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Application of the Invader® RNA Assay to the Polarity of Vertebrate mRNA Decay

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Abstract

The inability of structural elements within a reporter mRNA to impede processive decay by the major 5' and 3' exonucleases has been a major obstacle to understanding mechanisms of vertebrate mRNA decay. We present here a new approach to this problem focused on quantifying the decay of individual portions of a reporter mRNA. Our approach entails two parts. The first involves the use of a regulated promoter, such as one controlled by tetracycline, to allow reporter gene transcription to be turned off when needed. Cells stably expressing the tetracycline repressor protein are transiently or stably transfected with tetracycline-regulated beta-globin genes in which the sequence element under study is cloned into the 3'-UTR. The second involves the quantification of beta-globin mRNA using the Invader RNA assay, a sensitive and quantitative approach that relies on signal amplification instead of target amplification. Because the Invader RNA assay does not depend on downstream primer binding the use of multiple probes across the reporter beta-globin mRNA allows for quantification of the decay of individual portions of the mRNA independent of events acting at other sites.

Keywords

mRNA decay; Invader RNA assay; invasive cleavage assay; transcriptional pulse; transcriptional turn-off

1. Introduction

Measuring the decay rate of a particular mRNA fundamentally involves two steps: turning off reporter gene transcription and quantitation of the decrease over time of its encoded mRNA. In the past, transcriptional inhibitors such as actinomycin D and DRB have been used to stop transcription; more current methods use regulated systems whereby the transcription of the gene of interest can be specifically controlled. Traditionally, mRNA detection has relied on northern blotting, ribonuclease protection assay and RT-PCR. The method that we use combines the inducible tet-repressor system and a novel method for mRNA quantification, the invasive cleavage assay, or Invader RNA assay.

Various methods to measure mRNA decay have been described. One approach is to radioactively label mRNAs and measure the approach to steady state or decay. Other approaches involve using transcriptional inhibitors such as actinomycin D or DRB (5,6-dichloro-1 β -D-ribofuranosylbenzimidazole) to stop transcription and then measure the decay of the mRNA transcript (1). Actinomycin D interferes with transcription by intercalating with

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DNA, and DRB inhibits transcription by inhibiting pTEFb. Both transcriptional inhibitors can have significant effects on normal cell physiology leading to possible artifacts. Most current studies use a regulated promoter such as the serum-inducible c-fos promoter or a promoter flanked upstream by multiple tetracycline operator elements to produce either a pulse of transcription or allow for decay from steady-state by turning off transcription (2). These methods have the advantages of specifically controlling expression of a single gene with minimal effects on normal cell physiology.

Using this system there are several ways to measure reporter mRNA half life. Transcriptional pulsing has been previously described in detail (2). Briefly, this approach involves inducing reporter mRNA expression for a defined period, for example by removing tetracycline from the medium, followed by its re-addition to repress transcription. Provided that the pulse length is shorter than the half life of the mRNA this results in production of a homogeneous population of reporter mRNAs. In situations where the mRNA half-life is very short or the amount of mRNA induced during a pulse of transcription is insufficient for subsequent quantitation this system can be used to study decay from steady state by maintaining cells in medium lacking tetracycline and following decay after its addition.

Methods commonly used to quantify mRNA decay include northern blotting, ribonuclease protection assay (RPA) and RT-PCR with radiolabeled primers or real-time PCR. The advantage of northern blotting is that it tracks decay of the full-length mRNA and as such can provide evidence for deadenylation and in rare cases for endonuclease cleavage. RPA is more sensitive and better for quantifying changes in less abundant mRNAs than northern blotting, and RT-PCR and real-time PCR offer even greater sensitivity and flexibility when dealing with limited amounts of RNA. PCR-based methods require a reverse transcription step that by definition depends on the integrity of a downstream primer binding site, in addition to which PCR can be subject to target amplification artifacts. The Invader RNA assay (Third Wave Technologies, Inc., www.twt.com) offers a new way to quantify mRNA decay (3). This assay is applicable to small quantities of input RNA, quantitative, highly sensitive and free from potential artifacts associated with PCR target amplification.

