubilizing and is still very viscous, increase the solubilization by pipetting the sample repeatedly up and down. After 5 min of incubation at room temperature, proceed to the next step.

If not ready to proceed immediately with the rest of the protocol, the homogenized sample may be stored at room temperature for several hours or at -70°C for 1 month or longer.

5. Add 0.2 ml of chloroform per ml of TRI-mix used.

Scale the volumes up or down where appropriate (for example, if only 200 µl of TRI-mix was used, then only add 40 µl of chloroform at this step).

6. Vigorously mix by hand for 15 sec.
7. Incubate for 3 min at room temperature.
8. Centrifuge for 15 min at 12,000 × g, 4°C.

The chloroform causes the solution to separate into two phases, and, after centrifugation, the organic (yellow) phase is at the bottom of the tube and the aqueous (clear) phase, which contains the extracted RNA, is at the top. There will also be a visible white interface that contains DNA and some denatured proteins.

9. Move the upper aqueous layer to a fresh microcentrifuge tube.

The aqueous phase should be ~600 µl. When the aqueous phase is transferred to a fresh tube, it is important to avoid disturbing this interphase or pipetting any of this material. To be absolutely certain of avoiding contamination of the aqueous phase with interphase, leave a small amount of aqueous phase behind when pipetting.

10. Add 1 µl of glycogen to each sample.

The glycogen acts as a carrier of the RNA, promoting efficient precipitation, preventing small amounts of RNA from sticking to the side of the tube and aiding in detection of the RNA pellet.

Precipitate the RNA

11. Precipitate the RNA by adding 2 volumes of isopropanol. Mix well by inversion.
12. Incubate for 10 min at room temperature.
13. Centrifuge for 10 min at 12,000 × g, 4°C.
14. Remove the supernatant, leaving a white glycogen pellet.
15. Resuspend the precipitated pellet in 80 µl 75% ethanol to wash.
16. Briefly vortex.
17. Centrifuge 5 min at 12,000 × g, 4°C.
18. Remove the ethanol supernatant.
19. Repeat wash steps 15 to 18.

20. Air dry the RNA pellets for no longer than 5 to 10 min.

Note that over-dried pellets will be difficult to resuspend.

21. Dissolve the RNA pellet in 45 μ l of RNase-free water or in RNase-free Tris-EDTA buffer heated briefly to 55° to 65°C in a heat block to aid solubilization if desired.

Note that remnants of non-inactivated DEPC may inhibit RT reactions.

Remove DNA contamination

The RNA samples are treated with RNase-free DNase I. This DNase treatment is especially critical if the primers for the qPCR step cannot be designed to fall across exon-exon boundaries or in separate exons.

22. Add 5 μ l of 10 \times TURBO DNase Buffer to 45 μ l of RNA solution from step 21 and mix by pipetting.

23. Add 1 μ l TURBO DNase (kept on ice), then gently mix the solution.

24. Incubate for 30 min at 37°C.

25. Add 5 μ l DNase inactivation reagent, then mix well by flicking the tube.

26. Incubate for 5 min at room temperature, flicking the tube two to three times to redistribute the inactivation reagent.

27. Centrifuge 10 min at 10,000 $\times g$, room temperature.

28. Transfer the supernatant to a fresh sterile microcentrifuge tube, being very careful not to disturb the DNase I inactivation reagent pellet.

The pellet can sequester divalent cations and change buffer conditions, which would be detrimental to downstream applications such as RT-qPCR.

29. Proceed to spectrophotometric quantification of RNA (Support Protocol 3).

Synthesis of Mammalian cDNA Using Total Cellular RNA

This protocol uses 1 μ g of total RNA in the production of single-stranded complementary copy DNA (cDNA) through reverse transcription (RT). RT coupled to PCR (RT-PCR) is commonly used to detect and quantitate levels of mRNA, pre-mRNAs, or other types of RNA. The method involves using a primer annealed to the RNA of interest. The primer is usually a synthetic oligo(dT)₁₅₋₁₈, random hexamer (dN)₆, or a synthetic DNA oligonucleotide that is complementary to a specific transcript (a gene-specific primer). The primer:RNA hybrid serves as a template during RT, which generates the first strand, a single-stranded cDNA copy of a portion of the target RNA molecule. Using random hexamer priming, it is possible to obtain representative cDNA copies of sequences from the entire length of the mRNAs and pre-mRNAs in a population. This cDNA can be used as a template for PCR.

Materials

1 μ g of total RNA (Basic Protocol 4)

RNase-free water: filtered or DEPC treated as described in APPENDIX 2A

150 ng/ μ l random hexamers (e.g., Applied Biosystems, cat. no. N8080127)

Reagents for RT master mix (Table 27.4.5)

10 \times AffinityScript RT buffer (Agilent Technologies)

100 mM DTT

10 mM each dNTP

RNasin

BASIC PROTOCOL 5

**RNA-Based
Methods in Cell
Biology**

27.4.29

AffinityScript Multiple Temperature Reverse Transcriptase (Agilent Technologies)

Aerosol-barrier pipet tips

Nuclease-free 1.5-ml microcentrifuge tubes

Thermomixer heat block (e.g., Eppendorf Thermomixer Compact)

1. Mix 1 μg total RNA, nuclease-free water, and 300 ng random hexamers (2 μl of 150 ng/ml random hexamers) to a final volume of 13.7 μl in a nuclease-free microcentrifuge tube.
2. Incubate for 5 min at 65°C.

This is a separate RNA denaturation step before cDNA synthesis and it is only required if the template RNA has a high degree of secondary structure. Such a denaturation step (5 min at 65°C) should be performed before adding the RT buffer and reverse transcriptase to the reaction mix.

3. Mix gently and incubate for 10 min at room temperature to cool down.
4. Add the components shown in Table 27.4.5, in the order shown, to each annealed primer/template generated in steps 1 to 3.
5. Mix gently and incubate for 10 min at 25°C for initial primer extension.

This step is important when using random hexamers or oligo(dT) primers because otherwise the primers may dissociate from the template when the samples are incubated immediately at >37°C.

6. Incubate for 1 hr in a 50°C heat block, for cDNA synthesis.

Incubation at 37°C will work for most templates but it can be optimized between 37° and 55°C if necessary. Increasing the temperature can be helpful if the template has strong secondary structures, and may also improve specificity if gene-specific primers are used. 30 min incubation is sufficient in most cases. The cDNA synthesis incubation time can be extended up to 60 min if the target sequence is located near the 5' end of a long transcript and oligo (dT) priming is used, or if the target sequence is rare. However, extended times at high temperatures induce RNA degradation.

7. Incubate for 15 min in a 70°C heat block, for inactivation of the reverse transcriptase at the end of the reaction to prevent it from interfering with subsequent qPCR reactions.
8. Dilute the freshly synthesized cDNA to 8 ng/ μl by adding 105 μl of RNase-free water. Place the cDNA on ice for subsequent use or store it at – 20°C for later use.

Table 27.4.5 Components and Amounts for the RT Reaction^a

Components	Amount per reaction	Volume per reaction
AffinityScript buffer (10 \times)	1 \times	2.0 μl
DTT (100 mM)	10 mM	2.0 μl
dNTPs (10 mM each)	0.4 mM each	0.8 μl
RNasin	40 U	0.5 μl
AffinityScript Multi-Temp Reverse Transcriptase	50 U	1.0 μl
Total/reaction		6.3 μl

^aDue to the small volumes that are required for each component, it is highly recommended to make a master mix of the above components tailored to the number of RNA samples that need to be reverse transcribed. This will minimize variations between the samples due to pipetting errors.

Quantitative PCR

All real-time PCR instruments combine a thermal cycler to drive the DNA amplification, an optical system to excite fluorophores and capture emitted fluorescence from the detection chemistry, and specialized software to collect and analyze the quantitative data generated. The instrument described in this protocol (Corbett RotorGene 6200 system) uses a rotary format where reaction vessels are heated and cooled with air. It can be purchased along with a robot that accurately pipets the PCR master mix and the samples into the polypropylene V-well tubes. Having a robot will standardize the PCR reaction setup and reduce pipetting errors. After pipetting, the wells have to be efficiently sealed, to prevent evaporative losses which can in turn lead to variable results.

Materials

- 8 ng/μl cDNA samples from Basic Protocol 5
- TaqMan or SYBR Green assay components (see Strategic Planning and Table 27.4.6):
 - SYBR Green Assay: forward and reverse primers and 2× KAPA SYBR FAST Universal qPCR Master Mix (KAPA Biosystems; <http://www.kapabiosystems.com>)
 - TaqMan Assay: forward and reverse primers, TaqMan Probe and 2× Brilliant II Fast chemistry qPCR master mix (Agilent Technologies)
 - DNase-free and RNase-free highly pure deionized water
 - Aerosol-barrier pipet tips
 - Real-time PCR machine, accessory machines, and analysis software (we use the Corbett RotorGene 6200 system, robot, and heat sealer; <http://corbettlifescience.com/>)

1. Turn on the real-time PCR instrument and sealing equipment (if necessary) to warm it up.
2. Mix the PCR components as shown in Table 27.4.6 for either a TaqMan or a SYBR Green assay in to the appropriate vessels for the specific qPCR-instrument in use.

Table 27.4.6 The Required Components and Volumes for Both TaqMan and SYBR Green Assays^a

TaqMan assay	Volume per reaction	SYBR Green assay	Volume per reaction
qPCR standard water	3.75 μl	qPCR standard water	6.20 μl
2× Brilliant II Fast chemistry qPCR master mix (Agilent Technologies)	7.50 μl	2× KAPA SYBR FAST Universal qPCR Master Mix (KAPA Biosystems)	10.00 μl
20× TaqMan probe/primer mix (made by mixing 16 μM forward primer, 16 μM reverse primer, and 4 μM TaqMan probe.	0.75 μl	forward primer 10 μM reverse primer 10 μM	0.40 μl 0.40 μl
8 ng/μl cDNA sample	3.00 μl	8 ng/μl cDNA sample	3.00 μl
Total	15.00 μl		20.00 μl

^aImportantly, these PCR master mixes contain nucleoside triphosphates, dye, buffer, magnesium, and a DNA polymerase. Importantly, the DNA polymerase must be heat-stable, robust, and efficient, and in the case of TaqMan assays, it must also contain 5'-3' exonuclease activity. Such master mixes are recommended for qPCR assays, as they are optimized and consistent in composition. Avoid multiple freeze-thaw cycles with these master mixes. The 2× master mixes, primers, and probes and water volumes should be assembled as a master mix tailored to the number of reactions that are to be carried out in total; this reduces inaccuracies due to pipetting very small volumes. Quantitative PCR standard water means highly pure deionized water that is nuclease-free.

Table 27.4.7 Cycling Parameters for Both TaqMan and SYBR Green Assays^a

Step of TaqMan assay	Temp.	Time	Cycles	Steps of SYBR Green assay	Temp.	Time	Cycles
Initial denaturation	95°C	120 sec	1×	Initial denaturation	95°C	120 sec	1×
Denaturation	95°C	5 sec	40×	Denaturation	95°C	5 sec	40×
Primer annealing and elongation	60°C	20 sec		Primer annealing and elongation	60°C	20 sec	
				Dissociation	60°C to 99°C 1°C/cycle	90°C premelt conditioning step. Then 5 sec /cycle	

^aAn initial denaturation step is needed to ensure efficient reactivation of some hot-start DNA polymerases and complete denaturation of the template. Denaturation at 95°C is used in most commercial kits. The temperature and incubation time can be changed according to the requirements of the amplicon and reaction buffer. For most amplicons, a combined annealing and extension step at 60°C works well if the primers have been designed to anneal efficiently at 60°C. If the T_m of the primers is low, it is often better to use separate annealing and extension steps. 35 to 45 cycles of amplification are sufficient for most applications even when the template is present at a very low copy number. More than 45 cycles tends to increase the amount of nonspecific products. The melting curve step is carried out to monitor the specificity of the amplified PCR product when using DNA-binding dyes such as SYBR Green as the detection chemistry. An additional extension step can be performed before the melting curve step to ensure that all amplification products are in dsDNA form. Specific products can be distinguished from the nonspecific products by the difference in their T_m 's (see text for further details).

Keep the qPCR master mixes at –20°C until needed, and then thaw the master mixes and store on ice. Following this initial thawing of the master mixes, the unused portion can be stored at 4°C for up to 3 months or returned to –20°C for longer-term storage. Protect the SYBR Green master mix and TaqMan probes from light.

3. After the reactions are assembled, seal the vessels.
4. Program the real-time PCR instrument for the cycling parameters shown in Table 27.4.7 for either a TaqMan or a SYBR Green assay.
5. Perform PCR amplification under optimized conditions.

The fluorescence of the samples in most chemistries is collected after each extension step to monitor the progress of the reaction.

6. Acquire the data and calculate the relative mRNA levels. If a SYBR Green assay has been performed, analyze the data by first inspecting the melting curve and making sure that the products formed in each sample have a uniform melting transition at the expected temperature for the amplicon. For evaluation of a qPCR run, set the threshold manually in a region where the amplification rate of the cDNAs is the same in all samples. Measure the mRNA levels by calculating the relative change of the target sequence compared to a reference sequence, using the following formula:

$$x = E^{(b - a)}$$

where, x = expression level of the RNA of interest relative to a reference sample; E = amplification efficiency (see below); $a = C_t$ of RNA of interest in the sample of interest; and

$b = C_t$ of RNA of interest in the reference sample.

Finally, divide the relative mRNA levels of the gene of interest by the relative mRNA levels of the co-transfected or endogenous normalizer gene to generate the relative normalized mRNA levels, which can be compared to each other.

For accurate measurements, it is important to determine the amplification efficiency experimentally. Theoretically, the amount of DNA is doubled with every PCR cycle,

meaning the efficiency (E) is equal to 1. However, in practice, such an ideal efficiency is rarely reached due to suboptimal PCR conditions. To determine the effective E for a specific primer/probe set, a serial dilution should be made with cDNA to generate a standard curve, in which the C_t values (y axis) are plotted against the log of the cDNA concentration (x axis). E can be derived from the slope of this standard curve using the following formulas (Rasmussen et al., 2001):

$$\text{Exponential amplification} = [10^{(-1/\text{slope})}].$$

$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1.$$

THE USE OF TETHERED FUNCTION ASSAYS TO STUDY NMD

This approach is flexible and overcomes numerous obstacles in the study of mRNA-binding proteins, which is why such assays have been used to show the role of proteins in control of mRNA transport, translation, localization, stability (Coller and Wickens, 2002, 2007; Keryer-Bibens et al., 2008), and, of particular importance here, in NMD (see Table 27.4.8). The Tethered Function Assay (also see Background Information) is so useful for examining the process of NMD because mRNA regulatory events generally occur through multi-protein complexes formed via protein-protein and protein-RNA interactions. In such cases, RNA binding may occur via one critical protein, which tethers the activity of another protein to the mRNA. Thus, the active protein may not directly contact the RNA. An advantage of the tethered approach is its ability to examine the activity independently of RNA binding. In many cases, putative RNA-binding proteins have been identified, but their respective RNA targets are unknown. Moreover, this assay is useful in NMD because many of the NMD proteins are essential for cell viability, and traditional genetic techniques are complicated by pleiotropic effects. The Tethered Function Assay allows the function of the protein to be examined on just one mRNA species in an otherwise wild-type cell.

NOTE: Essential details to this protocol are included in the Commentary (see sections entitled “Basic Protocol 7: Using the Tethered Function Assay to Investigate NMD” in Background Information, Critical Parameters and Troubleshooting, Anticipated Results, and Time Considerations) and must be read in conjunction with this protocol before proceeding with any experiment.

Methodology

This basic protocol is a set of guidelines to aid in the establishment of the Tethered Function Assay. Each necessary component of the assay is methodically considered and explained below so that the assay can be established and optimized based on the ultimate aims of the investigator. The guidelines closely follow Figure 27.4.6A, where the method to establish a Tethered Function Assay has been summarized and is accompanied by an elaborate example in Figure 27.4.6B. This assay utilizes the skills that have been outlined in Basic Protocols 1, 2, 4, 5, and 6 of this unit. Please refer to these protocols when necessary.

