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Assays for determining poly(A) tail length and the polarity of mRNA decay in mammalian cells

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Abstract

This chapter describes several methods for measuring the length of the mRNA poly(A) tail and a novel method for measuring mRNA decay. Three methods for measuring the length of a poly(A) tail are presented: the poly(A) length assay, the ligation-mediated poly(A) test (LM-PAT) and the RNase H assay. The first two methods are PCR-based assays involving cDNA synthesis from an oligo(dT) primer. The third method involves removing the poly(A) tail from the mRNA of interest. A major obstacle to studying the enzymatic step of mammalian mRNA decay has been the inability to capture mRNA decay intermediates with structural impediments such as the poly(G) tract used in yeast. To overcome this we combined a standard kinetic analysis of mRNA decay using a tetracycline repressor-controlled reporter with an Invader® RNA assay. The Invader RNA assay is a simple, elegant assay for the quantification of mRNA. It is based on signal amplification, not target amplification, so it is less prone to artifacts than other methods for nucleic acid quantification. It is also very sensitive, able to detect attomolar levels of target mRNA. Finally, it requires only a short sequence for target recognition and quantitation, therefore it can be applied to determining the decay polarity of a mRNA by measuring the decay rates of different portions of that mRNA.

I. Introduction: Poly(A) Tail Length Assays

A. Poly(A) Length Assay

This is a straightforward and fast assay which can be completed in a day. The first step involves the synthesis of cDNA from the RNA sample using an oligo(dT) primer. The next step is to perform PCR on the cDNA using an end-labeled target mRNA-specific primer. Following PCR, samples are resolved on a polyacrylamide gel. mRNAs with short tails yield a compact band while mRNAs with long tails yield PCR products of a variety of lengths which appear as a smear on the gel. Enough cDNA is produced for the PCR step to be repeated a number of times.

1. Materials for the Poly(A) Length Assay—Two *oligonucleotides*: An oligo(dT) primer/adapter (5′-GGGGATCCGCGGTTTTTTTTTT) and a mRNA-specific primer. The mRNA-specific primer should be located upstream of the polyadenylation site at a location that will give a convenient size for resolution on a 6% polyacrylamide gel, approximately 100–300 bases.

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M-MLV reverse transcriptase (Invitrogen)

T4 polynucleotide kinase (Roche)

Standard molecular biology reagents and equipment (heat block, thermocycler, PCR reagents, equipment for polyacrylamide gel electrophoresis)

2. Methods for the Poly(A) Length Assay

RNA preparation: RNA may be purified from cells using any number of techniques. However, it is important that contaminating DNA is removed from the sample prior to cDNA synthesis and that the final RNA sample is sufficiently concentrated. In our lab, we typically isolate RNA using TRIzol (Invitrogen), followed by treatment with DNase, purification by organic extraction and precipitation with isopropanol.

A 60 mm dish of cells should give enough RNA to repeat the poly(A) assay several times. Prior to harvesting the cells, wash the plate 2 or 3 times with PBS. Then add 1 ml TRIzol and incubate the plate on a shaker or rocker for 5 min at room temperature to lyse the cells directly on the plate. Transfer the TRIzol to a microcentrifuge tube and continue to purify the RNA according to the manufacturer's directions. Glycogen may be added during the isopropanol precipitation step to improve the yield of the RNA precipitation. Dissolve the RNA pellet in RNase-free water and quantitate it by measuring the absorbance at 260 nm. Next, RNA preparations should be treated with DNase to remove any contaminating DNA. To do this, bring the volume of the RNA preparation to 300 μl and add 30 μl 10x DNase reaction buffer and 10 μl RQ DNase (RNase-free, Promega) then incubate at 37°C for 15 min. After incubation, purify the RNA by adding 30 μl 5 M ammonium acetate and 350 μl phenol/chloroform/isoamyl alcohol, followed by vortexing for 10 sec and centrifugation at maximum speed for 5 min to separate the phases. Remove the top layer containing the RNA and place it in a clean microcentrifuge tube. Add an equal volume of chloroform/isoamyl alcohol and repeat the extraction. Following centrifugation, transfer the top layer to a clean tube and precipitate the RNA by adding an equal volume of isopropanol to the RNA. Place the tube at −20°C for at least 15 min and then pellet the RNA by centrifuging it at maximum speed for 20 min. Following centrifugation, remove the supernatant and discard it. Wash the pellet by adding 1 ml of 70% ethanol to the tube, briefly vortexing and then centrifuging again at maximum speed for 5 min. After centrifugation, remove the supernatant from the pellet and discard. Centrifuge the tube briefly to collect any remaining ethanol from the sides of the tube and then remove it by pipetting. Briefly air dry the pellet and then dissolve it in RNase-free water. The typical RNA recovery from this treatment is 60%, so dissolve the pellet in a volume of water to yield a final concentration greater than 1 μg RNA per μl and then quantitate the RNA sample.