The Invader RNA assay consists of two isothermal reactions run in the same well of a microtiter plate. In the primary reaction, three oligonucleotides (a stacking oligo, a primary probe oligo and an Invader oligo) bind adjacent to one another at a specific locus on the target mRNA (Fig. 1). The primary probe oligonucleotide consists of two portions, one which binds the mRNA, and a second which is non-complementary to the target mRNA and forms a 5' flap. The Invader oligo binds the target mRNA immediately downstream of the primary probe. The nucleotide located at the 5' end of the Invader oligo does not bind the mRNA target or the primary probe, but instead overlaps the junction of the primary probe and the target mRNA. This pushes on the 5'-most bond between the primary probe and the target mRNA to create a bulged secondary structure. The primary probe oligo is cleaved by a modified thermostable form of tTh polymerase termed Cleavase® to release the 5' flap. The stacking oligo serves to modulate the T_m of the primary probe to 60°C, so that at the reaction temperature of 60°C, the primary probe oligo freely cycles on and off the target mRNA while the Invader oligo, which has a higher T_m, remains bound. Thus, multiple primary probes are cleaved per target, and the number of free flaps produced in this reaction is a product of the length of incubation and target abundance. In the secondary reaction, three additional oligos are added: an Arrestor oligo, a FRET-labeled secondary probe oligo (containing both a fluorophore and a quencher molecule) and a Secondary Reaction Template (SRT) oligo. The cleaved flap of the primary probe from the primary reaction acts as an Invader oligo in conjunction with the SRT and the FRET-labeled secondary probe oligo in another cleavage reaction in which the FRET-labeled secondary probe oligo is cleaved to separate the fluorophore on the flap from the quencher molecule. As in the primary reaction, the cleaved 5' probe flap from the primary probe remains bound to the SRT

while the FRET-labeled oligo cycles on and off, resulting in linear fluorescence signal generation. The Arrestor oligo binds uncleaved probe oligo from the primary reaction. This assay can be run in biplex format to allow the simultaneous detection of two targets from any one sample. This is helpful when using internal controls. Here we describe a system based on the common reporter, human β -globin mRNA, that takes advantage of the unique properties of the Invader RNA assay to study the polarity of mRNA decay.

2. Materials

2.1. In vitro transcript generation

1. pG-glo-PLEB-cDNA full length-pA14, a plasmid containing human beta-globin cDNA downstream of the SP6 promoter. After linearizing with SstII transcription with SP6 polymerase generates full-length β -globin mRNA with a 14 residue poly (A) tail.
2. SstII restriction enzyme to linearize plasmid.
3. PCR clean up kit (Qiagen).
4. Megascript kit (Ambion).
5. DNase I
6. Phenol/chloroform/isoamyl alcohol (25:24:1). Caution this is corrosive and is a hazard to eyes, skin, upon ingestion and inhalation. Avoid contact with this and wear gloves, lab coat and protective eyewear. Light sensitive.
7. Isopropanol.
8. DEPC (diethylpyrocarbonate)-treated water. Caution, DEPC is irritating to eyes, skin and mucus membranes. It is a suspected carcinogen. Light sensitive. Open DEPC only in fume hood, wear gloves. See Note 1.
9. Reagents for agarose gel electrophoresis.
10. Ethidium bromide. Caution, possible carcinogen. Avoid inhalation or skin contact. Light sensitive.

2.2. Cell culture

1. LM(tk- tTA) cells, murine fibroblasts stably expressing the tTA protein for Tet off regulation of reporter gene.
2. pTet-CMV_{min}-glo-SPA, pTet-CMV_{min}-glo-ARE[*cfos*]-SPA, plasmids containing beta-globin reporter genes with or without the cfos ARE in the 3' UTR, respectively, under control of tetracycline-responsive promoter (see Note 2).
3. Transfection control plasmids, constitutively expressing a second reporter mRNA such as β -galactosidase or luciferase.
4. Cell culture media. LM (tk- tTA) cells are grown in 90% Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine.
5. Transfection reagent, Superfect (Invitrogen) for plasmid transfection; Dreamfect (OZ Biosciences) for siRNA transfection.
6. Tetracycline or doxycycline, at 1 mg/ml in 70% ethanol, stored at -20°C . Tetracycline stored in this manner is stable two weeks, doxycycline for 4 weeks. Both are light sensitive.