Decide on the Tethering System You Would Like to Use for Studying NMD in Your Laboratory

In Tethered Function Assays, the polypeptide of interest is tethered to a reporter mRNA via a heterologous RNA-protein interaction, and, so far, four main tethering systems have been used with success by a number of laboratories. Table 27.4.9 summarizes the salient features of each tethering system; for a more detailed exploration of each system refer to Coller and Wickens (2007). The two most common systems involve tethering proteins of bacteriophage origin: the coat protein from the MS2/R17 bacteriophage (Bardwell and Wickens, 1990; Coller et al., 1998; Graveley and Maniatis, 1998) and the 1–22 peptide

BASIC PROTOCOL 7

Table 27.4.8 Using the Tethered Function Assay to examine NMD: Details and Simplified Results of Most of the Tethered Function Assays that Have Been Used to Study Aspects of NMD Reported to Date

Proteins tethered	Reporters	Effects	Ref.
MS2 tagged hUPF1, hUPF2, hUPF3a, and hUPF3b	<p>β-globin transcript wild-type lacking MS2 coat binding sites (bs) in the 3'UTR</p> <p>β-globin transcript with a PTC at position 39, lacking MS2 coat bs in the 3'UTR</p> <p>β-globin transcript wild-type with either 2, 4, 6, or 8 MS2 coat bs in the 3'UTR</p> <p>β-globin transcript wild-type where the termination codon UAA has been mutated to a UAC codon, with 6 MS2 coat bs in the 3'UTR</p> <p>β-globin transcript wild-type lacking MS2 coat bs but instead has a 645 nt fragment of the GAPDH ORF inserted into the 3'UTR</p>	Tethered UPFs transform a normal message in to a message subject to NMD	Lykke-Andersen et al. (2000)
MS2 tagged Y14, RNPS1, DEK, REF-2-1, SRm160	Same reporters were used as detailed above in Lykke-Andersen et al. (2000)	Tethered RNPS1 and to a lesser extent Y14 stimulate NMD on a normal message	Lykke-Andersen et al. (2001)
λ N-tagged hUPF3b and Y14	<p>β-globin transcript wild-type lacking BoxB binding sites (bs) in the 3'UTR</p> <p>β-globin transcript with a PTC at position 39, lacking BoxB bs in the 3'UTR</p> <p>β-globin transcripts wild-type with either 2, 3 or 5 BoxB bs in the 3'UTR</p> <p>β-globin transcripts wild-type where the natural termination codon UAA has been mutated to a CAA codon and UAA stop codons have been created at locations further downstream in the 3'UTR within the 5 BoxB bs</p> <p>β-globin transcripts wild-type containing either a functional or non-functional IRE in the 5'UTR, with 5 BoxB bs in the 3'UTR</p> <p>β-globin transcript wild-type which has extended second and third exons, lacking BoxB binding sites (bs) in the 3'UTR</p>	Characterization of Y14/hUPF3b interaction and demonstration that it is essential for NMD while the UPF2/UPF3 interaction is not	Gehring, et al. (2003)
λ N-tagged Y14 and Magoh	Same reporters were used as detailed above in Gehring et al., 2003	Y14/Magoh interaction is essential for NMD	Fribourg et al. (2003)
MS2 tagged UPF1-3, Y14, RNPS1, DEK, REF-2-1, SRm160	<p><i>Renilla</i> luciferase transcript wild-type with 5' end situated 6 MS2 coat bs immediately preceding the <i>Renilla</i> luciferase ORF and just after the start codon</p> <p>β-globin transcript wild-type with 6MS2 coat bs in the 3'UTR</p>	Y14, Magoh, RNPS1 and UPF1-3 stimulate translation when tethered to the 5' end of the ORF	Nott et al. (2004)
λ N-tagged Y14, SMG5, SMG6 and SMG7 and mutations thereof	β -globin transcripts wild-type where the natural termination codon UAA has been mutated to a CAA codon and UAA stop codons have been created at locations further downstream either in the within the 5 BoxB bs or at the end of the 5 BoxB bs in the 3'UTR GFP transcript wild-type with 5 BoxB bs in the 3'UTR	SMG7 elicits mRNA decay when tethered to a reporter transcript. This effect does not require SMG6 but does need DCP2 and XRN1	Unterholzner and Izaurralde (2004)

continued

Table 27.4.8 Using the Tethered Function Assay to examine NMD: Details and Simplified Results of Most of the Tethered Function Assays that Have Been Used to Study Aspects of NMD Reported to Date, *continued*

Proteins tethered	Reporters	Effects	Ref.
	GFP transcript wild-type with 5' end situated 5 BoxB bs (containing stop codons) just preceding the GFP ORF		
MS2 tagged UPF1, UPF2, UPF3	Firefly luciferase transcript wild-type lacking MS2 coat binding sites (bs) in the 3'UTR Firefly luciferase transcript wild-type with 8 MS2 coat binding sites (bs) in the 3'UTR	RNAi of CBP80 abrogates the reduction in reporter mRNA that is induced by tethering UPF2 and UPF3 but it does not alter the effect by tethering UPF1, while 4E-BP1 inhibits the effect of tethering UPF1 but not UPF2 and UPF3	Hosoda et al. (2005)
MS2 tagged PABPC1	β -globin transcript wild-type lacking MS2 coat binding sites (bs) in the 3'UTR β -globin transcripts with a PTC at position 39, with one MS2 coat bs located either 15 codons or 44 codons downstream from the PTC	Tethering PABPC1 to 15 codons downstream of a PTC repressed NMD but tethering PABPC1 at 44 codons downstream of the PTC had no such effect.	Silva et al. (2008)
MS2 tagged PABPC1	Mini μ transcripts with a PTC at position 310, with 6 MS2 coat bs located either 50 nt downstream of the PTC much further downstream from the PTC TCR- β transcripts with a PTC at position 68, with 6 MS2 coat bs located either 50 nt downstream of the PTC or much further downstream from the PTC (see Fig. 27.4.6)	Tethering of PABPC1 50 nts downstream of the PTC repressed NMD while tethering PABPC1 further downstream had no such effect (see Fig. 27.4.6).	Eberle et al. (2008)
MS2 tagged PABPC1 and λ N-tagged Y14, RNPS1 and Magoh	β -globin transcript wild-type with 4 BoxB bs and 4MS2 coat bs respectively in the 3'UTR β -globin transcript wild-type with 4MS2 coat bs and 4 BoxB bs respectively in the 3'UTR	Demonstrated that PABPC1 can overcome the effect of tethered Y14, RNPS1 or Magoh when positioned closer to the termination codon	Ivanov et al. (2008)
MS2 tagged PNRC2	This study used β -globin reporter genes from Lykke-Andersen et al. (2000)	Tethering PNRC2 to the mRNA 3'UTR elicits mRNA decay	Cho et al. (2009)
λ N-tagged eIF4AIII, Upf1, Y14 and Ago2	Constructs composed of <i>Renilla</i> luciferase cDNA, no stop codon and β -globin wild-type with 5 BoxB bs in the 3'UTR Constructs composed of <i>Renilla</i> luciferase cDNA, no stop codon and β -globin containing a PTC at position 39, with 5 BoxB bs in the 3'UTR	Tethering of Ago2 inhibited CT efficiently and thus NMD	Choe et al. (2010)
λ N-tagged Ago2, Ago2F2V2	This study used the same reporter constructs as detailed in Choe et al., 2010 in addition to a further control reporter <i>Renilla</i> luciferase cDNA with a stop codon, lacking any BoxB bs in the 3'UTR	Tethering of Ago2 inhibited CT efficiently and thus NMD This effect requires the cap-associating activity of Ago2	Choe et al. (2011)

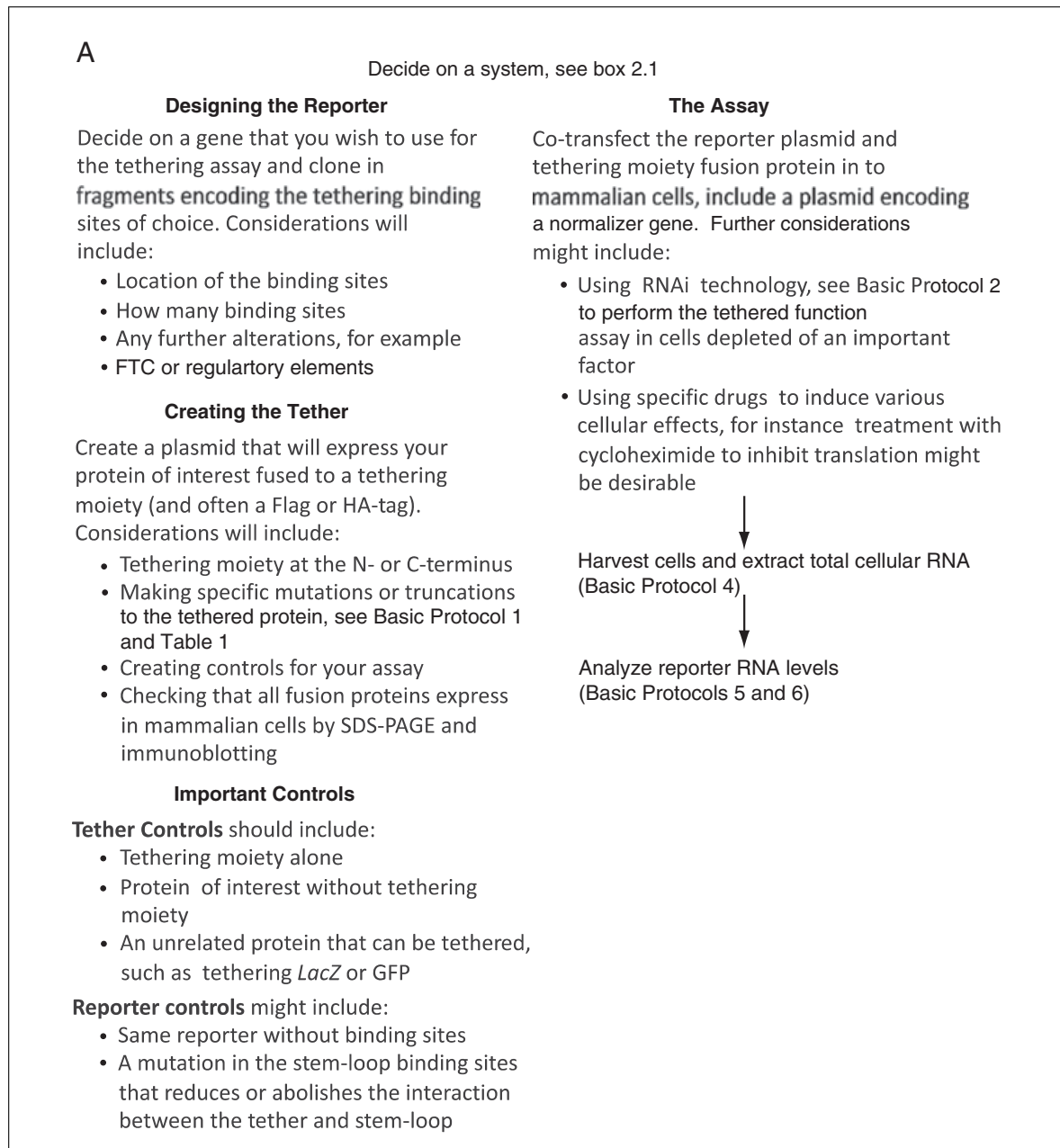


Figure 27.4.6 (continued) Establishing a Tethered Function Assay system to study NMD. **(A)** Flow diagram for the steps that need to be taken and the deliberations that need to be made when setting up a Tethered Function Assay to examine NMD from cloning the reporter and plasmids to performing the actual assay and interpreting the results. **(B)** Flow diagram exemplifying the steps taken to perform a tethering assay to examine the role of PABPC1 in NMD (taken from Eberle et al., 2008) to mirror the flow diagram described in (A) and is explained in the text.

of lambda N protein (λ N) (De Gregorio et al., 1999). These tethering systems are most popular because the tether in each case is small, thus minimizing potential interferences to the test protein, and also because they bind to their target RNA stem-loops with high specificity and selectivity. The U1A protein and iron responsive protein (IRP) have also been used successfully as tethers (De Gregorio et al., 1999; Finoux and Seraphin, 2006), but both of these proteins are relatively large—38 kDa for U1A and 97 kDa for IRP—so they have not been as commonly used in Tethered Function Assays.

B

Example: Using the MS2 tethered function assay to examine if PABPC1 inhibits NMD in mammalian cells

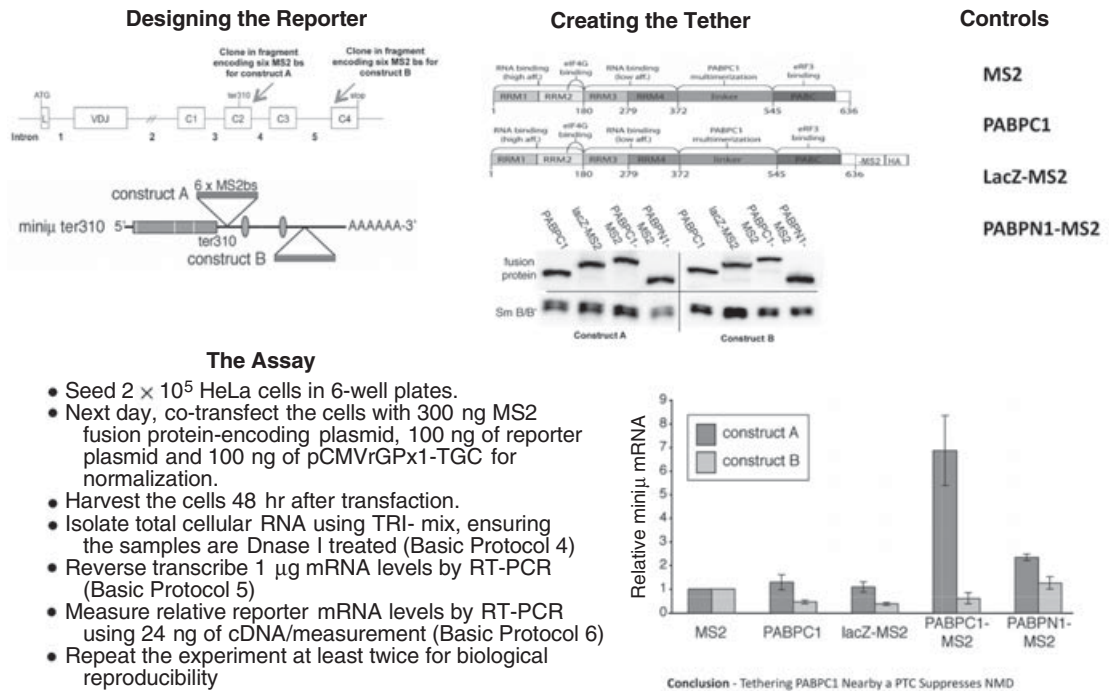


Figure 27.4.6 (legend appears on previous page)

The MS2 system, unlike the others, allows a high quantity of tethered proteins to be present on the mRNA because the MS2 coat interacts with its target sequence as an obligate dimer (Valegard et al., 1994). Thus, for every stem-loop present in the mRNA reporter, two tethered proteins can bind. Also, MS2 binds cooperatively to two stem-loops, further increasing the occupancy of sites (Witherell et al., 1990). In certain cases, this might be favorable because the more protein that is bound, the better; both of these features contribute to a strong signal in the functional assay, and this can be achieved without greatly increasing the overall length of the mRNA reporter, an undesired situation in some applications. On the other hand, the MS2 coat protein is not the simplest selection when it is obligatory to carefully control the number of tethered protein molecules bound.