cDNA synthesis: In this step, the oligo(dT) primer/adapter is annealed to polyadenylated mRNAs and then extended in a cDNA synthesis reaction. Those RNAs with short poly(A) tails will have a limited number of sites for oligo(dT) binding and will give cDNA products of a uniform size. Those RNAs with long poly(A) tails will have multiple sites for binding the oligo (dT) primer/adapter and will give cDNA products of heterogeneous size. For this assay, we have found that the M-MLV reverse transcriptase gives satisfactory results. However, engineered reverse transcriptases (ie. Superscript RT) may give better results in some circumstances, therefore this is a factor to consider when optimizing the assay.

To synthesize the cDNA, first denature the RNA and anneal the oligo(dT) primer/adapter to the sample. Combine 10 μg (or more) RNA and 300 ng oligo(dT) primer/adapter in RNAse-free water to a volume of 16.5 μl and heat to 85°C for 5 min and then cool quickly on ice. For the reverse transcription reaction, add 6 μl 5x first strand buffer, 3 μl 0.1 M DTT, 2 μl 10 mM dNTPs, 0.5 μl RNase Inhibitor (Invitrogen) and 2 μl M-MLV reverse transcriptase (Invitrogen)

and incubate at 42°C for 1.5 hours. Following the incubation, inactivate the reverse transcriptase by heating the reaction to 85°C for 10 min. The cDNA may be used immediately as the template for PCR or stored at −20°C.

Second primer end-labeling: In this step, the upstream, target-specific primer for PCR is 5′ end labeled with γ-^{32}P- ATP by T4 polynucleotide kinase (PNK). The primer should be located about 100 nt upstream of the polyadenylation site so that the PCR products will be between 100 nt and 300 nt, a size easily resolved on a 6% polyacrylamide gel. Primer labeling can be performed during the cDNA synthesis; it is convenient to label the size marker at this time also.

To perform the end-labeling reaction, combine 250 ng primer, 2.5 μl 10x polynucleotide kinase buffer (supplied with the enzyme), 3 μl γ-^{32}P-ATP, 1 μl T4 PNK (Roche) and RNase-free water to 25 μl and incubate the reaction at 37°C for 15 to 60 min. Following incubation, heat inactivate the PNK by incubating the reaction at 65°C for 5 min. The labeled oligo may be purified on a G25 column or with a silica spin-column (ie. Qiagen Nucleotide Removal Kit), but purification is not required as any unincorporated γ-^{32}P-ATP will run far ahead of the PCR products on a gel and not interfere with their visualization. The labeled oligo may be used immediately for PCR or stored at −80°C.