7. Cytoplasmic isolation buffer:
 - 0.14 M NaCl
 - 1.5 mM MgCl₂
 - 0.01 M Tris-HCl pH 8.0
 - 0.0025% NP20
 - 0.01 M DTT (dithiothreitol)
 - RNase OUT (Invitrogen)
 - DEPC-treated water to desired volume.Add DTT and RNase OUT just prior to use. Store buffer at 4°C.
8. Kit for RNA purification such as TRIzol (Invitrogen), Absolutely RNA RTPCR miniprep kit (Stratagene), RNA miniprep kit (Qiagen).

2.3. RNA Invader assay

1. Invader RNA assay generic kit (Third Wave Technologies, Madison, WI).
2. Primary oligonucleotide mix (see Table 1 for specific sequences):
 - Primary probe oligonucleotide, 40 µM.
 - Invader oligonucleotide, 20 µM.
 - Stacking oligonucleotide, 12 µM.
 - made in Te buffer to desired volume.
3. Secondary oligonucleotide mix (see Table 1 for specific sequences):
 - Arrestor oligonucleotide at 53.4 µM in Te buffer.
4. FRET-labeled oligonucleotide mix:
 - Secondary reaction template, 2.0 µM
 - Fluorescent oligonucleotide, 13.4 µM
5. Te buffer (EDTA inhibits the Invader RNA assay): 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA.
6. DEPC-treated water.
7. tRNA, diluted to 20 ng/ml in DEPC-treated water.
8. Stop buffer (optional): 10 mM Tris-HCl pH 8.0, 10 mM EDTA.
9. 0.2 ml thin walled strip tubes with individually attached caps (USA Scientific, Enfield, CT).
10. 96-well polypropylene round-bottom microplates.
11. Chill-Out 14 liquid wax (MJ Research, San Francisco, CA)
12. Thermal adhesive sealing film for PCR (Fisher).
13. 8-channel 0.1–10 µl multichannel pipettor and tips.
14. 0.1–100 µl electronic repeat pipettor and tips.
15. Hybridization oven or 96-well format thermocycler.

16. Fluorescence plate reader with appropriate filters attached to a computer with Magellan™ and Microsoft Excel™ software. We use the Tecan Genios fluorescence plate reader.

3. Methods

3.1. Primary reaction oligonucleotide design, synthesis and preparation

1. For a description of the functions of each oligonucleotide, see Note 3.
2. Choose the region for quantification on the target mRNA of interest. For this study we developed probes targeting each of the 3 exons of human β -globin mRNA (Table 1).
3. Using a sequence of the top strand, in the 5'-3' direction of the spliced and cleaved mRNA, pick a location where the cleavage site (the site of secondary structure) will be located (see Note 4).
4. Determine if the surrounding ~50 nt region is likely fall in a highly structured region of the mRNA (see Note 5).
5. Determine the length of sequence each oligonucleotide needs to cover in order to get the appropriate T_m of 60°C or 80°C, as oligo sequences are strictly a function of T_m (see Note 6).
6. Check that the resulting oligos are free of common pitfalls:
 - Length is too short to be sequence-specific. This can happen in GC-rich regions. Primer/dimer formation.
 - Secondary structure within the oligo, eg. runs of 4 or more Gs.
 - 2 base invasion: the nucleotide upstream of the cleavage nucleotide in the probe (located in the flap region) must not bind to the target molecule.
 - G as cleavage site in probe, as for some reason it is bad. Try to get this to be a C (would be a G in the mRNA sequence).
7. Add a flap sequence to the 5' end of the probe molecule (see Note 7).
8. Synthesize oligos at 1 μ M scale, be sure to use HPLC purification (see Note 8).
9. Probe and Invader oligos are DNA oligos; the stacking oligo is an RNA oligo. The probe oligo has a 3' amino modifier C7, the stacking oligo is 2' O-methylated throughout (see Note 9).
10. To prepare oligo mixes, dissolve oligos in 300 μ l Te and measure a 1:200 dilution at A_{260} . Dilute oligos in Te to make primary oligo mix:
 - Primary probe oligonucleotide, 40 μ M.
 - Invader oligonucleotide, 20 μ M.
 - Stacking oligonucleotide, 12 μ M.

3.2. Secondary reaction oligonucleotide design, synthesis and preparation

1. For a description of the functions of each oligonucleotide, see Note 10.
2. Design an arrestor oligo to be complementary to the uncleaved primary probe oligo. This is an RNA oligo and should be 2'-O methylated throughout, and purified by desalting.