Therefore, some laboratories have elected to use the λ N-peptide rather than MS2 coat protein, because the tether is smaller and binds 1:1 to its RNA target. However, despite this bias, it should be noted that tethering via the λ N peptide or the MS2 coat protein appeared to produce similar results. Indeed, functional studies of the factors required for NMD have used both systems (Lykke-Andersen et al., 2000, 2001; Gehring et al., 2003, 2005; Kunz et al., 2006), and, as nonconflicting results were attained, these studies also show that the functionality of the complex formed around the tethered protein is independent of the tethering protein. It is clear that each of the tethering systems has certain advantages in particular situations. Therefore, in designing experiments using the tethering assay, it is highly recommended that these characteristics be fully taken into account within the constraints of the structural requirements for the protein to be fused with the tethering moiety.

Table 27.4.9 Summary of the Important Features of Four Different Tethering Systems

System	Tether	Size of tether	RNA binding site	Interaction specificity	Interaction characteristics
Bacteriophage MS2 coat protein binds to MS2 binding sites	MS2 bacteriophage coat protein	14 kDa	21 nt RNA stem-loop	$K_d = 1 \text{ nM}$	MS2 coat interacts as a dimer and binds cooperatively to two stem-loops. Certain mutations in the stem-loop can increase or decrease the interaction affinity. U-C alteration increases affinity by over 50 fold. Only the loop nt's are important for the interaction.
λ N-peptide binds to stem-loops called BoxB	Bacteriophage λ N peptide	22 amino acids	19 nt RNA stem-loop	$K_d = 1.3 \text{ nM}$	λ N peptide binds 1:1 with its target. nt in both the stem and loop are important for the interaction.
U1A protein binds to stem loop II	U1A protein	38 kDa	30 nt RNA stem-loop	$K_d = 5 \text{ nM}$	U1A binds 1:1 with its target
IRP binding protein binds to tem loops called iron response elements	IRP	97 kDa	30 nt RNA stem-loop	$K_d = 90 \text{ pM}$	IRP binds 1:1 with its target. Can be complicated by the fact that this binding site will exist in the 5'UTR and 3'UTR of various physiological mRNA in eukaryotic cells.

^aThe description of each system, the name of the tether, the size of the tether, the number of nt required for each binding site in the reporter mRNA, and the interaction specificity of tether and its binding site are detailed in the table. Some important features of the interaction characteristics of the tether and its binding site are also described for each system.

Choosing the Reporter mRNA to Use in Your Assays

The Tethered Function Assay can be adapted to measure the effect of a tethered protein in many steps of mRNA metabolism and function. The adaptability comes mainly from the choice of reporter mRNA and the final assay performed. The choice of reporter mRNA obviously is dictated by the effect to be assayed. With regard to examining NMD, prevalent choices have included nonmammalian genes such as *Renilla* luciferase, firefly luciferase, and green fluorescent protein (GFP). The former two are popular because the mRNA level can be indirectly examined by measuring the luciferase activity in a luminometer. Genes that are found in eukaryotes such as β -globin and TCR- β have also been employed, whereby the labs have adapted genes that had previously been used to study NMD. To this end, several studies have also looked at PTC+ mRNAs, as in the example in Figure 27.4.6. The inherent behavior of the reporter mRNA is an important consideration. To determine whether a tethered protein stabilizes an mRNA, the mRNA must be unstable in the absence of the protein; conversely, to determine whether a tethered protein destabilizes the mRNA, the mRNA reporter must be stable without the protein. Therefore, it is helpful to select as a reporter mRNA where the *cis*-acting sequences are well characterized.

The Location of Tethered Binding Sites

Next, it must be decided where the tethered sites should be located within the coding region or in the UTRs. From Table 27.4.8, it can be seen that the tethering binding sites have been placed in various different regions, mostly to suit the study and the specific questions the study is trying to answer. For instance, in two studies, the tethering binding sites were placed very close to the PTC and, as a comparison, further downstream from the PTC because the researchers wanted to examine if the physical distance between the termination codon and PABPC1 is a crucial determinant for PTC recognition in human cells (see Fig. 27.4.6) (Eberle et al., 2008; Silva et al., 2008). However, in most cases,

the tethering site(s) have been placed within the 3' UTR of the reporter mRNA. Often, this has been the region of the endogenous target mRNAs to which the studied protein bound. In addition, it is known that the exact location of several 3' UTR regulators is not critical for their function, implying that precise spatial positioning is not critical. Also, the 3' UTR has fewer constrictions than the 5' UTR, whereby tethering a protein near to the 5' end of the mRNA can inhibit translation independently of its normal function(s) (Stripecke et al., 1994; Grskovic et al., 2003). Finally, it is worth examining the sequence into which the tethering binding sites are embedded. This may be of importance, and some of the variability as to the number of sites required could be due to the presence of inessential sequences. For example, Witherell et al. (1990) showed that binding of MS2 coat protein can be prevented by occlusion of the binding site(s) by alternative structures (Witherell et al., 1990).

The Number of Tethered Binding Sites

Another feature to take into account when designing a reporter is the number of tethered binding sites to include. In several cases, using the MS2 bacteriophage coat as the tether, two stem-loops have been adequate to observe an effect (Coller et al., 1998; Gray et al., 2000; Ruiz-Echevarria and Peltz, 2000; Minshall et al., 2001) and only one BoxB sequence was enough in the initial study with the BoxB system (De Gregorio et al., 1999). However, in the early use of each of these tethering systems to study NMD, more binding sites were used, and this was systematically worked out. Accordingly, Lykke-Andersen et al. (2001) used 2, 4, 6, and 8 MS2 sites inserted into the 3' UTR of the β -globin gene, and Gehring et al. (2003) used 2, 3, or 5 BoxB elements within the same reporter (see Table 27.4.8 and references therein). In the absence of tethered NMD factors, no effect of the number of binding sites on the steady-state level of the reporter mRNA was noted in either of these studies. Since these original studies, 6 to 8 MS2 binding sites and 5 BoxB binding sites have been used on average in NMD studies with these systems.

Considering the Tether to Use in Your Assays

In choosing which protein to use as the tether, it is essential to consider affinity and specificity for the RNA tag, subcellular localization, and impact of the tether on the activity of the test protein.

Mutagenesis of tethered proteins

Since the effects of a tethered protein are examined on a single reporter mRNA, the effects of many manipulations of the protein sequence can be examined readily and conclusively. This can reveal novel molecular properties in the protein. In most of the studies detailed in Table 27.4.8, NMD factors with specific mutations have been tethered to gain information about the function of the NMD protein. See Table 27.4.1 for a comprehensive list of a multitude of known mutations made in NMD factors.

N-terminal or C-terminal fusions

The two independent studies examining the role of poly(A)-binding protein C1 (PABPC1) on NMD actually had the MS2 tethering moiety at opposite ends of the PABPC1: in the Silva et al. (2008) report, it was at the N terminus of PABPC1, whereas the opposite was true for Eberle and colleagues (see Table 27.4.8, Fig. 27.4.6 and references therein). The same conclusions were drawn from the two studies despite this difference. However, the relative positions of the tethering protein and the protein of interest is not always so straightforward. It can be observed that tethering the MS2 coat protein to the one terminus can result in much more activity and/or better expression than if the tether were located at the opposite terminus. It is unclear why this is the case for some proteins, and it has to be determined on a case-by-case basis by testing both orientations.

Trans effects

Another key issue to consider is that the fusion protein may have *trans*-acting effects. Often, the Tethered Function Assay is performed in a wild-type background with the endogenous copy of the test protein present. It is possible that the presence of the tethering moiety may produce a dominant negative allele that hampers the function of the normal protein *in vivo*. Such an event would seriously confound the results from the assay. Therefore, controls to ensure that any observed effects occur only *in cis* with respect to the mRNA reporter are vital.

Designing all the necessary controls for your assay

Several controls are critical in Tethered Function Assays, and should always be performed. Starting with controls concerning the reporter mRNA, it is important to compare the stability of the reporter with the binding sites to the same reporter lacking the binding sites to certify that the tethered binding site does not affect the mRNA on its own. Another control concerning the reporter mRNA is to make specific mutations in the RNA stem-loop which affect the tether-stem-loop interaction. This is a particularly well known control for the MS2 system, and examines the specificity of the system, i.e., whether the changes in reporter RNA levels are really due to the test protein being tethered to the reporter. In a similar theme, several controls can be carried out concerning the tether that also control for any effects being specific to the fact that the test protein was tethered to the RNA. The tethering moiety alone should be tethered (MS2 alone in the example in Fig. 27.4.6), the tethering moiety fused to an unrelated but similarly sized protein should be tethered (in the example in Fig. 27.4.6, LacZ-MS2 was used as a control, and it was truncated to be of a similar size to the test protein, PABPC1), and the test protein without the tethering moiety should also be tethered (just PABPC1 alone in Fig. 27.4.6). All of these outlined controls will test that the tethering moiety alone does not have any influence, that any observed effects occur only when the protein is bound to the mRNA, and for possible *trans*-acting effects. Finally, experiment-specific controls can also be included. For instance, in the example in Figure 27.4.6, PABPN1 was also tethered, and this served as a control for this specific experiment because it is generally thought that only the cytoplasmic PABP has an influence on cytoplasmic translation events and that the nuclear PABP does not.

Adapting your assay: Identifying mRNA localization functions and visualizing tagged mRNAs in vivo

Several adaptations of the Tethered Function Assay have been developed to tag an mRNA for analysis in living cells, and this technique has also recently been used to study NMD. Although this is not a Tethered Function Assay per se because the protein that is fused to the tethering moiety is just a tag, it is still the same fundamental system. The initial use of such a method followed the localization of ASH1 (absent, small, and homeotic discs 1) mRNA to the bud tip in *S. cerevisiae* (Bertrand et al., 1998). This was achieved by tethering GFP fused in-frame to the MS2 coat protein to a recombinant ASH1 mRNA containing six MS2 binding sites in the 3' UTR. The localization of this RNA was then monitored in real time in living cells by time-lapse fluorescence microscopy. This approach is widely used and has been well reviewed (Beach et al., 1999; Singer et al., 2005) and further developed (Larson et al., 2011). It has also very recently been used to study NMD (de Turris et al., 2011). In this study, an *in vivo* approach to measure synthesis and release kinetics at the transcription sites of PTC- and PTC+ Ig mini μ reporter genes stably integrated into the genome of U2OS cells was developed to monitor PTC-specific differences. These Ig mini μ reporter genes contained 24 MS2 binding sites in the 3' UTR (see Fig. 27.4.10), which allowed for the visualization of transcription sites *in vivo* upon binding of these stem-loops by the MS2 coat protein fused to a yellow fluorescent protein (YFP). Using this method revealed that PTC+ transcripts are specifically retained at the transcription site.

ANNEALING THE OLIGONUCLEOTIDES

This is a support protocol that elaborates on step 3 of the Strategic Planning guideline, accompanying Basic Protocol 2, entitled “Creating pSUPER vector system for expression of short interfering RNA to knock down NMD genes” (see Strategic Planning at beginning of unit). In that specific step, the forward and reverse strands of the oligonucleotides that contain the siRNA-expressing sequence targeting your gene of interest (designed based on steps 1 and 2 of the Strategic Planning guideline mentioned above) are annealed so that they can be effectively ligated into the *Bgl*III and *Hind*III sites of the pSUPER vector at later steps in the strategic planning procedure.

Materials

Forward and reverse oligonucleotides
Annealing solution (see recipe)
Thermomixer heat block (e.g., Eppendorf Thermomixer Compact)

1. Mix the following components in a sterile microcentrifuge tube:
 - 1 μ l of 100 μ M forward oligonucleotide
 - 1 μ l of 100 μ M reverse oligonucleotide
 - 48 μ l annealing solution (100 mM sodium acetate, 20 mM Tris pH 7.5, 2 mM EDTA)
 - 50 μ l highly pure deionized water.
2. Incubate the mixture for 4 min at 95°C using a Thermomixer.
3. Incubate for 10 min at 70°C using a Thermomixer.
4. Slowly cool the annealed oligonucleotides to room temperature before using them or moving them to 4°C. For longer storage keep them at –20°C.

HARVESTING CELLS FOR EVENTUAL PROTEIN ANALYSIS

This support protocol accompanies Basic Protocol 2 “pSUPuro-based RNAi to knock down NMD factors.” As is the standard procedure, the extent of gene knockdown should be tested on the mRNA and on the protein level. For the former, gene knockdown verification on the mRNA level can be monitored by RNA isolation followed by RT-qPCR (see Basic Protocols 4 to 6 and Fig. 27.4.7A), or by northern blotting. Usually, mRNA analyses are easier to perform, since a gene-specific probe for northern blotting or quantitative PCR can be designed from available sequence data. For the latter, gene knockdown confirmation on the protein level is a key measurement in RNAi experiments and can be monitored by performing epitope tagging, reporter gene assays, or most commonly, western blotting (see below and Fig. 27.4.7B). However, these analyses require that the gene product is known and that a specific antibody for it is available (for the majority of the known NMD factors, several antibodies are commercially available), that the protein has a biochemical activity that can be measured, or that the protein has been tagged. Carrying on where Basic Protocol 2 leaves off, the support protocol below explains how to collect whole-cell extract samples (from the cells that been depleted of an NMD factor by RNAi) for eventual verification of the gene knockdown by SDS-PGE followed by western blotting.

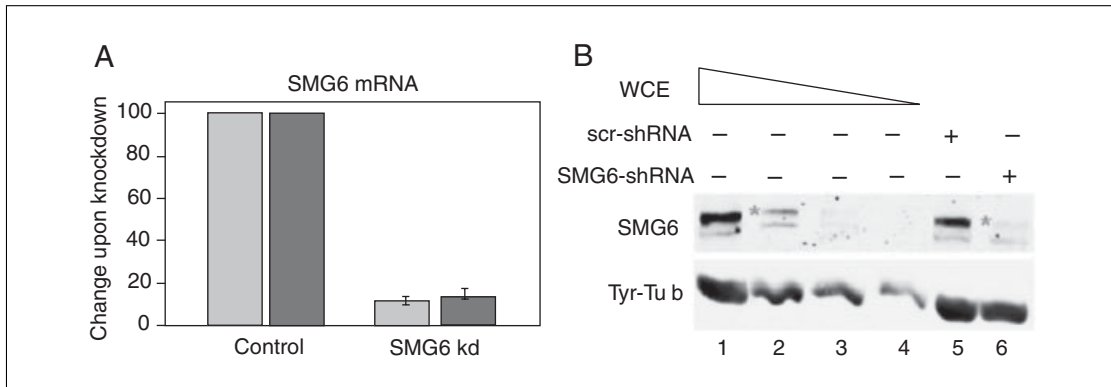


Figure 27.4.7 SMG6 knockdown levels monitored at RNA and protein level. **(A)** The histogram shows relative SMG6 mRNA levels measured by RT-qPCR in Ig mini μ wild-type cells (light gray bars) and Ig mini μ ter310 cells (dark gray bars) after knockdown of SMG6 or a control knockdown using a scrambled shRNA. All mRNA levels were normalized against 18S rRNA. Average values and standard deviations from two independent experiments are displayed (Data from de Turris et al., 2011). **(B)** Western blot monitoring SMG6 protein levels in whole cell extract (WCE). The asterisks (*) mark the SMG6 specific band. Control WCE (-) from Ig mini μ wild-type cells (equivalent cells) and three-fold serial dilutions of the untreated cells were loaded in lanes 1 to 4. Treated Ig mini μ wild-type (lane 5 and 6) (+) or (-) plasmids expressing scrambled-shRNA (scr-shRNA) or SMG6-targeting shRNA (SMG6-shRNA). Tyrosine-Tubulin (Tyr-Tub) was used as loading control.