PCR: The cDNA is used as a template for amplification by a PCR reaction with the 5′ end labeled target-specific primer and any unincorporated oligo(dT) primer/adapter. As noted above, cDNAs from mRNAs with short poly(A) tails will give PCR products of a more uniform size due to the limited sites for oligo(dT) priming whereas cDNAs from mRNAs with long heterogeneous tails will yield PCR products with heterogeneous sizes due to the availability of multiple sites for oligo(dT) priming. An example of this is shown in Fig. 1A, which uses this assay to show the impact of a poly(A)-limiting element (PLE, Das Gupta et al., 1998) on the length of the poly(A) tail on luciferase mRNA in transfected murine fibroblasts. This PCR is performed in hot-start tubes and is the step in the poly(A) assay that requires the most optimization. Some important variables to consider include the location of the target mRNA-specific oligonucleotide, the amount of cDNA template in the reaction, the concentration of the $MgCl_2$ in the reaction, the kind of Taq DNA polymerase in the reaction, the annealing temperature of the cycling reaction, the length of the extension time in the cycling reaction, and the number of cycles of the PCR reaction. When optimizing the PCR reaction, it is helpful to have in vitro transcripts of the target mRNA with A20 and A100 tails to serve as positive controls for short and long tails, respectively.

The following protocol serves as a good starting point. In a hot-start tube, combine 3 μl cDNA, 2.5 μl 10x PCR buffer, 3 μl 50 mM $MgCl_2$, and 3 μl 1.25 mM dNTPs. Heat the tube at 85–95° C for a few seconds to melt the wax, then put the tube on ice to allow the wax to solidify. After the wax solidifies, add 2 μl 5′-labeled primer, 11 μl RNase-free water and 0.5 μl Taq DNA Polymerase (Invitrogen). Incubate the reaction at 94°C for 2 min, then perform 25 cycles of 94°C for 1 min, 60°C for 30 sec and 72°C for 2 min. Finish the PCR reaction with a final extension at 72°C for 2 min. PCR products may be immediately run on a polyacrylamide gel or stored at −20°C.

Resolution of PCR products by electrophoresis: The final step is to combine 3 μl of each PCR reaction with 3 μl formamide loading buffer (95% formamide, 0.025% xylene cyanol and bromophenol blue, 18 mM EDTA and 0.025% SDS), heat to 95°C for 5 min and resolve on a 6% polyacrylamide gel. For proper resolution of the PCR products, the gel itself should be the length and thickness of a sequencing gel. Pre-run the gel in 1x TBE at 60 W for 30 min before adding the samples, and run the gel at 60 W for about 70 min after the samples are loaded, or until the lower dye front is at the bottom of the gel and the upper dye front is midway down

the gel. Remove one glass plate and transfer the gel to a piece of Whatman paper and dry the gel under heat (80°C) and vacuum for about 70 min. When the gel is dry, expose it to film or a phosphor screen overnight. If one uses a storage phosphor screen and an instrument such as a phosphorimager the graphing function of ImageQuant can be used to obtain a graphical distribution of poly(A) tail lengths that is useful for determining the length distribution of the poly(A) tail (see Fig. 1B). mRNAs with a short tail will give a discrete peak, while a mRNA with a long tail will generate PCR products in a range of sizes that can appear as a smear of bands either with or without several peaks at 25 nucleotide intervals, which correspond in size to the distribution of poly(A)-binding protein.

B. Ligation-mediated poly(A) test (LM-PAT)

This assay differs from the poly(A) length assay in several ways. First, the molar ratio of RNA to oligo(dT) is much lower so that the entire length of each poly(A) tail (with the exception of some nucleotides at the 5′-most and 3′-most ends) is saturated with oligo(dT) primers. Second, the oligo(dT) primer has a 5′ phosphate to allow the ligation of adjacent primers. Third, an additional oligo(dT) primer-adapter at the 5′ end is ligated to the 3′-most oligo(dT) primer. Although there are a few additional steps as compared to the poly(A) length assay, this is still a relatively fast assay, and, like the poly(A) length assay, enough cDNA is synthesized to repeat the PCR analysis several times. This assay should be less biased to short tails than the poly(A) length assay.

1. Materials: LM-PAT assay—

Three *oligonucleotides*; oligo(dT)$_{12–18}$, the oligo(dT) primer/adapter (see **I.A.1**), and a target mRNA-specific primer.