3. Design a secondary probe oligo. For published secondary probe sequences see references (3,5). This is a DNA oligo containing a fluorescent tag on the 3' end and a quencher molecule at the third position. This oligo should be HPLC purified.
4. Design the SRT. This oligo should hybridize with both the secondary probe oligo and the primary probe flap oligo to create the secondary structure recognized by Cleavase as a cleavage substrate. The 3'-most 3–5 nucleotides of this DNA oligo should be 2'-O methylated.

3.3. In vitro transcript generation

1. For a description of the in vitro transcript, see Note 11.
2. Linearize plasmid template by restriction digestion.
3. Check linearization by running a portion of the product on an agarose gel and staining with ethidium bromide prior to UV visualization. This must be gel purified if more than one band is seen.
4. Clean up linearized plasmid. We use the PCR Clean up kit (Qiagen) according to manufacturer's directions.
5. Perform transcription. We use the Megascript kit (Ambion). Each transcription reaction is composed of 5 µl plasmid (1 µg), 2 µl 10× RNA polymerase buffer, 2 µl each ATP, CTP, GTP or UTP solution, 2 µl RNA polymerase and water to 20 µl. Incubate overnight at 37°C.
6. Remove plasmid template from in vitro transcript by adding 1 µl DNase I and incubating for 15 min at 37°C.
7. Extract with phenol/chloroform/isoamyl alcohol. First bring transcription reaction up to 100 µl volume with DEPC-treated water, then add 100 µl phenol/chloroform/isoamyl alcohol.
8. Precipitate with isopropanol.
9. Dissolve pellet in 50 µl DEPC-treated water.
10. Check integrity of in vitro transcript by running on agarose gel and staining with ethidium bromide prior to UV visualization. If more than one band is observed or if there are shorter products detected the transcript must be gel purified prior to use.
11. Quantify by measuring at A₂₆₀.
12. Dilute in vitro transcript to 100 amol/µl. Store aliquots at –80°C.

3.4. Transcription pulse assay

1. For general considerations of experimental design, see Notes 12 and 13.
2. On the first day, plate cells in normal growth medium plus 50–100 ng/ml tetracycline or doxycycline to repress reporter gene expression (see Notes 14, 15 and 16).
3. To induce reporter gene expression, remove the growth medium and wash cells with PBS.
4. Replace with medium without tet or dox and grow cells for about 4 hours (see Note 17).
5. To shut off reporter gene expression add tet or dox back to the medium at 1000 ng/ml.

6. Cells may be removed from the incubator at desired timepoints following tet or dox readdition for protein or RNA isolation.

3.5. Transcription turn off assay

1. For general considerations of experimental design, see Notes 12 and 13.
2. Plate cells in normal growth medium including antibiotics. Omit tetracycline or doxycycline to permit reporter gene expression to steady state.
3. At desired time, repress reporter gene expression by the addition of tetracycline or doxycycline to 1000 ng/ml.
4. Cells may be removed from the incubator at desired timepoints following tet or dox addition for protein or RNA isolation.

3.6. RNA isolation

1. Remove growth medium from cells and wash plate twice with PBS.
2. Add 1 ml PBS to each plate and use a cell scraper to suspend the cells. The suspended cell solution is transferred to a 1.7 ml microcentrifuge tube.
3. Pellet cells by centrifuging for 1 min at 1000×g, 4°C.
4. Remove the supernatant and resuspend the cells in 200 µl of cytoplasmic extraction buffer.
5. Incubate cells on ice for 10 min.
6. Remove the nuclei by centrifuging the lysed cell suspension for 5.5 min at 12,000×g, 4°C.
7. Remove the supernatant containing cytoplasmic extract to a fresh tube. Cytoplasmic extract and the nuclear pellets can be stored frozen at −80°C until further use at this point.
8. Purify RNA from cytoplasmic or nuclear fractions using RNA isolation protocol of choice.
9. Quantify RNA yield by measuring A₂₆₀ in a spectrophotometer.