Materials

- Cells (any particular kind)
- Modified radio immunoprecipitation assay (RIPA) buffer (see recipe), ice cold
- 2 \times SDS-PAGE sample buffer (APPENDIX 2A)
- Refrigerated shaker
- Refrigerated centrifuge
- Additional reagents and equipment for counting cells

1. Count the desired number of cells (50,000 to 200,000 cells).
2. Lyse the cells in ice-cold modified RIPA buffer using 1 μ l of buffer per 2 \times 10⁴ cells
3. Incubate for 30 min with slight shaking at 250 rpm at 4°C.
4. Centrifuge the lysate for 15 min at 12,000 \times g, 4°C.
5. Mix the recovered supernatant with 1 volume of 2 \times SDS-PAGE sample buffer.
6. Store the samples at -20°C until they are required.

The results of an SMG6 knockdown experiment done according to this protocol using pSUPuro SMG6 (Fig. 27.4.1C) is shown in Figure 27.4.7. To examine the SMG6 knockdown efficiency at the protein level, SDS-PAGE followed by western blotting with an anti-SMG6 antibody was carried out (Fig. 27.4.7B); for analysis of the SMG6 mRNA levels, RT-qPCR was performed (Fig. 27.4.7A).

SUPPORT PROTOCOL 3

Analysis of Nonsense- Mediated mRNA Decay

27.4.42

SPECTROPHOTOMETRIC QUANTIFICATION OF RNA

Successful completion of the procedures outlined in this unit demand the isolation of high-quality total RNA. In this case, quantifying RNA using A₂₆₀ spectrophotometry values can stand as a proxy for quality determination. Desired values will be A₂₆₀/A₂₈₀ = ~1.9 to 2.1.

Materials

RNA samples to be quantified
Solution used to dissolve RNA pellets in Basic Protocol 4
NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific)

1. Switch the settings of the NanoDrop spectrophotometer to RNA, which will set the absorbance to 280 nm (A_{280}).
2. Use 2 μ l of the solution that was used to dissolve the RNA pellet as a blank.
3. Use 2 μ l of each RNA sample to assess the RNA quantity.

Record the readings at 260 nm, 270 nm, and 280 nm if possible. If the optical density at 270 nm is greater than that at 260 nm, this indicates phenol contamination of the RNA (in this case, re-precipitate the RNA to remove the phenol contamination). The ratio of the measurements at 260 nm and 280 nm indicate the purity of each sample. Ratios of 1.9 to 2.1 are very pure, whereas lower ratios indicate possible protein contamination. Although this procedure will measure the amount of RNA, it does not tell anything about the quality of the preparation, i.e., whether the RNA is intact or partially degraded. This is important for downstream applications such as RT-qPCR, because a significantly degraded RNA sample will not give accurate quantification of the target mRNAs. The presence of contaminating proteins may inhibit the qPCR reaction overall, and contaminating DNA can lead to unwanted amplification products

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Annealing solution

100 mM sodium acetate
20 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
2 mM EDTA
Store up to 2 years at room temperature

Antibiotic stocks

All stock solutions of antibiotics for bacterial work are made up in deionized distilled water and filter sterilized using Terumo syringes and MillexGP 0.22- μ m filter membranes (Millipore). Stock solutions are aliquotted and stored up to 6 months at -20°C .

Normally ampicillin stocks are prepared at 100 mg/ml and kanamycin stocks are prepared at 100 mg/ml.

5-Bromo-4-chloro-3-indolyl- β -D-galactoside (Xgal) stock, 50 mg/ml

100 mg 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Xgal)
2 ml *N,N'*-dimethylformamide
Store in aliquots in glass tubes covered with aluminum foil up to 6 to 12 months at -20°C

If the solution turns pink, then it should be discarded.

DMEM –/–

Measure out 950 ml deionized, distilled water using a 1-liter mixing container. Add 12 g DMEM/F-12 plus glutamine powder (Invitrogen, cat. no. 32500-035; store closed at 4°C) to the water at room temperature. Add 1.125 g NaHCO_3 powder or 15 ml of 7.5% sodium bicarbonate solution (Invitrogen, cat. no. 25080060). Dilute

continued

to 1 liter with water and stir until dissolved; do not over-mix. Adjust the pH to 0.2 to 0.3 below 7.2 (because the pH normally rises 0.1 to 0.3 upon filtration) using 1 N NaOH and/or 1 N HCl added slowly with stirring. After the pH has been adjusted, keep the container closed. Sterilize immediately by membrane filtration using a 0.2- μ m filter and, if possible, a positive-pressure system. Store in the dark up to 12 months at 4°C.

DMEM+/-

450 ml DMEM -/- (see recipe)
50 ml FBS (10% v/v final; mycoplasma and virus-screened)

During use, the medium should be stored predominantly at 4°C up to 4 months in the dark, but should be supplemented after 3 weeks with a 1 \times final concentration of L-glutamine, added from 100 \times L-glutamine solution (Invitrogen, cat. no. 25030-124). Also see http://www.invitrogen.com/etc/medialib/en/filelibrary/pdf.Par.54294.File.dat/MediaPreparation_O064037.pdf.

DMEM+/+

450 ml DMEM -/- (see recipe)
50 ml FBS (10% v/v final; mycoplasma and virus-screened)
5 ml 100 \times (5000 U/ml penicillin/5000 mg/ml streptomycin) penicillin-streptomycin solution (1 \times final)

During use, the medium should be stored predominantly at 4°C up to 4 months in the dark, but should be supplemented after 3 weeks with a 1 \times final concentration of L-glutamine, added from 100 \times L-glutamine solution (Invitrogen, cat. no. 25030-124). Also see http://www.invitrogen.com/etc/medialib/en/filelibrary/pdf.Par.54294.File.dat/MediaPreparation_O064037.pdf.

Isopropyl β -D-thiogalactopyranoside (IPTG) stock, 0.1 M

Dissolve 1.2 g IPTG and make up to 50 ml using distilled deionized water. Store up to several weeks in aliquots at 4°C or up to several months at -20°C.

Modified RIPA buffer

50 mM Tris-Cl, pH 7.5 (*APPENDIX 2A*)
150 mM NaCl
1 mM EDTA
1% (v/v) Triton X-100
0.25% (w/v) sodium desoxycholate
Store up to 2 years protected from light at 4°C
Add 1 \times protease inhibitor cocktail just prior to use

Puromycin dihydrochloride, 0.5 mg/ml stock

Store 25 mg of puromycin dihydrochloride solid at -20°C, protected from moisture until ready to reconstitute using 50 ml of PBS (*APPENDIX 2A*) to create a 0.5 mg/ml stock solution. Filter sterilize the solution using a Terumo syringe and MillexGP 0.22- μ m filter membranes (Millipore). Store in aliquots up to 2 months at -20°C.

TRI-mix

94.53 g/liter (800 mM) guanidine thiocyanate
30.45 g/liter (400 mM) ammonium thiocyanate
8.20 g/liter (100 mM) sodium acetate
380 ml/liter (38% v/v) phenol
50 ml/liter (5% v/v) glycerol
1.0 g/liter (0.1% w/v) 8-quinolinol, pH 5.0
Store up to 2 years at 4°C

COMMENTARY

Background Information

Basic Protocol 1: Creation of PTC+ mRNAs and mutant NMD proteins by site-directed mutagenesis

Site-directed mutagenesis using oligonucleotides was first reported in 1978 (Hutchinson et al., 1978). Michael Smith, its inventor, shared the Nobel Prize in Chemistry with Kary Mullis, who invented PCR. Using site-directed mutagenesis, the information in the genetic material can be altered. This reprogrammed DNA molecule can, e.g., direct the synthesis of a protein with an exchanged amino acid. Michael Smith's method has become one of molecular biology's most important instruments. Not surprisingly, this method is also used extensively in NMD research—it forms the key foundations of nearly all experiments carried out in NMD from simple analyses to other approaches outside the scope of this unit, such as protein-protein and protein-RNA interaction studies. The importance of this method is evident from the long list of mutant NMD proteins (see Table 27.4.1) that have been generated by crucial amino acid changes and also by the ability to make genes carrying mutations that will give rise to PTCs in the mRNA and hence trigger NMD (see Fig. 27.4.4A).

Basic Protocol 2: pSUPuro-based RNAi to knock down NMD factors

RNAi enables sequence-specific, experimentally induced silencing of virtually any gene by tapping into innate regulatory mechanisms that are conserved among virtually all eukaryotes, with the notable exception of *Saccharomyces cerevisiae*. Historically, observations relating to RNA interference were known by other names, including co-suppression, post-transcriptional gene silencing, and quelling. Only after these apparently unrelated processes were fully understood did it become clear that they all actually could be explained by RNAi. In 2006, Andrew Fire and Craig Mello were awarded The Nobel Prize in Physiology or Medicine for their research on RNAi in *C. elegans*. Soon after the initial discovery by Fire and Mello (Fire et al., 1998) that dsRNAs can be used to knock down the activity of individual genes, many RNAi-based methods were (and continue to be) added to the tool-boxes of various organisms. These methods have truly revolutionized the field of functional genomics because of their relative ease, and most significantly because RNAi, unlike more traditional genetic screening methods,

provides a powerful reverse genetic approach, especially for organisms in which genetics is difficult, as is the case with mammalian systems.

At its core, RNAi is a response to dsRNA (Fire et al., 1998). This unusual structure, often reflective of the presence of viral replication intermediates or genetic elements, is recognized and deconstructed into small RNAs that are a defining feature of this family of pathways. In the classical response, the dsRNA processing step is carried out by a RNase III family enzyme called Dicer (Bernstein et al., 2001; Jinek and Doudna, 2009). Dicer cleaves long dsRNA molecules into short dsRNA fragments of ~21 nts in each strand that are called siRNAs. This siRNA is flanked by two nt 3'overhangs on both sides (Elbashir et al., 2001). Each siRNA is subsequently unwound into two ssRNAs, namely the passenger strand and the guide strand. The passenger strand will be degraded, and the guide strand is incorporated into the RISC, which has an Argonaute family protein (AGO proteins) as its core component (Hock and Meister, 2008; Nowotny and Yang, 2009). RISC uses the sequence of its bound guide RNA to search for complementary substrates, which it engages via Watson-Crick base pairing. In animal cells, AGO2, a component of RISC, cleaves the mRNA in a metal-assisted reaction in the middle of a perfectly complementary siRNA-mRNA duplex. The endonucleolytically cleaved target mRNA is then released, freeing RISC to act on additional targets as a multiple turnover enzyme (Haley and Zamore, 2004). Hence, this process works efficiently even under limited molar concentrations of siRNA, and in certain organisms (e.g., *C. elegans*), the siRNAs can even spread systemically throughout the organism. This manner of post-transcriptional gene silencing is used for knockdown experiments as described here.

In contrast, endogenous microRNAs (miRNAs) do not form perfectly base-paired duplexes with their target mRNAs, and silence gene expression through different mechanisms that result in translation inhibition and/or recruitment of deadenylation and decapping factors (Bartel, 2009). MicroRNAs are produced from longer primary transcripts called pri-miRNAs. These contain regions that fold into short, duplexed structures that are initially processed within the nucleus by Drosha, another conserved RNase III family nuclease. This liberates a short hairpin structure, known as

pre-miRNA, that is exported to the cytoplasm. Further processing of pre-miRNAs by Dicer yields something akin to an siRNA that becomes loaded onto RISC. MicroRNAs recognize their targets via interactions that depend disproportionately on a region spanning nt 2 to 8 of the miR strand (guide strand). This seed region is also likely important for initial target recognition by siRNAs, particularly in organisms where the miRNA and siRNA are mediated by identical components (e.g., mammals) (Bartel, 2009).

The use of RNAi in NMD research has advanced the field tremendously from uncovering the proteins involved in the process via various genetic screens, to enhancing our understanding of small mechanistic details of the mechanism, to larger genome-wide implications. Indeed, genome-wide studies in yeast, flies, and human cells have recently shown that 3% to 10% of all mRNAs are changed by the RNAi-mediated depletion of the central NMD factor UPF1 (reviewed in Rehwinkel et al., 2006; Nicholson et al., 2009). Interestingly, these studies identified many mRNAs with intact ORFs as NMD targets, which changed the definition of NMD and denoted a role for NMD beyond RNA quality control as a post-transcriptional regulator of gene expression.

Basic Protocol 3: NMD complementation assays

Wide-ranging efforts in bioinformatics have been focused toward the design of successful reagents that maximize gene knockdown but minimize off-target effects. However, an siRNA might trigger a number of potential unspecific events such as the degradation of partially complementary mRNA due to cross-hybridization, leading to unspecific RNAi, or to translational arrest due to an miRNA-like effect where a siRNA hybridizes to a mRNA with one or few mismatches. Thus, it is of paramount importance to ensure that the phenotypic effects observed as a result of siRNA presence in cells are due to silencing of the target gene only. It has been estimated from studying the genomes of *H. sapiens*, *C. elegans*, and *S. pombe* that about 10% of possible siRNAs will have substantial off-target effects. Rescue experiments in which the RNAi effect is reversed through expression of a target gene refractory to silencing by a particular siRNA can give confirmation that an observed effect is specifically due to knocking down the target of interest. Beyond assessing the specificity of RNAi-induced NMD factor silencing by rescuing a RNAi-mediated-loss-

of-function phenotype by expressing a RNAi-resistant version of the NMD target gene, complementation assays are also useful for examining exactly what part or function of the protein is necessary to rescue NMD by exogenously expressing RNAi resistant mutant versions of the NMD factor (see Table 27.4.1) and observing if it can still rescue the loss of NMD after the endogenous NMD factor has been depleted by RNAi.

Basic Protocols 4-6: Extraction of RNA and analysis of RNA levels by reverse transcription-quantitative PCR

In order to meticulously detect and quantify gene expression from small amounts of RNA, amplification of the gene transcript is essential. RT-qPCR is a powerful tool for the detection and quantification of small amounts of specific RNA species. It is widely used because of its high sensitivity, specificity, good reproducibility, wide dynamic range, and speed. Specifically, RT-qPCR assays measure the amount of PCR product after each PCR cycle. The change in concentration of the target DNA is detected using fluorescent molecules that interact with DNA. These can be fluorescent dyes that intercalate with double-stranded DNA (e.g., SYBR Green, see Fig. 27.4.2B) or fluorescently tagged oligonucleotide probes that change signal intensity as a result of DNA amplification (e.g., TaqMan probes, see Fig. 27.4.2A). The fluorescent signal is monitored during the reaction and its intensity correlates to the amount of product formed (Mackay et al., 2002; Bustin, 2005; Wong and Medrano, 2005; Fleige and Pfaffl, 2006; Kubista et al., 2006). The course of the experiment can be broken down into four phases: the linear ground phase, early exponential phase, late exponential phase, and plateau phase. The linear ground phase is during the early cycles (first 10 to 15 cycles); the DNA duplication is not detected because of DNA quantities that produce insufficient signal/background ratio. Baseline fluorescence is calculated during this phase. The target DNA sequence starts to double in concentration with each cycle, causing the signal level to increase exponentially after it has become detectable above background. The amplification efficiency (E) of a well-optimized qPCR assay is very high (near 1) at the beginning of the reaction and remains relatively stable during the early exponential phase of the amplification. In qPCR, quantification is performed during this early exponential phase, when reaction efficiency is still stable. In later cycles, the

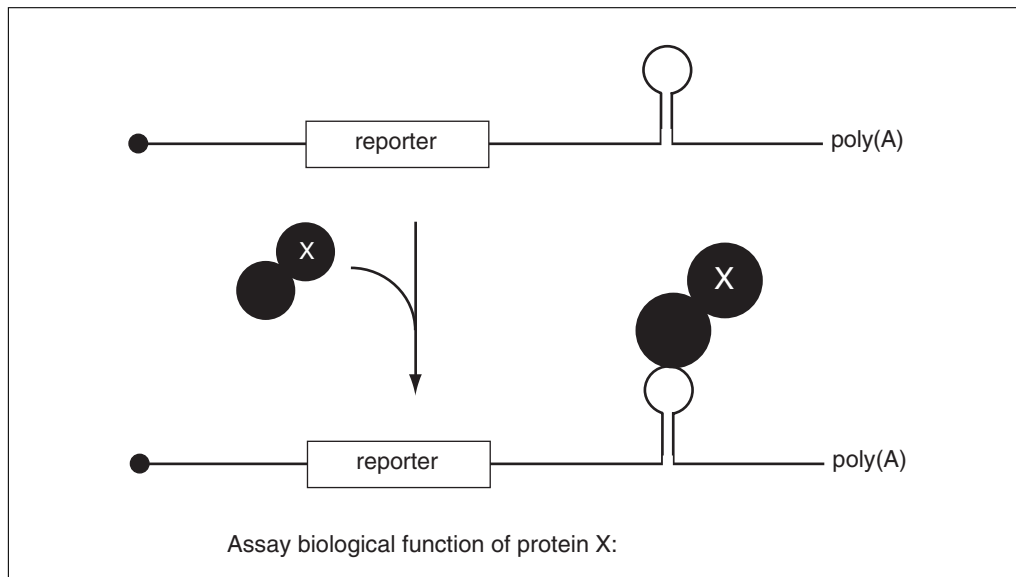


Figure 27.4.8 Tethered Function Assay. The design of the Tethered Function Assay is relatively straightforward. To determine the effects of protein X on mRNA metabolism, a chimeric protein is expressed *in vivo* in which protein X is continuous with a tethering polypeptide. The tethering protein is an RNA-binding protein that recognizes an RNA tag sequence with high specificity and affinity. The effect of the fusion protein on mRNA metabolism is determined by co-expressing the chimera with an mRNA reporter (such as *LacZ* or Firefly luciferase) into which a tag RNA sequence has been embedded. The fusion protein's effects on mRNA metabolism are assayed by conventional means such as western blotting, northern blotting, RT-PCR, etc. (altered diagram of the original shown in Collier and Wickens, 2002).

signal levels off (late exponential and plateau phase) and the intensity of the fluorescence is no longer related to the starting template copy number. This saturation results from the reaction running out of critical components, such as primers, fluorescence dye, and dNTPs (Bustin, 2000). Saturation can also occur due to competition from re-annealing reactions, the changing concentration ratios of the components, or the decreasing ratio of enzyme units to DNA substrate molecules.