Superscript II reverse transcriptase (Invitrogen)

T4 DNA ligase (Invitrogen)

Standard equipment and reagents for molecular biology (heat blocks, thermocycler, reagents for PCR, equipment for polyacrylamide gel electrophoresis)

2. Methods: LM-PAT assay

RNA preparation: The preparation of RNA suitable for LM-PAT analysis is the same as the preparation of RNA for the poly(A) length assay (see A2).

Primer annealing and primer ligation: A broad range of RNA concentrations can be used with this assay (typically 20 ng- 1 μg) but the optimum concentration of RNA for a given experiment may be empirically determined by performing several LM-PAT assays with different concentrations of the same RNA sample. The addition of too much sample RNA will interfere with the saturation of the poly(A) tail with oligo(dT) binding and ligation of adjacent oligo(dT) primers, which can cause a “laddering” effect upon electrophoresis, or poor PCR amplification (Salles and Strickland, 1999).

To anneal oligo(dT)$_{12–18}$ to polyadenylated RNA, dilute the RNA sample to a concentration of 180–200 ng/μl and combine 1 μl diluted RNA, 4.5 μl RNase-free water and 2 μl oligo (dT)$_{12–18}$ (at 10 ng/μl, with 5′ phosphorylated end) in a microcentrifuge tube. Heat denature the reaction to 65°C for 10 min and then immediately transfer it to 42°C. While the tube is at 42°C, add 4 μl 5x first strand buffer, 1 μl RNase Inhibitor (Invitrogen), 2 μl 0.1 M DTT, 1 μl 10 mM dNTP, 1 μl 10 mM ATP, 3 μl RNase-free water and 1.5 μl T4 DNA ligase (Invitrogen) to ligate the adjacent oligo(dT)$_{12–18}$ that is annealed to the poly(A) tails. These reagents should be mixed together and prewarmed before adding to the RNA-oligo(dT) sample. A large amount of DNA ligase is required in this step to offset the enzyme’s half life at this temperature (Salles

and Strickland, 1999). After the addition of the mix, combine the reagents thoroughly by pipetting and continue to incubate the reaction for an additional 30 min. While the reaction is still at 42°C, ligate the oligo(dT) primer/adapter to the 5′-phosphorylated oligo(dT)$_{12–18}$ primer by adding 1 μl of the oligo(dT) primer/adapter (200 ng/μl) to the reaction and immediately transferring it to 12°C. After a few minutes at 12°C, centrifuge the tube briefly to collect the entire reaction at the bottom of the tube and incubate it at 12°C for 2 hours. The 10-fold excess of the oligo(dT) primer/adapter and the low temperature favor the oligo(dT) primer/adapter over the oligo(dT)$_{12–18}$ primer for annealing and ligation to the 3′ end of the poly(A) tail, such that the majority of cDNA products will have the GC-rich adapter sequence at the 5′ end.

Reverse transcription: The ligation reaction is placed at 42°C for 2 min, followed by addition of 1 μl Superscript II reverse transcriptase (Invitrogen) and incubation at 42°C for 1 hr. The brief incubation at 42°C before addition of the reverse transcriptase serves to decrease, but not eliminate, annealing of the oligo(dT) primer/adapter to the 5′ end of the poly(A) tail. Both ligase and the reverse transcriptase are inactivated by incubating the reaction at 65°C for 20 min. The cDNA synthesis is now complete and the cDNA is ready for use as a template in PCR amplification or it may be stored at −20°C.

Target-specific primer end-labeling, PCR amplification and resolution of PCR products via polyacrylamide gel electrophoresis: These steps are similar to those described for the poly(A) length assay (see A2).

C. RNase H assay

The previously described methods for poly(A) length measurement rely on cDNA synthesis from an oligo(dT) primer and PCR amplification of the cDNA products, and are thus indirect measurements of the poly(A) tail length. In contrast, the RNase H assay involves the removal of the poly(A) tail from the mRNA and the direct comparison of the deadenylated mRNA with its polyadenylated control by northern blotting. While this assay is more time-consuming than the poly(A) length assay or the LM-PAT assay, it can be useful, in particular when the PCR step in the previous two methods is proving problematic.