3.7. Invader RNA assay

1. See Notes 18–20 for information on experimental design.
2. Dilute in vitro transcript in tRNA at 20 ng/ml for use as standard curve (see Notes 21–23). Be sure to thoroughly vortex and spin down each sample between dilutions.
3. Transfer each standard to one tube in a 8-well tube strip. Store on ice.
4. Prepare samples by diluting RNA isolated from transcription pulse- or transcription turn off-treated cells in water or in 20 ng/ml tRNA (see Notes 24–26).
5. Thoroughly vortex each sample, briefly centrifuge to collect the contents and transfer each sample to a well of an 8-well tube strip. Store on ice.
6. Prepare primary reaction mix by combining (for each well) 4 µl RNA Primary Buffer, 0.25 µl beta-globin primary oligo mix, 0.25 µl GAPDH primary oligo mix (if using, otherwise add Te), and 0.5 µl Cleavase enzyme (see Note 27). Add Cleavase enzyme last. This is then mixed on a vortex mixer, centrifuged and placed on ice.

7. Prepare secondary reaction mix by combining (for each well) 2 μ l RNA secondary buffer, 0.75 μ l β -globin secondary oligo, 0.75 μ l FAM oligo and 1.5 μ l GAPDH secondary oligo (if the experiment uses this as an internal control; otherwise add Te). Vortex the mixture, centrifuge to collect and transfer into an 8-tube strip. Place on ice (see note 28).
8. Load 96-well round-bottom plate with 5 μ l primary reaction buffer per well using the electronic repeat pipettor.
9. Add 5 μ l standards or 5 μ l sample RNA to the plate with 8-channel micropipettor.
10. Overlay each reaction with 10 μ l Chill-Out wax using electronic repeat pipettor.
11. Cover plate with adhesive film.
12. Incubate at 60°C for 90 min in a thermocycler with 96-well plate format heating element or in a hybridization oven.
13. After 90 min, remove plate (if using hybridization oven), remove adhesive film and add 5 μ l secondary buffer mix to each sample with 8-channel micropipettor.
14. Incubate again 60°C for 60 min, or for up to 90 min, depending on signal strength desired.
15. Remove plate, remove adhesive film and add 10 μ l stop solution (optional).
16. Read plate in fluorescence plate reader immediately, using Magellan II software. For preliminary settings set: gain=50, # flashes=10, lag time =0 μ sec, integration time=50 μ sec, plate definition file=NUN96ft.pdf. See Note 29.

3.8. Data analysis, standard curves

1. Export data to Excel.
2. For each set of triplicates or quadruplicate points on the standard curve, calculate mean, standard deviation and percentage coefficient of variation (see Note 30).
3. Subtract the mean value for the 0 amol in vitro transcript/well from each of the other standard curve means to generate net values.
4. For all standard samples >0 amol IVT/well, calculate FOLD>zero by dividing the average gross signal for any particular sample by the average gross signal for 0 amol IVT/well.
5. For all standard samples >0 amol IVT/well, calculate SD>zero by dividing the net signal for any particular sample by the standard deviation of the 0 amol IVT/well sample.
6. For all standard samples > 0 amol IVT/well, calculate a Student's T Test comparing that sample to zero. This gives the confidence that the value is different than zero. Use the TTest function in Excel and include all 4 zero signal values and all 4 sample signal values. Select 2 tails, type 1.
7. For all standard samples, calculate 95% confidence values. Use the Confidence function in Excel, choose alpha = 0.05, size as 4 (quadruplicates) or 3 (triplicates), and the worksheet cell containing the standard deviation of the sample with which you are working.
8. For all samples >0.01 amol calculate a Student's T Test to compare it to the next lowest standard point. Use the TTest function in Excel as above, but select the 4 signal

values for the sample being analyzed, and the 4 signal values for the sample below it. Other selections remain the same.

9. Plot the standard curve of net counts vs. amol IVT/well using linear scale (not logarithmic). Examples of a standard curve showing a linear relationship between input RNA and output of relative light units is shown in Fig. 2. Figs. 3 and 4 provide examples of Invader sets that do not yield any light output or show a high background. Neither of these sets can be used for quantifying mRNA. Discard low points that have either TTest scores above 0.05 or FOLD>zero scores less than 1.15. The lowest value that can be plotted is the limit of detection (LOD) of the assay. Discard high points that are plateaued. The linear range of the assay is the range of values between the lowest and highest plotted points. Fit with a linear trendline ($y=mx+b$) and calculate the R^2 value.