The cycle threshold [C_t ; also referred to as quantification cycle (C_q) or crossing point (CP)] is the metric used for analyzing qPCR results. The C_t value represents the number of cycles needed to reach a set threshold fluorescence signal level. The greater the amount of starting template, the fewer cycles it takes to attain a detectable fluorescence level. The exact level used for this threshold should be chosen so that it captures data during the early exponential phase and is the same for all samples analyzed in a run. To determine C_t values in practice, background fluorescence levels are first subtracted from raw data. The background value is typically based on the relatively stable fluorescence level of the first few cycles. Then, a fluorescence threshold is chosen either manually or using an instrument-based algorithm. The data analysis searches data curves for each

sample and interpolates a C_t that represents where that sample crossed the threshold. Thus, the specific C_t obtained is a relative value. It is relative to the starting template copy number (Heid et al., 1996), but it is also specific for the instrument and reagents used, the efficiency of the PCR amplification, the efficiency of cleavage or hybridization of the fluorogenic probe, and the sensitivity of detection. Quantitative PCR can be used to quantify nucleic acids by two methods: relative quantification and absolute quantification. Relative quantification is based on internal reference genes to determine fold-differences in expression of the target gene. Absolute quantification gives the exact number of target DNA molecules by comparison with DNA standards; both are further explained below and the former is exemplified in Basic Protocol 6.

In comparison to traditional end-point PCR, qPCR results can be obtained more quickly and with less variability due to the use of very sensitive fluorogenic chemistries and the elimination of post-PCR detection procedures. Also, qPCR reduces the risk of laboratory contamination because the tubes need not be opened post-PCR. qPCR assays are also well-suited to more challenging applications such as high-throughput analysis. However, due to its high sensitivity, it demands a sound

experimental design and a meticulous understanding of quantification and normalization techniques for accurate results to be obtained.

Basic Protocol 7: Using the Tethered Function Assay to Investigate NMD

The basic design of the Tethered Function Assay

Many steps in the control of gene expression are dependent upon RNA-binding proteins, most of which are bi-functional, meaning they both bind to RNA and interact with other protein partners in a functional complex. Due to the importance of RNA-binding proteins in gene regulation, one of the major tasks in molecular cell biology is to determine the biological role of these proteins and the mechanisms by which they achieve their various functions. Certainly, RNA-binding proteins are involved in a wide spectrum of both regulated and constitutive functions in the cell, and much groundwork has been done by biochemical studies, but the real evidence of biological function requires analyses *in vivo*. However, this is not trivial, because in many instances the natural mRNA target for a given protein is unknown; any assay of function must therefore be performed independently of the natural RNA-protein interaction. Also, since post-transcriptional regulatory steps are often coupled, genetic analysis of functions *in vivo* can be complicated by subsidiary effects. Furthermore, mutations in many critical RNA-binding proteins can have pleiotropic effects on the cell, making it very difficult to deduce which functions are direct. Therefore, a different approach to study the functional properties of these proteins *in vivo*, independently of their RNA-binding ability, is to attach or tether them to specifically engineered reporter mRNAs whose fate can be easily followed. This powerful technique is called a Tethered Function Assay and is based on the use of two recombinant molecules—firstly, a hybrid mRNA comprising the coding region of the protein under study fused in-frame to the RNA-binding domain of a well characterized RNA-binding protein, and, secondly, a recombinant reporter mRNA encoding an easily measurable protein and the sequence element (tether) recognized with high affinity by the exogenous RNA-binding domain of the fusion protein. When co-expressed in a cell, the fusion protein should be bound (i.e., tethered) to the reporter mRNA via the exogenous RNA-binding domain, thereby imposing the functional consequences of its association

with a mRNA on the reporter mRNA (see Fig. 27.4.8). One of the main advantages of this system as a means of determining protein function is that a prior knowledge of a protein's RNA-binding specificity is not required, and also that high levels of the endogenous protein will not necessarily be a problem, as only the function of the tethered protein is monitored. This technique has been expertly covered in numerous reviews (Coller and Wickens 2002, 2007; Keryer-Bibens et al., 2008).

Critical Parameters and Troubleshooting

Basic Protocol 1: Creation of PTC+ mRNAs and mutant NMD proteins by site-directed mutagenesis

Deciding which site-directed mutagenesis technology to use

Site-directed mutagenesis has become so valuable an experimental tool that it has been extensively improved since its discovery, and several companies are continuously trying to streamline and enhance the process. This is particularly true of the QuikChange kits (Agilent Technologies), some of which have been examined here. Importantly, as highlighted here, they have engineered various alterations to their basic system to tailor each kit to specific needs. For instance, if the template DNA is very large, the QuikChange II XL is tailored to help in this situation, and this was necessary for the example given in Figure 27.4.5A. Similarly, if it is desirable to make multiple mutations within the same DNA plasmid, like in Figure 27.4.4B, then instead of doing multiple rounds of QuikChange using pairs of complementary primers, the QuikChange Multi-Site protocol will save time due to the use of their DNA enzyme blend described in Figure 27.4.5. In actuality, it can work out more cheaply to simply order one primer and use the QuikChange Multi-Site kit than to order two primers for every alteration and use the QuikChange II kit. The technology and choice offered by Agilent is very popular, as their systems require no specialized vectors, unique restriction sites, or multiple transformations. However, for simple mutations, a kit is perhaps not entirely necessary and many other companies (Finnzymes, BD Biosciences Clontech, Invitrogen, Promega, and Takara Bio) also offer site-directed mutagenesis technologies.

Primer design and use

The primers are central to the procedure, which is why extensive guidelines have been

included in the protocol. The primers should always be in excess, and it may be beneficial to keep the primer quantity constant while varying the plasmid DNA content in the reaction. Particularly in reactions where two complementary primers are being used and low efficiency is being observed, run 2.5 μ l of the reaction on an agarose gel. If a strong primer-dimer band is observed, most likely primer-primer annealing is favored over primer-template annealing, and primer-DNA template quantities should be evaluated. If, after extensive troubleshooting using all the advice given in the protocol, the mutagenesis efficiency is still poor, strongly consider re-designing the primers.

High-quality and high-fidelity DNA polymerase

The PCR reaction goes around the entire plasmid DNA, and so it is essential to use a high-fidelity polymerase in order to minimize the chances of introducing unwanted mutations in both your gene and the vector backbone. A hot-start formulation of the enzyme is desirable, as the proofreading capability of most of these enzymes may otherwise degrade the primers during the reaction setup.

Highly competent bacterial cells are necessary for transformation step

This process does not amplify the DNA exponentially, but linearity and yields are complicated by the fact that the product DNA must undergo the nick repair and are not supercoiled, resulting in reduced efficiency of bacterial transformation. Therefore, the use of highly competent bacterial cells is crucial for this step in the protocol.

Controls

While many of the companies have designed site-directed mutagenesis kits that are faster, with fewer, less-complicated steps, there are still a wide range of factors that can hinder an efficient reaction, and, depending on the specific reaction, optimization and/or troubleshooting will have to be carried out to obtain positive results. Inclusion of good controls in the mutagenesis reaction can give information as to where in the protocol there might be a problem. It is highly recommended to use the mutagenesis control included in the QuikChange kits. In brief, the positive control is based on the pBluescript plasmid, which has one premature stop codon (or in the case of the Multi-Site kit, several premature stop codons) in the *LacZ* coding region, preventing the pro-

duction of the gene product β -galactosidase. In a positive reaction, the control primer(s) will mutagenize these stop codons back to their appropriate codons and thus allow for expression of the *LacZ* gene, producing β -galactosidase, which in presence of IPTG and Xgal will produce blue-colored colonies. Similarly, including a positive control such as transforming pUC18 in the transformation step can positively control for this part of the protocol too.

The nature of the mutations

The variety of alterations to the DNA sequences can be extensive for a wide variety of experimental reasons, but when specific changes are made to the coding region of an NMD protein, it is valuable to think carefully about exactly what kind of effect the desired change is aiming to have on the protein itself in the cell. In some cases, it might be desirable to make a neutral change, meaning a mutation to the nt sequence that will not change the amino acid sequence at the protein level. This is desirable when making genes RNAi resistant, as featured in Figure 27.4.1D. However, to change functionally important amino acids by site-directed mutagenesis of the DNA sequence requires careful consideration and depends on the desired effect. If the goal is to inactivate an enzyme as illustrated in Figure 27.4.4B, change the chemical properties of conserved amino acids in the catalytic center, here for example aspartic acid (D), to the similarly sized but polar and uncharged asparagine (N) or to the smaller and nonpolar alanine (A). Bear in mind that certain amino acid changes in the polypeptide chain may be detrimental to correct folding, and hence stability, of the protein. Therefore, one should always check that similar amounts of protein are expressed from the mutated and the wild-type gene.

Basic Protocol 2: pSUPuro-based RNAi to knock down NMD factors

Design of oligonucleotides

The design of the shRNA-encoding oligonucleotides is essential, and should be based upon the correct nt sequence of the exact gene intended for depletion. Moreover, many studies have examined the degree of complementarity needed to elicit an effective silencing response (Siomi and Siomi, 2009). This is important especially for designing specific silencing triggers. Therefore, the trigger should be designed to be fully complementary to the target. If the gene levels are not suitably decreased, which is a common and poorly

understood problem, it is worthwhile to try several targets. Due to the unavoidable risk of off-target effects in knockdown experiments, knockdown-associated phenotypes should be reproduced by use of a second target sequence against the same target mRNA. Alternatively, rescue experiments by expression of a recombinant version of the depleted protein from a RNAi-resistant mRNA can be performed to demonstrate the specificity of the observed phenotype (see below).

Transfection

The transfection protocol in this unit uses DreamFect, a transfection reagent based on the “triggered endosomal escape” technology and which is lipid based. However, certain cell types may not achieve high levels of transfection efficiency with this reagent. If so, there are several other transfection reagents available. For instance, we find that Fugene HD (Promega) works more efficiently than Dreamfect for this protocol in U2OS cells. In general, it is very important to optimize this part of the protocol for your cell type and for the specific experiment, because if there is low transfection efficiency, then massive cell death will occur under puromycin selection.

Toxicity of the gene knockdown

A genetic knockout of many of the NMD factors is lethal; thus, knocking down these genes for prolonged periods of time will also be detrimental to the cells. If you are experiencing that the knockdown is particularly toxic to your cells, you can shorten the duration of the experiment by reducing the puromycin selection time from 48 hr to 24 hr, which may allow analysis of the cells before they die. For instance, we find that, in particular, depletion of SMG6 and UPF1 are quite toxic for human cell lines.

Cell type

For this protocol, the very common HeLa cell line has been used. This method can be adapted to other cell types. However, the transfection will have to be optimized as previously explained, and the optimal conditions for puromycin selection will have to be determined by titrating the puromycin concentration. Puromycin is an aminoglycoside antibiotic produced by *Streptomyces alboniger*. It specifically inhibits peptidyl transfer on both prokaryotic and eukaryotic ribosomes through the *pac* gene encoding a puromycin *N*-acetyl-transferase (PAC), and the different cells have different sensitivity levels for such an antibiotic. Unfortunately, we have found

that HEK293-T cells do not work well with this protocol due to their high sensitivity to puromycin.

Controls

As with all experiments, controls are vital to interpret the results. In this method, the set of experiments carried out using the pSUPuro plasmid encoding the shRNA targeting the knockdown of a particular NMD factor should also be carried out in parallel using a pSUPuro plasmid expressing no shRNAs (pSUPuro empty) or a pSUPuro plasmid expressing a scrambled sequence which is predicted to target no gene in the used species. Finally, off-target effects can arise when an introduced shRNA has a base sequence that can pair with and thus reduce the expression of multiple genes at a time.

Basic Protocol 3: NMD complementation assays

The mutation(s) introduced to create RNAi resistance must match the RNAi target sequence

It cannot be stressed enough that the silent mutations that are made in the exogenously expressed NMD factor have to be based upon the exact target sequence used to knock down the NMD gene. The shRNA must lose its absolute complementarity to the specific target region in the exogenous mRNA due to these introduced mutations. Furthermore, if more than one shRNA is used to knock down the NMD gene, then silent mutations should be made in all the regions of the exogenous mRNA where the multiple shRNAs bind, making the exogenously expressed NMD factor resistant to all targets.