RNase H is an endoribonuclease that specifically hydrolyzes the phosphodiester bonds of RNA in RNA:DNA duplexes. In this application, the poly(A) tail is removed by the action of RNase H on the oligo(dT):poly(A) duplex. RNase H does not degrade single-stranded or double-stranded RNA or DNA. The size of a poly(A) tail on a given mRNA can be determined by running reactions with and without RNase H side by side on a gel and comparing the mobilities of the two samples. If the size of the mRNA of interest is so large that a removing the poly(A) tail does not give a measurable shift in electrophoretic mobility a second deoxyoligonucleotide can be annealed upstream of the poly(A) tail, and the products generated by RNase H cleavage at this site are compared side-by-side in identical reactions ±oligo(dT).

1. Materials: RNase H assay—Two *oligonucleotides*: oligo(dT)$_{12–18}$ and a target mRNA-specific primer (optional). The target mRNA-specific oligo should be located no more than 400 nt upstream of the polyadenylation site such that cleavage of the RNA:DNA hybrid between the primer and the mRNA leaves a mRNA product that is large enough to be visualized by northern analysis but small enough that removal of the poly(A) tail makes an observable difference in its electrophoretic mobility.

RNase H (United States Biochemicals, Inc.)

Standard molecular biology equipment and reagents (heat blocks, equipment for agarose gel electrophoresis, RNA transfer and northern blotting)

2. Methods: RNase H assay

RNA purification: RNA is purified for RNase H assay as for the poly(A) length assay or LM-PAT.

Primer annealing and RNase H reaction: One or two oligonucleotides may be annealed in this step, depending on the length of the mRNA body, eg. whether or not it is necessary to shorten the length of the mRNA body to see the effect of removing the poly(A) tail on its electrophoretic mobility. All reactions should be prepared in duplicate; one with the addition of oligo(dT), the other without.

The RNA is first heat denatured, followed by a slow annealing of the oligonucleotides and then RNase H is added to degrade the RNA:DNA hybrids. Combine 10 μg RNA, 5 μl oligo(dT) primer (at 600 ng/μl), 5 μl internal primer (at 600 ng/μl, optional) and 4 μl 5x RNase H buffer in a volume of 18.8 μl. Heat the reaction to 85°C for 5 min to denature the RNA, then incubate it at 42°C for 10 min followed by slow cooling to 32°C. This is readily accomplished by performing the 42°C incubation in a water-filled 250 ml beaker in a water bath and then transferring the beaker from the water bath to the benchtop where it should cool at a rate of 1° C per min. When the reaction reaches 32°C, add 0.5 μl RNase Inhibitor (Invitrogen) and 0.7 μl RNase H (USB) and incubate it at 37°C for 1 hour. The choice of RNase inhibitor is important here, as some inhibit a wide variety of RNases and may therefore interfere with the degradation of the specific RNA:DNA duplexes.

Purify and concentrate the RNA by organic extraction and ethanol precipitation: To purify the RNA from the RNase H reaction, first bring the volume of the RNase H reaction to 150 μl with RNase-free water and add an equal volume of phenol/chloroform/isoamyl alcohol. Extract the RNA by vortexing the reactions for 10 sec and then centrifuge them for 5 min at maximum speed to separate the layers. Following centrifugation, transfer the top layer containing the RNA to a clean microcentrifuge tube and add 1 μl glycogen (20 μg/μl), 10% volume 5 M ammonium acetate and 2.5x volume 95% ethanol. Precipitate at −20°C for at least 20 min and then centrifuge the samples for 20 min to pellet the RNA. Following centrifugation, remove the supernatant and dissolve the RNA pellet in a volume of buffer appropriate for electrophoresis.