3.9. Data analysis, mRNA decay

1. Use only signal values that fall within the linear range of the standard curves run simultaneously with the experiment.
2. Subtract the 0 amol IVT/well signal from the standard curve to calculate net signal values for all experimental values.
3. For biplex assays, divide the reporter gene signal in each well by the cotransfected control signal. This step is omitted when assaying a single mRNA in each reaction.
4. Calculate the mean and standard deviation for each set of quadruplicates or triplicates.
5. Using time=0 hours as 100%, calculate percent remaining. Percent remaining = $[(\text{signal of sample at time=x})/(\text{signal of sample at time=0})] \times 100$.
6. Plot as percent remaining (y axis, logarithmic) vs. time (x axis, linear).
7. Fit the line and calculate the half life.

3.10. Data analysis, calculating amols target per well

1. For each replicate in a set of triplicate or quadruplicate samples, plug the net RLU value into the equation describing the standard curve to get amol/well.
2. Calculate mean and standard deviation for each set of triplicates or quadruplicates.

4. Notes

1. All steps should be performed in a manner that is consistent with preventing RNase contamination and protein degradation. If applicable, treat solutions with DEPC (autoclaving afterward to remove excess DEPC); wipe surfaces with RNaseAway (Invitrogen), wear gloves, use plasticware that is certified RNase-free, and perform manipulations on ice. For all steps use either double-distilled water or water that has been processed through a purification apparatus such as a Millipore system.
2. Alternately, one may co-transfect cells lacking tetracycline repressor with the a tet-regulated plasmid expressing the reporter mRNA and a plasmid expressing the tTA protein.
3. The primary oligonucleotide mix is composed of three oligos: a primary probe oligo, an Invader oligo and a Stacking oligo. The primary probe oligo is composed of two regions, an assay specific region (ASR) and a flap region. The ASR is ≥ 10 bases in length to ensure specificity, and anneals to the target mRNA. The flap region does not hybridize to the target mRNA and is a synthetic sequence designed only for use

in the assay. The Stacking oligo binds the target mRNA at the 3' end of the probe ASR and stacks coaxially with it. The function of the Stacking oligo is to improve assay performance by increasing the T_m of the primary probe, and thus the assay reaction temperature. Together, the primary probe and Stacking oligo should have a T_m of 60°C. The T_m may be slightly higher, but a T_m of $\geq 65^\circ\text{C}$ is detrimental to the assay performance. Because the T_m of the primary probe oligo is the temperature at which the assay is run, the primary probe oligo cycles on and off the target freely during the reaction. The Invader oligo binds the target mRNA immediately downstream (on the mRNA) from the primary probe oligo. The 3' nucleotide of the Invader oligo does not hybridize with mRNA target or another oligo, but instead through steric effects, pushes on the 5'-most bond between the primary probe oligo and the mRNA target. This creates a unique bulged secondary structure in the primary probe oligo which is recognized as a cleavage substrate by the Cleavase enzyme. The Cleavase enzyme then cuts the probe molecule to release the flap region from the ASR. The T_m of the Invader oligo is $\sim 80^\circ\text{C}$ to ensure that it is stably bound to the mRNA target throughout the assay. Because the difference in T_m between the stacking and probe oligos and the Invader oligo is key to the success of the assay, the difference between them should always be $\sim 20^\circ\text{C}$.

4. This is not typically a critical location, but can be strategically chosen to quantify splice variants, spliced vs. unspliced mRNAs or SNPs if desired.
5. Methods to determine mRNA secondary and tertiary structure include computer modeling and RT-ROL (4), an experimental method. It might be necessary to design several possible sites for analysis and test each.
6. The T_m of the probe set is key to success with the Invader assay. A number of programs are available to determine the T_m of the hybrids, and this must be taken into account when determining the length or locations of particular oligonucleotides, or when either of these parameters are changed. Remember the combined T_m of the Stacking oligo and the primary probe oligo should be 60°C.
7. For published probe flap sequences, see references 3 and 5.
8. High purity oligonucleotides are essential for good results.
9. We have had success using oligonucleotides from IDT.
10. In the primary reaction, the Cleavase enzyme recognizes a distinct bulged secondary structure caused by the simultaneous hybridization of Stacking, primary probe and Invader oligos with the target mRNA. This recognition of the secondary structure leads to cleavage by Cleavase of the primary probe to release the flap portion. In the secondary reaction, the primary probe flap released in the first reaction becomes the Invader oligo in the formation of another secondary structure that serves to amplify the signal and couple cleavage with fluorescence production. The secondary reaction is performed by adding secondary oligos to the primary reaction. An Arrestor oligo is added to bind up uncleaved primary probe oligo. A secondary probe molecule, containing a fluorophore molecule and a quencher molecule, is added to bind a Synthetic Reaction Template which simultaneously binds the primary probe flap to form the bulged structure. Cleavage of the secondary probe liberates the fluorophore from the quencher molecule and the resulting free fluorophore can be determined by reading in a fluorescence plate reader.
11. The in vitro transcript serves as a control for the assay. It is used in optimizing the assay and in standard curves that must always be performed when running the assay. The in vitro transcript ideally spans the entire length of the mature mRNA, and