Expression levels of the exogenous RNAi-resistant factors

It is important to ensure that the exogenously expressed RNAi resistant NMD proteins and any functional mutants thereof have similar expression levels to the endogenous protein, and in particular to each other. This will require titrating the amount of plasmid DNA transfected for each RNAi resistant expression plasmid so that similar protein levels are achieved. It is recommended to keep the expression levels of the exogenously expressed NMD proteins similar to the endogenously expressed NMD proteins to make the experiment physiologically relevant. Likewise, it is important to keep the expression levels of the different RNAi-resistant NMD factors (wild-type and mutants thereof) very similar to each

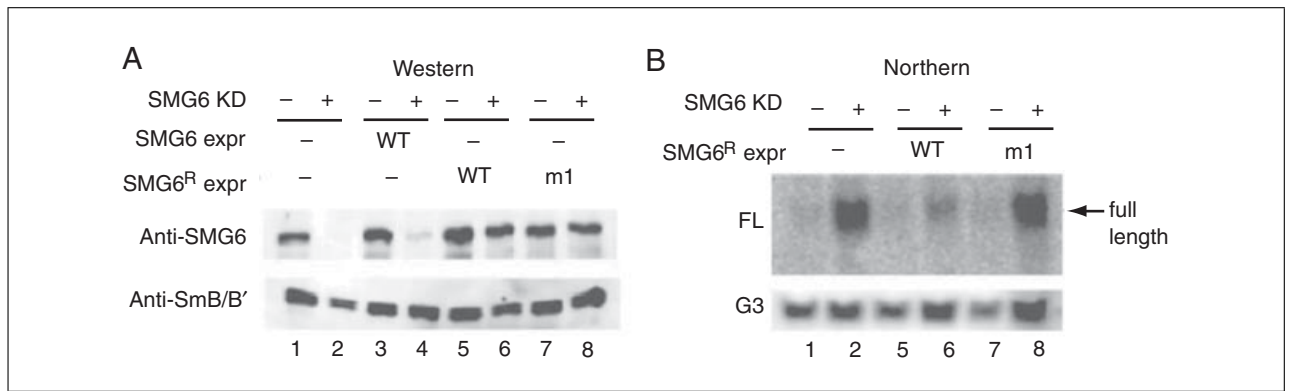


Figure 27.4.9 SMG6 complementation assay showing that SMG6 endonuclease activity is required for the degradation of TCR β PTC+ RNA by NMD. **(A)** Western blotting analysis demonstrating RNAi-mediated knockdown of SMG6 and the ectopic expression of RNAi resistant HA-tagged versions of SMG6 (wild-type (WT) and m1 HA-SMG6R) at equal levels in HeLa-TCR β PTC+ cells. Lanes 1 and 2 depict an efficient SMG6 knockdown of endogenous SMG6, lanes 3 and 4 depict an efficient knockdown of both endogenous and exogenous SMG6, lanes 5 and 6 show that the exogenously expressed RNAi resistant SMG6 is expressed even under knockdown conditions, and the same is true for lanes 7 and 8, where an exogenously expressed SMG6 PIN mutant is tested. **(B)** Northern blotting analysis of total RNA isolated from HeLa-TCR β PTC+ cells shown in lanes 1, 2, 5, 6, 7, and 8 of the western blot to the right. The northern blot was hybridized with a probe spanning the VDJ region of TCR β gene. Lane 2 shows that the depletion of SMG6 has abolished NMD. Lane 6 shows that expression of the exogenous RNAi-resistant wild-type SMG6 can rescue the loss of NMD, shown by the reduced RNA level, whereas the exogenous expression of a RNAi-resistant SMG6 that is endonucleolytically inactive is not able to rescue NMD, as portrayed in lane 8 (data from Eberle et al., 2009).

other to allow for a fair interpretation of the results. Indeed, in Figure 27.4.9, only 500 ng of the pcDNA3-HA-SMG6 was transfected, whereas 2000 ng of its PIN mutant counterpart had to be transfected to gain a similar protein level.

A loss of function has to be observed in order to be rescued

An mRNA containing a PTC should be degraded by the NMD pathway, unlike its wild-type counterpart, and this should be observed by very low levels of PTC+ mRNA compared to its wild-type counterpart. However, if a key protein necessary for NMD is depleted from cells by RNAi, this abolishes NMD, and in this situation the PTC+ mRNA is not degraded by NMD and thus has stabilized mRNA levels (see Fig. 27.4.9B). This is how to monitor the effect the knockdown has and how to observe a loss of function. Therefore, it is critical to also transfect a plasmid that will express a PTC-containing mRNA and its wild-type counterpart in the cells.

Controls

As with every experiment, a set of controls is necessary for a complete experiment and for proper interpretation of the results. Four controls are needed: a co-transfection control, a control for the vector-based RNAi (use of pSUPuro empty), a control for NMD (comparing wild-type RNA to a PTC+ RNA),

and a control for the exogenously expressed RNAi-resistant proteins (comparing RNAi resistant SMG6 to SMG6 that is not RNAi resistant). Here, the former control should be further explained, as the others have already been discussed. Co-transfection controls are useful to remove variability due to transfection efficiency, and in the above protocol, a plasmid expressing GFP is included in every transfection for this reason. The importance of appropriate normalization controls in quantitative polymerase chain reaction (qPCR) experiments has become more apparent as the number of biological studies using this methodology has increased. In developing a system to study gene expression from transiently transfected plasmids, it became clear that normalization using chromosomally encoded genes is not ideal, as it does not take into account the transfection efficiency and the significantly lower expression levels of the plasmids.

Basic Protocols 4-6: Extraction of RNA and analysis of RNA levels by reverse transcription-quantitative PCR

RNA extraction and RT reaction

Use an RNA isolation procedure that produces high-quality total RNA from all samples to be analyzed. In this protocol, quantifying RNA using A_{260} values was outlined whereby spectrophotometry values can indicate RNA quality ($A_{260}/A_{280} = 1.9$ to 2.1).

Further quality checks may include using a lab-on-a-chip instrument such as the Agilent Technologies 2100 Bioanalyzer (RNA integrity number, RIN = 10 examines intact RNA and RIN 2 examines degraded RNA) or electrophoretic separation on a high-resolution agarose gel (look for sharp ethidium bromide-stained 18S and 28S rRNA bands; smearing of these bands can indicate RNA degradation). It is also important to digest the purified RNA with DNase I to remove contaminating genomic DNA, which, depending on the qPCR assay design, can act as a template during PCR and lead to spurious results. Complementary DNA synthesis can greatly affect the overall qPCR results, and it has a large capacity for introducing variation. The reverse transcriptase itself, and also DTT, are PCR inhibitors and may affect the PCR (Tichopad et al., 2004). This can be avoided by precipitating the cDNA (Liss, 2002), and DTT can be omitted from the RT reaction altogether (Gibson et al., 1996). However, it should be mentioned that the method described here has not encountered problems due to DTT. Of course, another crucial parameter of the RT reaction that affects the cDNA levels is the choice of primers used in the reaction. Gene-specific primers generally allow for a higher RT efficiency of the specific target RNA than using oligo(dT) or random priming, but in practice they are seldom used because the specific target RNA is the only one that can be quantified in such a cDNA sample. Using oligo(dT) primers for the RT generates cDNA of all polyadenylated RNAs (i.e., mainly mRNA), but it has the drawback that the reverse transcriptase may not be able to transcribe all the way through very long transcripts, and thus sequences towards the 5' end of mRNAs may be under-represented in these cDNA pools. For most applications, random priming using a randomized pool of hexa- or nonameric oligonucleotides is suitable for the RT reaction. In theory, random priming should produce a cDNA pool that represents the entire transcriptome proportionally, and hence every desired RNA species can be measured by qPCR from such a cDNA. In reality, another source of variation is the RNA structure and concentration and the reverse transcriptase used. Naturally, the RNA is neither linear nor naked but instead will have secondary structures and proteins bound to it which can interfere with the RT, inducing enzyme pausing, skipping, and dissociation (Liss, 2002). Therefore, a high-quality robust reverse transcriptase should be used

that can deal with such issues. For instance, in this protocol, AffinityScript Multiple Temperature Reverse Transcriptase (Agilent Technologies) has been specifically mentioned. It is a desirable enzyme because it has an optimal, broad reaction temperature range from 37°C to 55°C. It is an engineered version of a Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase. The recombinant enzyme is purified to near homogeneity from *E. coli*. It has no detectable exonuclease, endonuclease, or RNase activity, and has increased thermal stability. Finally, it has 10-fold higher affinity to primer:template complexes compared to conventional M-MuLV reverse transcriptases.

Template amount and dynamic range

The amount of template DNA or RNA required for successful amplification is dependent on the detection method, the complexity of the sample, and the copy number of the target gene. The optimum amount of starting DNA template is typically 10^4 copies or more, which should result in a C_t value of 20 to 25 cycles. With optimal amplification efficiency, a single template molecule can be detected after 35 to 40 cycles.

The importance of replicate reactions

It is important to run duplicate or preferably triplicate reactions when performing a qPCR assay. If the C_t values are very high (meaning the amplicon is appearing late in the reaction), the use of replicates is especially important to make sure the PCR reaction is working well. If data from replicate reactions varies by more than 0.5 cycles, the reactions should be repeated. If the C_t values consistently vary by >0.5 cycles in replicates, the assay should be re-optimized and the primers redesigned.

Detection chemistry

The choice of detection chemistry to be used is specific to the experiment and laboratory. Of course, DNA-binding fluorophores such as SYBR Green are the cheapest and most flexible choice, but since the specificity lies entirely in the set of primers, it makes the assay prone to false positives (Simpson et al., 2000). Here, it is vital to perform dsDNA melting curve analysis at the end of each PCR run to ensure that only a single PCR amplicon of the expected length and T_m is produced (this can also be achieved using gel electrophoresis). The qPCR instrument can be set to perform post-PCR melting curve analysis by gradually increasing the temperature (see Table 27.4.7) and monitoring the fluorescence

as a function of the temperature. A sharp drop in fluorescence occurs when the temperature is high enough to denature dsDNA and the fluorophore molecule is released. The T_m is calculated by the instrument software from the melting curve data by plotting the negative first derivative versus temperature. The T_m of a DNA fragment is dependent on its length, G+C composition, sequence, strand complementarity, and concentration, and on buffer components such as salts, dyes, and PCR enhancers. The characteristic T_m of primer-dimers is normally lower than that of target DNA sequence due to the smaller size of primer-dimers. This difference between the T_m 's allows detection of potentially interfering primer-dimers or other nonspecific amplification products. Since the melting curve is quick and easy to run after every reaction, SYBR Green detection has become widely used as a means to detect specific target DNAs. If the melting curve analysis reveals the presence of primer-dimers or other unwanted PCR products, then more suitable primers should be designed. Indeed, it might prove to be difficult to design a specific PCR assay for certain sequences, and in this case using a sequence-specific probe-based method would be a better choice. Ultimately, using this method will be more expensive, but it will significantly increase the specificity and enable quantification even in the presence of nonspecific DNA amplification, as only the DNA containing the probe sequence will be detected. Of the many probe-based chemistries available, a well established system such as the 5' nuclease TaqMan probes may be the best selection. This system has very good guidelines and protocols that should be error-proof if followed correctly.

Primers and probes

Undoubtedly, the primers and probes designed for RT-qPCR assays are key to the assay, which is why comprehensive guidelines were outlined and several online resources are specified below. Despite such guidelines, they may need to be redesigned if they perform poorly, such as by giving low amplification efficiencies or nonspecific amplification products. Primer concentration may also have to be considered, and it will be specific to the primers being used, but the working concentration should be in the range of 50 to 1000 nM. A large excess of primers can lead to mispriming and promote amplification of unwanted products. On the other hand, too-low primer concentrations can adversely affect the ampli-

fication rate. Thus, optimization of the primer concentration can result in better qPCR assays.

Controls

Several types of controls are used in qPCR assays to ensure the reliability and integrity of the assay. Two important controls are described below.

A *no-template control (NTC)* should be used in all PCR assays to monitor possible contamination and primer-dimer formation. This control includes all the reaction components except the actual template DNA, and thus no product should be amplified.

A *minus RT control (-RT)* should be used to control for the possible presence of contaminating DNA. It contains all the reaction components and a mock reverse-transcribed RNA sample instead of cDNA; hence, no PCR product should be detected in the amplification phase. If PCR amplification is seen, it is probably due to contaminating DNA that can serve as a template in the PCR.

Quantification of qPCR data

There are two common quantification strategies for RT-qPCR applications. Absolute quantification utilizes serially diluted standards of known template concentration to generate a standard curve to obtain the copy number or concentration of a sample. Relative quantification determines the relative change in RNA expression levels of a target gene versus a control. The latter has been outlined in this protocol, and is commonly used in RT-qPCR assays, especially for gene expression studies. Relative quantification is used to determine the ratio between the quantity of a target molecule in a sample and in a reference sample also known as a calibrator (Livak and Schmittgen, 2001). It is widely used when analyzing variation in expression levels between different samples. A reference sample can be, for example, a zero time point in time-course studies, an untreated sample, or a reference cell line to which others are compared. Results from the quantification are typically reported as relative differences between samples. The standard curve used for relative quantification can be a simple dilution series; it does not need to involve standards traceable to absolute, known concentrations like in the standard curves used for absolute quantification. Several mathematical models have been formulated for calculating the expression of a target gene compared to a suitable reference gene (reviewed in Wong and Medrano, 2005). These calculations are based on comparisons

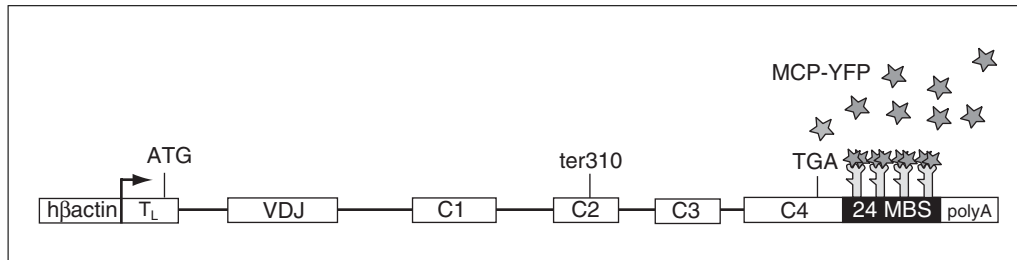


Figure 27.4.10 An *in vivo* approach to measure transcription in real-time on the single-cell level and to monitor differences in behavior between a PTC⁻ and a PTC⁺ gene. New versions of the genes explained in Figure 27.4.4 were created by exploiting the MS2 system (Bertrand et al., 1998; Darzacq et al., 2007). The insertion of 24 MS2 binding sites (MBS) in the 3' UTR of the Ig mini μ gene allowed for the visualization of transcription sites *in vivo* upon binding of these stem-loops by the MS2 coat protein (MCP) fused to yellow fluorescent protein (MCP-YFP) shown by gray stars. Stable transfection of the Ig mini μ genes either with the wild-type sequence or with a single point mutation generating a PTC at amino acid 310 in to U2OS cells allowed for live-cell imaging of active transcription sites (diagram from de Turris et al., 2011).

of separate quantification cycle values (C_t) at a particular fluorescence level. The following relative quantification methods all use a standard curve to calculate amplification efficiencies: Standard Curve Method (Livak, 1997), Comparative C_t Model (Livak and Schmittgen, 2001), Pfaffl Model (Pfaffl, 2001), and Q-gene (Muller et al., 2002).

Normalization: Relative gene expression data obtained with RT-qPCR should be normalized in order to correct for several variables, including the amount of cells or nucleic acid in the sample, instrument-dependent variation in fluorescence collection, and variation in reaction efficiency, nucleic acid integrity, and sample purity. A normalizer can be an exogenous control (such as measuring GFP mRNA levels from the example experiment in Table 27.4.4, Basic Protocol 3) or an endogenous control such as a reference gene. However, any variations in the normalizer can hide the actual expression-level changes and produce misleading results. It is important that, if reference genes are used as normalizers, the genes be well validated and results not be reported more precisely than the normalizer can be quantified. Normalizers can be simultaneously amplified in the same tube as the target gene as a multiplex assay, or separately amplified in parallel reactions, although the latter approach reduces the value of the normalizer, since tube-to-tube variations in pipetting and reaction rates are not normalized.

An external, artificial RNA molecule with known concentration can be added into the RNA sample and used as an exogenous reference for normalizing against experimental variation (such as the use of GFP in Basic Protocol 3). These added external molecules can

be cloned from other species or produced synthetically. They will be exposed to the same effects and experimental errors as the target RNA. However, this type of internal reference does not normalize for variability in the RNA extraction process (reviewed in Huggett et al., 2005). Hence, this method also requires extra validation to demonstrate that it is a suitable basis for normalization.

Reference genes should also be used to normalize for variation in sample preparation procedures and for variations in each RT reaction. A gene used as a reference should be expressed at a constant level across all samples and experimental conditions tested. If these requirements are not fulfilled, the normalization process can result in significant errors. The amplification efficiency of a reference gene should be the same as the efficiency of the target gene; otherwise, the data need to be corrected for variation. Reference genes are usually well known housekeeping genes. Some housekeeping genes frequently used as reference genes include GAPDH, β -actin, and 18S rRNA. It should be noted that some sample treatment methods may have a different effect on rRNA and mRNA expression. For example, rRNA is transcribed with a different polymerase than mRNA, so variations in polymerase activity can result in different effects in expression levels (reviewed in Huggett et al., 2005; Wong and Medrano, 2005). A big problem is that even the expression of housekeeping genes usually varies to some extent. That is why several reference genes are often required, and their expression levels need to be checked for each experiment. To this end, several Excel-based macros and programs are available for assessment of multiple reference

genes. Screening a panel of candidate reference genes under specified experimental conditions and using the statistical methods and software packages recommended under Internet Resources in this unit will yield the most stable reference gene or set of genes.