Visualization of RNA products: RNase H degradation products are visualized by northern blotting, and if one uses an upstream primer in the cleavage reaction to generate a smaller 3′ product it is best to use a hybridization probe that is specific for the 3′ end of the mRNA. Chapter (…) describes an asymmetric PCR protocol that is particularly useful for generating probes that are specific to a given portion of the mRNA. The products of RNase H digestion are separated on either agarose or polyacrylamide gels, transferred to a nylon membrane, and hybridized with a radioactively labeled probe. As with the PCR-based assays, the difference in the sizes of the poly(A) tails can be most easily determined by using a phosphorimager and analyzing differences in mobility of the fragments with and without the poly(A) tail using ImageQuant. Note that appropriate molecular weight standards should be run on the same gel. RNA from which the poly(A) tail has been removed with RNase H plus oligo(dT) will appear as a tight band corresponding to the distance between the upstream primer binding site and the 3′ end of the mRNA. Excluding oligo(dT) from the reaction will result in an RNase H cleavage product corresponding to the same 3′ sequence with poly(A). The difference between the mobility of this fragment and the fragment lacking poly(A) results from the presence or absence of the poly(A) tail and from this one deduces its size.

II. Introduction: Invader RNA assay

The methods available to quantify mRNA for studying mRNA decay include northern blotting, ribonuclease protection assay (RPA), RT-PCR and Invader RNA chemistry. All of these methods have different advantages and limitations. The advantage of northern blotting is the ability to monitor changes in the full-length mRNA (eg. deadenylation), and in some cases, to detect intermediates in the decay process. RT-PCR and RPA are more sensitive than northern blotting and are therefore useful for assaying less abundant transcripts, or when only a small amount of sample is available. When used with multiple probe sets to different portions of a target mRNA an Invader assay approach (Third Wave Technologies, Inc., www.twt.com) combined high sensitivity with the ability to offer insights into decay mechanics by quantifying changes in specific portions of mRNA.

The Invader assay (diagrammed in Fig. 2) consists of two reactions that are run sequentially in the same well at the same temperature (Eis et al., 2001). In the primary reaction, three oligonucleotides (stacking oligonucleotide, primary probe oligonucleotide and invasive oligonucleotide) specifically bind a region of the target mRNA. The primary probe oligonucleotide has two parts, an assay-specific region (ASR) that is complementary to a portion of the target mRNA and a ‘flap’ which is not complementary to the target mRNA. The invasive oligonucleotide binds the target mRNA immediately downstream of the bound ASR of the primary probe oligonucleotide. The 3′-most nucleotide of the invasive oligonucleotide does not bind the mRNA target or the primary probe, but instead pushes on the 5′-most bond between the primary probe and the target mRNA to create a bulged secondary structure. The thermostable Cleavase enzyme recognizes the specific one-base overlap structure and cleaves the primary probe oligonucleotide to release the 5′flap. The stacking oligonucleotide binds upstream of the complementary portion of the primary probe oligonucleotide and serves to modulate the Tm of the primary probe to 60°C. At the reaction temperature of 60°C, the primary probe oligonucleotide freely cycles on and off the target mRNA while the invasive oligonucleotide, which has a higher Tm, remains bound. Thus, multiple primary probes are cleaved per target, and the number of free flaps produced in the primary reaction is a product of the length of incubation and target abundance.

In the secondary reaction, three additional oligonucleotides are added: an arrestor oligonucleotide (not shown), a FRET-labeled probe oligonucleotide (containing both a fluorophore and a quencher molecule) and a secondary reaction template (SRT) oligonucleotide (a synthetic sequence). The cleaved flap of the primary probe from the primary reaction acts as an invasive oligonucleotide in conjunction with the SRT and the FRET-labeled probe oligonucleotide in another cleavage reaction in which the FRET-labeled probe oligonucleotide 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 (acting as an invasive oligonucleotide) remains bound to the SRT while the FRET-labeled probe oligonucleotide cycles on and off, resulting in linear fluorescence signal generation. The arrestor oligonucleotide binds uncleaved primary probe oligonucleotide. This assay can be run in biplex format to allow the simultaneous detection of two targets from any one sample, which is helpful for using internal controls.