includes a poly(A) tail. The length of the tail may be short so that the concentration of the in vitro transcript may be accurately determined.

12. Transcriptional pulse assay vs. transcriptional turn off assay. The transcriptional pulse assay has the advantage of generating a homogeneous population of transcripts which should decay in a homogeneous fashion. However, expression of the reporter gene may be higher in cells subjected to only transcriptional turn off. This may make it much easier to analyze decay in transcription turn off cells.
13. Transient vs. stable cells. Of course, cells must be stably transfected with tTA, but whether or not to construct stable cells that also express the reporter gene is another question. Although transiently transfected cells typically give higher expression of the gene of interest, stably transfected cells have a more consistent level of expression due to a lower copy number per cell. This may make it easier to analyze the effect of overexpression of a regulatory protein on decay, or to analyze the effect of knocking out a protein with siRNA on decay.
14. The level of tet or dox required to repress gene expression may be empirically determined via luciferase assay (using a tet-regulated luciferase reporter plasmid), northern, or RNase protection assay (perhaps using a specific tet-regulated reporter plasmid).
15. Tet has a half life of 12 hours in medium; dox has a 24 hour half life. If cells are grown for a few days, it will be necessary to add more antibiotic to maintain proper gene repression.
16. If using transiently transfected cells care should be taken to determine the number of cells that will be needed to reach the desired degree of confluency at the times cells are harvested. On the day of cell harvest the cells should nearly cover the plate but still be in log phase growth.
17. The length of the pulse may also be empirically determined as above. If it is too long, a significant portion of the target mRNA may be undergoing decay, thus complicating interpretation of the data.
18. Assay design: A standard curve is prepared from an in vitro transcript is required for each target mRNA. This is particularly important in establishing the linear range of the assay, which can vary between experiments even for the same oligonucleotides and target.
19. To facilitate statistical analysis of the data the assay should be run at least in triplicate, and preferentially in quadruplicate.
20. When using cells transiently transfected with the reporter gene, be sure to include a cotransfection control as well as loading control. The loading control is accomplished by doing the Invader RNA assay in biplex format, that is, assaying for the presence of two RNA targets in each sample, the reporter gene and a housekeeping gene (beta-actin, GAPDH). Due to the lack of availability of oligonucleotides for popular cotransfected controls such as luciferase, beta-galactosidase or green fluorescent protein, cotransfected controls must often be accomplished via an alternative method of RNA quantification such as northern blotting or RNase protection assay. It is not necessary to include a cotransfected control if using a cell line stably transfected with the reporter gene.
21. A typical standard curve includes in vitro transcript values of 0, 0.01, 0.02, 0.04, 0.16, 0.64, 2.5, 5.0, 10.0 and 20.0 amol in vitro transcript per well. Because the volume of each diluted in vitro transcript added to each well is always 5 μ l, the in vitro transcripts must be diluted to 1/5 of what will be in the well. For example, to add 5.0 amol in

vitro transcript, the in vitro transcript must be diluted to 1.0 amol/ μ l. 5 μ l of this is 5.0 amol in the well.