Basic Protocol 7: Using the Tethered Function Assay to Investigate NMD

Basic Protocol 7 is a comprehensive guide where each component of the assay has been methodically addressed to aid in the creation of a tethered function assay. Every aspect of the assay has been carefully discussed and all options have been explained so that the most suited and optimized assay can be established by the investigator. Two key points are worth reiterating. (1) It is of paramount importance that the outlined controls be created and included in the assay. Any results generated from such an assay will be meaningless without these stipulated controls. (2) It is also of great importance to verify that the NMD factor of interest fused to the desired tether can be expressed. Additionally, this assay can make use of the methods described in Basic Protocols 1, 2, 4, 5, and 6. Thus, the Critical Parameters and Troubleshooting sections accompanying each of these protocols should be read and considered when encountering any problems.

Anticipated Results

Basic Protocol 1: Creation of PTC+ mRNAs and Mutant NMD Proteins by Site-Directed Mutagenesis

The anticipated outcome is to generate the plasmid DNA containing the planned alterations and no other mutations as confirmed by DNA sequencing analysis. For changes made to plasmids below 10 kb, 80% to 100% of colonies should contain a correct plasmid. For longer plasmids or more complicated alterations, it may be necessary to optimize the protocol or screen more colonies.

Basic Protocol 2: pSUPuro-based RNAi to Knock Down NMD Factors

This method should ideally reduce the protein level of the targeted gene by >10-fold. In many cases, studying the effects of this decrease can reveal the physiological role of the gene product. However, since RNAi does not totally abolish expression of the gene, it is possible that even strongly reduced protein levels may still be sufficient to sustain the biological process under investigation. Therefore, it cannot be overstated that a negative result (i.e., no

change upon depletion of the target protein) in a knockdown experiment is inconclusive. This is a severe limitation of the RNAi method that fundamentally distinguishes it from genetic “knockout” techniques in which expression of a gene can be entirely eliminated.

Basic Protocol 3: NMD Complementation Assays

The NMD factor that is depleted by RNAi can be rescued by exogenously expressing the same NMD factor which has been made RNAi resistant. The depletion of the NMD factors should cause an abrogation of NMD, which will be reflected in a stabilization of PTC-containing reporters, but the inclusion of the RNAi-resistant exogenous NMD factor should rescue the loss of NMD, and thus PTC+ RNA will be rapidly degraded and thus have low RNA levels when analyzed.

Basic Protocols 4-6: Extraction of RNA and Analysis of RNA Levels by Reverse Transcription–Quantitative PCR

The projected outcome of Basic Protocol 4 is the production of highly pure RNA that is free of DNA contamination. The expected result of Basic Protocol 5 is the efficient reverse transcription of 1 µg of RNA into its complementary DNA (cDNA), which should be contaminant free and ready for use in qPCR. The anticipated outcome of Basic Protocol 6 is accurate extrapolation of the relative transcript abundance for each gene in each sample tested in the optimized qPCR procedure.

Basic Protocol 7: Using the Tethered Function Assay to Investigate NMD

Anticipating the results produced by such assays is impossible because the success and the results of such an approach are entirely case specific. However, these assays have been very successfully utilized for studying NMD, and will most likely continue to be worthwhile as more NMD factors are found. It should be borne in mind that while this is a powerful method, it is artificial, and only positive results are meaningful. Interpretations cannot be made when no effects have been observed.

Time Considerations

Basic Protocol 1: Creation of PTC+ mRNAs and Mutant NMD Proteins by Site-Directed Mutagenesis

After designing and ordering the oligonucleotide primers, it may take up to 1 week until you receive them. During this time, the high-quality template DNA can be prepared. The

PCR reaction, *DpnI* digestion, and transformation into competent bacteria can be completed in a day. Picking colonies to inoculate cultures and plasmid DNA preparation will take 2 days. The length of time for DNA sequencing analysis will depend upon specific laboratory facilities and may take 1 day to several weeks. The Lightning kits from Agilent Technologies have been specifically made to reduce the time aspect of this procedure, and the use of this kit has also been outlined in Basic Protocol 1.

Basic Protocol 2: pSUPuro-based RNAi to knock down NMD factors

Once the pSUPuro plasmid expressing a shRNA against your gene of choice has been created and tested, which can take several weeks or even longer if initial target sequences work inefficiently and new targets need to be designed or further optimizations are required, no further time or money has to be invested in creating this tool again, and a research laboratory can build up a large repertoire of plasmids to knock down many genes. Then, the experiment itself, from seeding the cells to protein and RNA analysis, should take a minimum of 7 to 8 days each time.

Basic Protocol 3: NMD complementation assays

Construction of all the plasmids necessary for this experiment will initially take a comparatively long time because it will involve cloning and site-directed mutagenesis. The validation of the pSUPuro based knockdown and the titration of the expression plasmids to achieve similar expression levels will also take a reasonable length of time, depending on how much optimization may be required in both cases. After complete preparation of the plasmids, the experiment itself from seeding the cells to protein and RNA analysis should take a minimum of 7 to 8 days each time.

Basic Protocols 4-6: Extraction of RNA and analysis of RNA levels by reverse transcription-quantitative PCR

The extraction, isolation, DNase treatment, spectrophotometric quantification of RNA, and RT reaction should take less than 4 hr depending on sample numbers. The qPCR may initially take some time to optimize all the parameters, but after this initial optimization and with the use of the fast-chemistry qPCR master mixes detailed in Basic Protocol 6, the actual qPCR running time for a TaqMan assay can be only 60 min, and slightly longer (70 min) for SYBR Green assays due to the inclusion of the melting curve at the end of the run. In fact,

the Brilliant II Fast qPCR master mix is soon to be replaced by an even faster qPCR master mix, meaning that the run-time for TaqMan assays could take less than 60 min. Therefore, in Basic Protocols 4 to 6, extraction of total RNA from cells to an accurate measurement of mRNA levels by RT-qPCR can be carried out in one working day.

Basic Protocol 7: Using the Tethered Function Assay to investigate NMD

While the assay seems quite straightforward, several issues that have been discussed should be considered at the outset in designing a tethering experiment (see Fig. 27.4.6), and thus establishing this assay may take some time. Once it has been established, it can be expanded by tethering more factors, introducing mutations into the test proteins, or performing the assays in cells that are depleted of certain factors. In summary, the more elaborate the system, the longer it will take.

Literature Cited

- Andersen, C.L., Jensen, J.L., and Orntoft, T.F. 2004. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* 64:5245-5250.
- Arias-Palomo, E., Yamashita, A., Fernández, IS., Núñez-Ramírez, R., Bamba, Y., Izumi, N., Ohno, S., and Llorca, O. 2011. The nonsense-mediated mRNA decay SMG-1 kinase is regulated by large-scale conformational changes controlled by SMG-8. *Genes Dev.* 25:153-164.
- Bardwell, V.J. and Wickens, M. 1990. Purification of RNA and RNA-protein complexes by an R17 coat protein affinity method. *Nucleic Acids Res.* 18:6587-6594.
- Bartel, D.P. 2009. MicroRNAs: Target recognition and regulatory functions. *Cell* 136:215-233.
- Battaglia, M., Pedrazzoli, P., Palermo, B., Lanza, A., Bertolini, F., Gibelli, N., Da Prada, G.A., Zambelli, A., Perotti, C., and Robustelli della Cuna, G. 1998. Epithelial tumour cell detection and the unsolved problems of nested RT-PCR: A new sensitive one step method without false positive results. *Bone Marrow Transplant.* 22:693-698.
- Beach, D.L., Salmon, E.D., and Bloom, K. 1999. Localization and anchoring of mRNA in budding yeast. *Curr. Biol.* 9:569-578.
- Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409:363-366.
- Bertrand, E., Chartrand, P., Schaefer, M., Shenoy, S.M., Singer, R.H., and Long, R.M. 1998. Localization of ASH1 mRNA particles in living yeast. *Mol. Cell* 2:437-445.

- Bhattacharya, A., Czaplinski, K., Trifillis, P., He, F., Jacobson, A., and Peltz, S.W. 2000. Characterization of the biochemical properties of the human Upf1 gene product that is involved in nonsense-mediated mRNA decay. *RNA* 6:1226-1235.
- Brogna, S. and Wen, J. 2009. Nonsense-mediated mRNA decay (NMD) mechanisms. *Nat. Struct. Mol. Biol.* 16:107-113.
- Brummelkamp, T.R., Bernards, R., and Agami, R. 2002. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296:550-553.
- Buchwald, G., Ebert, J., Basquin, C., Sauliere, J., Jayachandran, U., Bono, F., Le Hir, H., and Conti, E. 2010. Insights into the recruitment of the NMD machinery from the crystal structure of a core EJC-UPF3b complex. *Proc. Natl. Acad. Sci. U.S.A.* 107:10050-10055.
- Bühler, M., Paillusson, A., and Muhlemann, O. 2004. Efficient downregulation of immunoglobulin mu mRNA with premature translation-termination codons requires the 5'-half of the VDJ exon. *Nucleic Acids Res.* 32:3304-3315.
- Bustin, S.A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* 25:169-193.
- Bustin, S.A. 2005. Real-time, fluorescence-based quantitative PCR: A snapshot of current procedures and preferences. *Expert Rev. Mol. Diagn.* 5:493-498.
- Carter, M.S., Daskow, J., Morris, P., Li, S., Nhim, R.P., Sandstedt, S., and Wilkinson, M.F. 1995. A regulatory mechanism that detects premature nonsense codons in T-cell receptor transcripts in vivo is reversed by protein synthesis inhibitors in vitro. *J. Biol. Chem.* 270:28995-29003.
- Chang, J.C. and Kan, Y.W. 1979. Beta 0 thalassemia, a nonsense mutation in man. *Proc. Natl. Acad. Sci. U.S.A.* 76:2886-2889.
- Chang, Y.F., Imam, J.S., and Wilkinson, M.F. 2007. The nonsense-mediated decay RNA surveillance pathway. *Annu. Rev. Biochem.* 76:51-74.
- Cho, H., Kim, K.M., and Kim, Y.K. 2009. Human proline-rich nuclear receptor coregulatory protein 2 mediates an interaction between mRNA surveillance machinery and decapping complex. *Mol. Cell* 33:75-86.
- Choe, J., Cho, H., Lee, H.C., and Kim, Y.K. 2010. microRNA/Argonaute 2 regulates nonsense-mediated messenger RNA decay. *EMBO Rep.* 11:380-386.
- Choe, J., Cho, H., Chi, S.G., and Kim, Y.K. 2011. Ago2/miRISC-mediated inhibition of CBP80/20-dependent translation and thereby abrogation of nonsense-mediated mRNA decay require the cap-associating activity of Ago2. *FEBS Lett.* 585:2682-2687.
- Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
- Chomczynski, P. and Sacchi, N. 2006. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: Twenty-something years on. *Nat. Protoc.* 1:581-585.
- Clerici, M., Mourão, A., Gutsche, I., Gehring, N.H., Hentze, M.W., Kulozik, A., Kadlec, J., Sattler, M., and Cusack, S. 2009. Unusual bipartite mode of interaction between the nonsense-mediated decay factors, UPF1 and UPF2. *EMBO J.* 28:2293-2306.
- Coller, J. and Wickens, M. 2002. Tethered function assays using 3' untranslated regions. *Methods* 26:142-150.
- Coller, J. and Wickens, M. 2007. Tethered function assays: An adaptable approach to study RNA regulatory proteins. *Methods Enzymol.* 429:299-321.
- Coller, J.M., Gray, N.K., and Wickens, M.P. 1998. mRNA stabilization by poly(A) binding protein is independent of poly(A) and requires translation. *Genes Dev.* 12:3226-3235.
- Darzacq, X., Shav-Tal, Y., de Turris, V., Brody, Y., Shenoy, S.M., Phair, R.D., and Singer, R.H. 2007. In vivo dynamics of RNA polymerase II transcription. *Nat. Struct. Mol. Biol.* 14:796-806.
- De Gregorio, E., Preiss, T., and Hentze, M.W. 1999. Translation driven by an eIF4G core domain in vivo. *EMBO J.* 18:4865-4874.
- Denning, G., Jamieson, L., Maquat, L.E., Thompson, E.A., and Fields, A.P. 2001. Cloning of a novel phosphatidylinositol kinase-related kinase: characterization of the human SMG-1 RNA surveillance protein. *J. Biol. Chem.* 276:22709-22714.
- de Turris, V., Nicholson, P., Orozco, R.Z., Singer, R.H., and Muhlemann, O. 2011. Cotranscriptional effect of a premature termination codon revealed by live-cell imaging. *RNA* 17:2094-2107.
- Doma, M.K. and Parker, R. 2007. RNA quality control in eukaryotes. *Cell* 131:660-668.
- Eberle, A.B., Stalder, L., Mathys, H., Orozco, R.Z., and Muhlemann, O. 2008. Posttranscriptional gene regulation by spatial rearrangement of the 3' untranslated region. *PLoS Biol.* 6:e92.
- Eberle, A.B., Lykke-Andersen, S., Muhlemann, O., and Jensen, T.H. 2009. SMG6 promotes endonucleolytic cleavage of nonsense mRNA in human cells. *Nat. Struct. Mol. Biol.* 16:49-55.
- Echeverri, C.J. and Perrimon, N. 2006. High-throughput RNAi screening in cultured cells: A user's guide. *Nat. Rev. Genet.* 7:373-384.
- Elbashir, S.M., Lendeckel, W., and Tuschl, T. 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* 15:188-200.
- Fernández, I.S., Yamashita, A., Arias-Palomo, E., Bamba, Y., Bartolomé, R.A., Canales, M.A., Teixidó, J., Ohno, S., Llorca, O. 2011. Characterization of SMG-9, an essential component of the nonsense-mediated mRNA decay SMG1C complex. *Nucleic Acids Res.* 39:347-358.