A. Materials: Invader RNA assay

In vitro transcript of the gene of interest—The in vitro transcript of the gene of interest serves as a control for the Invader RNA assay. It is used to optimize the assay and also to perform the 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 (eg. A20) so that the concentration of the in vitro transcript may be accurately determined. The plasmid serving as the template for in vitro

transcription should contain the cDNA of the gene of interest downstream of a promoter that will transcribe the cDNA in the sense direction, and upstream of a restriction site for plasmid linearization.

Prior to in vitro transcription, the plasmid template must be linearized by restriction enzyme digestion. It is essential to check for complete linearization by running a portion of the product on an agarose gel and staining with ethidium bromide or other nucleic acid staining reagent prior to UV visualization. The digestion product must be gel purified if it is not a single band. Following restriction enzyme digestion, the linearized plasmid should be purified. This can be accomplished with a spin column kit such as the PCR Clean up kit (Qiagen).

We use the Megascript kit (Ambion) for in vitro transcription. For each transcription reaction, combine 1 μg template DNA, 2 μl 10x RNA polymerase buffer, 2 μl each ATP, CTP, GTP or UTP solution, 2 μl RNA polymerase and RNase-free water to 20 μl and incubate overnight at 37°C. The plasmid template can be removed from the in vitro transcript by adding 1μl DNase I to the transcription reaction and incubating it for 15 min at 37°C. Following DNase treatment the in vitro transcript should be purified by organic extraction and concentrated by precipitation. To do this, first bring the volume of the transcription reaction up to 100 μl with RNase-free water, then add 100 μl phenol/chloroform/isoamyl alcohol, vortex for 10 seconds and centrifuge for 5 min at maximum speed. Remove the top (RNA-containing) layer to a clean microcentrifuge tube and precipitate with isopropanol. Following centrifugation to pellet the RNA, remove the supernatant and dissolve the pellet in 50 μl RNase-free water. Check the integrity of the in vitro transcript by running an aliquot of it on an agarose gel and quantify the in vitro transcript by measuring the absorbance at 260 nm. Dilute the in vitro transcript to a concentration of 100 amol/μl and store aliquots at −80°C.

Primary reaction oligonucleotide design, synthesis and preparation—The primary probe oligonucleotide 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 it 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 oligonucleotide binds the target mRNA at the 3′end of the probe ASR and stacks coaxially with it. The function of the stacking oligonucleotide is to improve assay performance by increasing the Tm of the primary probe, and thus the assay reaction temperature. Together, the primary probe and stacking oligonucleotide should have a Tm of 60°C. The Tm may be slightly higher, but a Tm of ≥65° C is detrimental to the assay performance. Because the Tm of the primary probe oligonucleotide is the temperature at which the assay is run, the primary probe oligonucleotide cycles on and off the target freely during the reaction. As noted above, the Tm of the invasive oligonucleotide is ~80°C to ensure that it is stably bound to the mRNA target throughout the assay. Because the difference in Tms between the stacking and primary probe oligonucleotides and the invasive oligonucleotide is key to the success of the assay, the difference between them should always be ~20°C.

The first step in designing the three oligonucleotides used in the primary reaction is to choose the region on the target mRNA one wishes to quantitate. To study the polarity of mRNA decay one needs at least three Invader probe sets; one toward the 5′ end of the target mRNA, one in the middle and one in the 3′ end. The polarity of the decay process is determined by comparing the rate of decay of each of these against an internal standard (we use GAPDH). The data in Fig. 3 (Murray and Schoenberg, 2007) show this approach used to study the decay of human β-globin mRNA without or with the c-fos AU-rich instability element added to the 3′-UTR. The sites within each of these regions that are chosen for each probe set should avoid known splice variants or SNPs unless one wishes to include these in the study. If the surrounding ~50 nt region has strong secondary or tertiary structure this will interfere with oligonucleotide