22. In vitro transcripts must be diluted in 20 ng/ μ l tRNA due to the very low concentration of the transcripts and the risk of significant sample loss.
23. Each standard will be measured in triplicate or quadruplicate, so prepare at least 15–20 μ l of each standard. Allow extra volume for pipetting error.
24. RNA samples should be diluted in water of the expression if the target gene is expected to be low such that a large amount of total cellular RNA will be added to each well (50–100 ng). RNA samples should be diluted in tRNA if the expression of the gene of interest is expected to be high such that a small amount of cellular RNA will be added to each well ($\leq$ 50 ng). It is not advisable to use more than 100 ng cellular RNA per well.
25. The volume of cellular RNA that will be added to each well is always 5 μ l. Therefore, cellular RNA must be diluted to a concentration 1/5 of the amount that will be added to each well. For example, to add 30 ng of cellular RNA to a well, the cellular RNA will be diluted to a concentration of 6 ng/ μ l, such that the addition of 5 μ l to a well gives 30 ng of sample RNA in the well.
26. Remember that each sample will be read in triplicate or quadruplicate, so prepare at least 15–20 μ l of each sample. Allow extra volume for pipetting error.
27. The volume of primary buffer mix to prepare depends upon the number of wells in the assay plus extra for pipetting error. If preparing a small number of wells ($\leq$ 20), use 20% extra mix volume. If preparing a large number of wells ($\sim$ 100 wells) it is sufficient to prepare enough for 4 extra wells. It is not usually necessary to prepare as large a volume as suggested in the assay manual.
28. This can be prepared before the assay or during the first incubation.
29. Adjust gain so that the “0 amol/well” sample of the standard curve has a value of approximately 100.
30. Percent coefficient of variation = [(standard deviation)/(average raw signal)] $\times$ 100. This value should be under 10%, preferably under 5%. This is a measure of the variation between replicates. The presence of an outlier can cause this value to be very high ($\geq$ 10%).

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Fig. 1.

Outline of the Invader RNA assay. The steps involved in the Invader RNA assay are shown. The stacking oligo abuts the probe oligo on the target mRNA and the invader oligo overlaps the junction of the probe oligo and the flap by 1 nucleotide. Cleavase enzyme cuts within the distorted structure formed by this complex to generate flaps in direct proportion to the concentration of target mRNA. These serve as the invader in a second reaction in which an oligo bearing a fluorescent tag and a quencher are separated by the cleavage site in a complex assembled on a generic second reaction template (SRT). Fluorescence is generated in direct proportion to the concentration of flaps generated in the first reaction.

Fig. 2. Standard curve for an optimal Invader set. This is a standard curve for the Invader set directed against exon 1 of human β -globin mRNA that is described in this chapter. Note that the line goes through zero and shows a linear increase in light output with increasing amount of *in vitro* transcript.

Fig. 3.

Standard curve for an Invader set that shows no relationship to input RNA. The Invader set examined in this standard curve lies just upstream of the exon 1 set shown in Fig. 2. However no change in light output is seen for this probe set when increasing amounts of the same RNA are added to the reaction.

Fig. 4.

Standard curve for an Invader set that does not go through zero. This is a standard curve for an Invader set in which light output is recorded with no input RNA. Although the amount of light detected increases with increasing transcript this cannot be used.

Table 1

Oligonucleotides used in the Invader RNA assay.

Probe set	Oligonucleotide	Sequence (5' to 3')
exon 1	Probe	aacgagcgcac CT CATCCAC-NH2
	Invader oligo	CAGGGCCTCACCACAAA
	Stacking oligo	<u>GUUCACCUUGCC</u>
	Arrestor oligo	<u>GUGGAUGAAGGUGCGC</u>
	FRET oligo	(6-FAM)-CAC-(EQ)-TGCTTCGTGG
	SRT	CCAGGAAGCAAGTGGTGCGCCTCGU <u>UAA</u>
exon 2	Probe	aacgagcgcac CTAA AGGCACC-NH2
	Invader oligo	GGTGAGCCAGGCCATCAA
	Stacking oligo	<u>GAGCACUUUCUUGC</u>
	Arrestor oligo	<u>GGUGCCUUUAGGUGCGC</u>
	FRET oligo	(6-FAM)-CAC-(EQ)-TGCTTCGTGG
	SRT	CCAGGAAGCAAGTGGTGCGCCTCGU <u>UAA</u>
exon 3	Probe	aacgagcgcac ACC AGCACG-NH2
	Invader oligo	GTGATGGGCCAGCACACAGC
	Stacking oligo	<u>UUGCCCAGGAG</u>
	Arrestor oligo	<u>CGUGCUGGUGUGCGC</u>
	FRET oligo	(6-FAM)-CAC-(EQ)-TGCTTCGTGG
	SRT	CCAGGAAGCAAGTGGTGCGCCTCGU <u>UAA</u>

Lowercase bases indicate 5' flap sequences. Cleavage base indicated by **boldface**. Underlined bases indicate 2' O-methylated nucleotides. A 3' NH2 was included to enhance assay performance.