- Finoux, A.L. and Seraphin, B. 2006. In vivo targeting of the yeast Pop2 deadenylase subunit to reporter transcripts induces their rapid degradation and generates new decay intermediates. *J. Biol. Chem.* 281:25940-25947.
- Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391:806-811.
- Fisher, C.L. and Pei, G.K. 1997. Modification of a PCR-based site-directed mutagenesis method. *BioTechniques* 23:570-574.
- Fleige, S. and Pfaffl, M.W. 2006. RNA integrity and the effect on the real-time qRT-PCR performance. *Mol. Aspects Med.* 27:126-139.
- Freshney, R.I. 2010. Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications" 6th ed. Wiley-Blackwell, Hoboken, New Jersey.
- Fribourg, S., Gatfield, D., Izaurralde, E., and Conti, E. 2003. A novel mode of RBD-protein recognition in the Y14-Mago complex. *Nat. Struct. Biol.* 10:433-439.
- Frischmeyer, P.A. and Dietz, H.C. 1999. Nonsense-mediated mRNA decay in health and disease. *Hum. Mol. Genet.* 8:1893-1900.
- Fukuhara, N., Ebert, J., Unterholzner, L., Lindner, D., Izaurralde, E., and Conti, E. 2005. SMG7 is a 14-3-3-like adaptor in the nonsense-mediated mRNA decay pathway. *Mol. Cell* 17:537-547.
- Gehring, N.H., Neu-Yilik, G., Schell, T., Hentze, M.W., and Kulozik, A.E. 2003. Y14 and hUpf3b form an NMD-activating complex. *Mol. Cell* 11:939-949.
- Gehring, N.H., Kunz, J.B., Neu-Yilik, G., Breit, S., Viegas, M.H., Hentze, M.W., and Kulozik, A.E. 2005. Exon-junction complex components specify distinct routes of nonsense-mediated mRNA decay with differential cofactor requirements. *Mol. Cell* 20:65-75.
- Gibson, U.E., Heid, C.A., and Williams, P.M. 1996. A novel method for real time quantitative RT-PCR. *Genome Res.* 6:995-1001.
- Glavan, F., Behm-Ansmant, I., Izaurralde, E., and Conti, E. 2006. Structures of the PIN domains of SMG6 and SMG5 reveal a nuclease within the mRNA surveillance complex. *EMBO J.* 25:5117-5125.
- Graveley, B.R. and Maniatis, T. 1998. Arginine/serine-rich domains of SR proteins can function as activators of pre-mRNA splicing. *Mol. Cell* 1:765-771.
- Gray, N.K., Collier, J.M., Dickson, K.S., and Wickens, M. 2000. Multiple portions of poly(A)-binding protein stimulate translation in vivo. *EMBO J.* 19:4723-4733.
- Grskovic, M., Hentze, M.W., and Gebauer, F. 2003. A co-repressor assembly nucleated by Sex-lethal in the 3'UTR mediates translational control of *Drosophila* msl-2 mRNA. *EMBO J.* 22:5571-5581.
- Haley, B. and Zamore, P.D. 2004. Kinetic analysis of the RNAi enzyme complex. *Nat. Struct. Mol. Biol.* 11:599-606.
- Heid, C.A., Stevens, J., Livak, K.J., and Williams, P.M. 1996. Real time quantitative PCR. *Genome Res.* 6:986-994.
- Hock, J. and Meister, G. 2008. The Argonaute protein family. *Genome Biol.* 9:210.
- Holbrook, J.A., Neu-Yilik, G., Hentze, M.W., and Kulozik, A.E. 2004. Nonsense-mediated decay approaches the clinic. *Nat. Genet.* 36:801-808.
- Holland, P.M., Abramson, R.D., Watson, R., and Gelfand, D.H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'→3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* 88:7276-7280.
- Hosoda, N., Kim, Y.K., Lejeune, F., and Maquat, L.E. 2005. CBP80 promotes interaction of Upf1 with Upf2 during nonsense-mediated mRNA decay in mammalian cells. *Nat. Struct. Mol. Biol.* 12:893-901.
- Huggett, J., Dheda, K., Bustin, S., and Zumla, A. 2005. Real-time RT-PCR normalisation: Strategies and considerations. *Genes Immun.* 6:279-284.
- Huntzinger, E., Kashima, I., Fauser, M., Saulière, J., Izaurralde, E. 2008. SMG6 is the catalytic endonuclease that cleaves mRNAs containing nonsense codons in metazoan. *RNA* 14:2609-2617.
- Hutchison, C.A., 3rd, Phillips, S., Edgell, M.H., Gillam, S., Jahnke, P., and Smith, M. 1978. Mutagenesis at a specific position in a DNA sequence. *J. Biol. Chem.* 253:6551-6560.
- Isken, O. and Maquat, L.E. 2007. Quality control of eukaryotic mRNA: Safeguarding cells from abnormal mRNA function. *Genes Dev.* 21:1833-1856.
- Isken, O., Kim, Y.K., Hosoda, N., Mayeur, G.L., Hershey, J.W., and Maquat, L.E. 2008. Upf1 phosphorylation triggers translational repression during nonsense-mediated mRNA decay. *Cell* 133:314-327.
- Ivanov, P.V., Gehring, N.H., Kunz, J.B., Hentze, M.W., and Kulozik, A.E. 2008. Interactions between UPF1, eRFs, PABP and the exon junction complex suggest an integrated model for mammalian NMD pathways. *EMBO J.* 27:736-747.
- Jinek, M. and Doudna, J.A. 2009. A three-dimensional view of the molecular machinery of RNA interference. *Nature* 457:405-412.
- Kadlec, J., Izaurralde, E., and Cusack, S. 2004. source asis for the interaction between nonsense-mediated mRNA decay factors UPF2 and UPF3. *Nat. Struct. Mol. Biol.* 11:330-337.
- Kashima, I., Yamashita, A., Izumi, N., Kataoka, N., Morishita, R., Hoshino, S., Ohno, M., Dreyfuss, G., and Ohno, S. 2006. Binding of a novel SMG-1-Upf1-eRF1-eRF3 complex (SURF) to the exon junction complex triggers Upf1 phosphorylation and nonsense-mediated mRNA decay. *Genes Dev.* 20:355-367.

- Kashima, I., Jonas, S., Jayachandran, U., Buchwald, G., Conti, E., Lupas, A.N., and Izaurralde, E. 2010. SMG6 interacts with the exon junction complex via two conserved EJC-binding motifs (EBMs) required for nonsense-mediated mRNA decay. *Genes Dev.* 24:2440-2450.
- Keryer-Bibens, C., Barreau, C., and Osborne, H.B. 2008. Tethering of proteins to RNAs by bacteriophage proteins. *Biol. Cell.* 100:125-138.
- Khajavi, M., Inoue, K., and Lupski, J.R. 2006. Nonsense-mediated mRNA decay modulates clinical outcome of genetic disease. *Eur. J. Hum. Genet.* 14:1074-1081.
- Kubista, M., Andrade, J.M., Bengtsson, M., Fo-rootan, A., Jonak, J., Lind, K., Sindelka, R., Sjoback, R., Sjogreen, B., Strombom, L., Stahlberg, A., and Zoric, N. 2006. The real-time polymerase chain reaction. *Mol. Aspects Med.* 27:95-125.
- Kunz, J.B., Neu-Yilik, G., Hentze, M.W., Kulozik, A.E., and Gehring, N.H. 2006. Functions of hUpf3a and hUpf3b in nonsense-mediated mRNA decay and translation. *RNA* 12:1015-1022.
- Kuzmiak, H.A. and Maquat, L.E. 2006. Applying nonsense-mediated mRNA decay research to the clinic: Progress and challenges. *Trends Mol. Med.* 12:306-316.
- Larson, D.R., Zenklusen, D., Wu, B., Chao, J.A., and Singer, R.H. 2011. Real-time observation of transcription initiation and elongation on an endogenous yeast gene. *Science* 332:475-478.
- Lee, S.K. and Kumar, P. 2009. Conditional RNAi: Towards a silent gene therapy. *Adv. Drug Deliv. Rev.* 61:650-664.
- Lewis, B.P., Green, R.E., and Brenner, S.E. 2003. Evidence for the widespread coupling of alternative splicing and nonsense-mediated mRNA decay in humans. *Proc. Natl. Acad. Sci. U.S.A.* 100:189-192.
- Liss, B. 2002. Improved quantitative real-time RT-PCR for expression profiling of individual cells. *Nucleic Acids Res.* 30:e89.
- Livak, K.J. 1997. Comparative Ct method. ABI Prism 7700 Sequence Detection System. User Bulletin no. 2. PE Applied Biosystems.
- Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} Method. *Methods* 25:402-408.
- Losson, R. and Lacroute, F. 1979. Interference of nonsense mutations with eukaryotic messenger RNA stability. *Proc. Natl. Acad. Sci. U.S.A.* 76:5134-5137.
- Lykke-Andersen, J., Shu, M.D., and Steitz, J.A. 2000. Human Upf proteins target an mRNA for nonsense-mediated decay when bound downstream of a termination codon. *Cell* 103:1121-1131.
- Lykke-Andersen, J., Shu, M.D., and Steitz, J.A. 2001. Communication of the position of exon-exon junctions to the mRNA surveillance machinery by the protein RNPS1. *Science* 293:1836-1839.
- Mackay, I.M., Arden, K.E., and Nitsche, A. 2002. Real-time PCR in virology. *Nucleic Acids Res.* 30:1292-1305.
- Maquat, L.E. 1995. When cells stop making sense: Effects of nonsense codons on RNA metabolism in vertebrate cells. *RNA* 1:453-465.
- Maquat, L.E., Kinniburgh, A.J., Rachmilewitz, E.A., and Ross, J. 1981. Unstable beta-globin mRNA in mRNA-deficient beta o thalassemia. *Cell* 27:543-553.
- Minshall, N., Thom, G., and Standart, N. 2001. A conserved role of a DEAD box helicase in mRNA masking. *RNA* 7:1728-1742.
- Muller, P.Y., Janovjak, H., Miserez, A.R., and Dobbie, Z. 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. *BioTechniques* 32:1372-1379.
- Nicholson, P., Yepiskoposyan, H., Metzke, S., Zamudio Orozco, R., Kleinschmidt, N., and Muhlemann, O. 2009. Nonsense-mediated mRNA decay in human cells: Mechanistic insights, functions beyond quality control and the double-life of NMD factors. *Cell Mol. Life Sci.* 67:677-700.
- Nott, A., Le Hir, H., and Moore, M.J. 2004. Splicing enhances translation in mammalian cells: An additional function of the exon junction complex. *Genes Dev.* 18:210-222.
- Nowotny, M. and Yang, W. 2009. Structural and functional modules in RNA interference. *Curr. Opin. Struct. Biol.* 19:286-293.
- Ochi, A., Hawley, R.G., Hawley, T., Shulman, M.J., Traunecker, A., Kohler, G., and Hozumi, N. 1983. Functional immunoglobulin M production after transfection of cloned immunoglobulin heavy and light chain genes into lymphoid cells. *Proc. Natl. Acad. Sci. U.S.A.* 80:6351-6355.
- Ohnishi, T., Yamashita, A., Kashima, I., Schell, T., Anders, K.R., Grimson, A., Hachiya, T., Hentze, M.W., Anderson, P., and Ohno, S. 2003. Phosphorylation of hUPF1 induces formation of mRNA surveillance complexes containing hSMG-5 and hSMG-7. *Mol. Cell* 12:1187-1200.
- Paillusson, A., Hirschi, N., Vallan, C., Azzalin, C.M., and Muhlemann, O. 2005. A GFP-based reporter system to monitor nonsense-mediated mRNA decay. *Nucleic Acids Res.* 33:e54.
- Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45.
- Pfaffl, M.W., Tichopad, A., Prgomet, C., and Neuvians, T.P. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* 26:509-515.
- Rasmussen, R., Meuer, S., Wittwer, C., and Nakagawara, K. (eds.) 2001. Quantification on the LightCycler, Springer-Verlag, Berlin. pp. 21-342.
- Rebbapragada, I. and Lykke-Andersen, J. 2009. Execution of nonsense-mediated mRNA decay: What defines a substrate? *Curr. Opin. Cell Biol.* 21:394-402.

- Redon, S., Reichenbach, P., and Lingner, J. 2007. Protein RNA and protein protein interactions mediate association of human EST1A/SMG6 with telomerase. *Nucleic Acids Res.* 35:7011-7022.
- Rehwinkel, J., Raes, J., and Izaurrealde, E. 2006. Nonsense-mediated mRNA decay: Target genes and functional diversification of effectors. *Trends Biochem. Sci.* 31:639-646.
- Ruiz-Echevarria, M.J. and Peltz, S.W. 2000. The RNA binding protein Pub1 modulates the stability of transcripts containing upstream open reading frames. *Cell* 101:741-751.
- Sambrook, J. and Russell, D. 2000. Molecular Cloning: A Laboratory Manual. 3rd Revised edition. Cold Spring Harbor Laboratory, New York.
- Serin, G., Gersappe, A., Black, J.D., Aronoff, R., Maquat, L.E. 2001. Identification and characterization of human orthologues to *Saccharomyces cerevisiae* Upf2 protein and Upf3 protein (*Caenorhabditis elegans* SMG-4). *Mol. Cell Biol.* 21:209-223.
- Silva, A.L., Ribeiro, P., Inacio, A., Liebhaber, S.A., and Romao, L. 2008. Proximity of the poly(A)-binding protein to a premature termination codon inhibits mammalian nonsense-mediated mRNA decay. *RNA* 14:563-576.
- Simpson, D.A., Feeney, S., Boyle, C., and Stitt, A.W. 2000. Retinal VEGF mRNA measured by SYBR green I fluorescence: A versatile approach to quantitative PCR. *Mol. Vision* 6:178-183.
- Singer, R.H., Lawrence, D.S., Ovary, B., and Condeelis, J. 2005. Imaging of gene expression in living cells and tissues. *J. Biomed. Optics* 10:051406.
- Siomi, H. and Siomi, M.C. 2009. On the road to reading the RNA-interference code. *Nature* 457:396-404.
- Stripecke, R., Oliveira, C.C., McCarthy, J.E., and Hentze, M.W. 1994. Proteins binding to 5' untranslated region sites: A general mechanism for translational regulation of mRNAs in human and yeast cells. *Mol. Cell Biol.* 14:5898-5909.
- Sun, X., Perlick, H.A., Dietz, H.C., and Maquat, L.E. 1998. A mutated human homologue to yeast Upf1 protein has a dominant-negative effect on the decay of nonsense-containing mRNAs in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 95:10009-10014.
- Tichopad, A., Didier, A., and Pfaffl, M.W. 2004. Inhibition of real-time RT-PCR quantification due to tissue-specific contaminants. *Mol. Cell Probes* 18:45-50.
- Unterholzner, L. and Izaurrealde, E. 2004. SMG7 acts as a molecular link between mRNA surveillance and mRNA decay. *Mol. Cell* 16:587-596.
- Valegard, K., Murray, J.B., Stockley, P.G., Stonehouse, N.J., and Liljas, L. 1994. Crystal structure of an RNA bacteriophage coat protein-operator complex. *Nature* 371:623-626.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:RESEARCH0034.
- Wang, W. and Malcolm, B.A. 1999. Two-stage PCR protocol allowing introduction of multiple mutations, deletions and insertions using QuikChange Site-Directed Mutagenesis. *BioTechniques* 26:680-682.
- Wilusz, J. 2009. RNA stability: Is it the endo' the world as we know it? *Nat. Struct. Mol. Biol.* 16:9-10.
- Witherell, G.W., Wu, H.N., and Uhlenbeck, O.C. 1990. Cooperative binding of R17 coat protein to RNA. *Biochemistry* 29:11051-11057.
- Wong, M.L. and Medrano, J.F. 2005. Real-time PCR for mRNA quantitation. *BioTechniques* 39:75-85.
- Yamashita, A., Ohnishi, T., Kashima, I., Taya, Y., and Ohno, S. 2001. Human SMG-1, a novel phosphatidylinositol 3-kinase-related protein kinase, associates with components of the mRNA surveillance complex and is involved in the regulation of nonsense-mediated mRNA decay. *Genes Dev.* 15:2215-2228.
- Zheng, L., Baumann, U., and Reymond, J.L. 2004. An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res.* 32:e115.
- Zipper, H., Brunner, H., Bernhagen, J., and Vitzthum, F. 2004. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Res.* 32:e103.

INTERNET RESOURCES

Basic Protocol 1: Creation of PTC+ mRNAs and mutant NMD proteins by site-directed mutagenesis

For designing all types of primers:

<http://www.geneinfinity.org/sp/sp.oligo.html>

For creating mutagenic primers:

<http://www.bioinformatics.org/primerx/>

Codon usage table:

<http://www.my-whiteboard.com/the-human-codon-usage-table/>

Basic Protocol 2: pSUPuro-based RNAi to knock down NMD factors

To design shRNA oligonucleotides:

<http://www.dharmacon.com/designcenter/design-centerpage.aspx>

<http://www.eu.idtdna.com/Scitools/Applications/shRNA/default.aspx>

<http://www.oligoengine.com>

Basic Protocols 4 to 6: Extraction of RNA and analysis of RNA levels by reverse transcription-quantitative PCR

For designing qPCR primers/probes:

<http://www.eu.idtdna.com/scitools/Applications/RealTimePCR/>

<http://www.primer3.sourceforge.net>

To BLAST designed primers/probes against databases to check their specificity:

<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>

A Web site that offers software products and services that are desirable for aiding with qPCR primer/probe design (as well as for many other techniques such as mutagenesis, cloning, PCR, etc.):

<http://www.PremierBiosoft.com>

Software's to help find the best normalizer for your experiment:

geNorm software (Vandesompele et al., 2002)

BestKeeper software (Pfaffl et al., 2004)

Norm-Finder software (Andersen et al., 2004)