binding and assay performance and these sites should be avoided. Methods to determine secondary and tertiary structure include computer modeling and RT-ROL (reverse transcription of random oligonucleotide libraries), an experimental method (Allawi et al., 2001). In practice one usually needs to design and test probe sets for several possible sites. The length of sequence of the target mRNA each oligonucleotide needs to cover in order to get the appropriate Tm of 60°C (for stacking and primary probe oligonucleotides) or 80°C (for invasive oligonucleotide) determines the sequence of each oligonucleotides as these are strictly a function of its Tm. The final step in probe set design is to check that each is free of the following pitfalls: the length is too short to be sequence-specific (this can happen in GC-rich regions), primer/dimer formation, secondary structure in the oligonucleotide, e.g. GGGG, 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), and a G at the cleavage site in the probe, as this is less efficiently cleaved. The preferred nucleotide at this location is a C (would be a G in the mRNA sequence). Primary probe oligonucleotides require a flap sequence at the 5′end and published probe flap sequences are available (Eis et al., 2001,Wagner et al., 2003).

These oligonucleotides should be synthesized at 1 μM scale and purified by HPLC as the purity of the oligonucleotide preps is essential for good results. The primary probe is a DNA oligonucleotide and has a 3′amino modifier C7. The invasive oligonucleotide is also a DNA oligonucleotide; the stacking oligonucleotide is an RNA oligonucleotide and is 2′O-methylated throughout. We have had success using oligonucleotides from IDT, Inc. Because EDTA inhibits the Invader RNA assay these are dissolved in 300 μl of buffer containing only 0.1 mM EDTA (Te buffer 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). The solution is diluted 1:200 and the concentration of each oligonucleotide is determined by its absorbance at 260 nm. The primary oligonucleotide mix consists of 40 μM primary probe oligonucleotide, 20 μM invasive oligonucleotide, and 12 μM stacking oligonucleotide diluted in Te.

Secondary reaction oligonucleotide design, synthesis and preparation—The secondary reaction is performed by adding three more oligonucleotides to the primary reaction. These oligonucleotides are the arrestor oligonucleotide, the FRET-labeled probe oligonucleotide and the synthetic reaction template (SRT). In the secondary reaction, the arrestor oligonucleotide binds up any uncleaved primary probe oligonucleotide remaining from the primary reaction. Then, the primary probe flap released in the first reaction becomes the invasive oligonucleotide for cleavage of the FRET-labeled probe simultaneously bound to the SRT (Fig. 2). Cleavase separates the fluorescent tag from the quencher (which is located in the ASR for this reaction) to generate a fluorescent signal that can be quantitated using a fluorescence plate reader.

The arrestor oligonucleotide should be designed to be complementary to the uncleaved primary probe oligonucleotide. This is an RNA oligonucleotide and is 2′-O methylated throughout, and purified by desalting. The FRET-labeled probe oligonucleotide is a DNA oligonucleotide containing a fluorescent tag on the 3′ end (we use 6-FAM) and a quencher molecule at the third position (we use Eclipse Dark Quencher). Published sequences for the design of the FRET-labeled probe oligonucleotide are available (Eis et al., 2001, Wagner et al., 2003). This oligonucleotide should be HPLC purified. The secondary reaction template (SRT) should be designed to hybridize with both the FRET-labeled probe oligonucleotide and the primary probe flap oligonucleotide to create the secondary structure recognized by Cleavase as a cleavage substrate. This is a DNA oligonucleotide and the 3′-most 3–5 nucleotides should be 2′-O methylated. There are two oligonucleotide mixes to prepare for the secondary reaction of the Invader RNA assay; one contains the arrestor oligonucleotide diluted to 53.4 μM in Te buffer and the other contains 2.0 μM secondary reaction template (SRT) and 13.4 μM FRET-labeled probe oligonucleotide in Te buffer.

Experimental design considerations—The essence of studying mRNA decay polarity is to terminate transcription and use the Invader RNA assay to quantify the decay of the 5′, middle and 3′ portions of the mRNA (Murray and Schoenberg, 2007). This is best done using a tetracycline repressor-based system where one can rapidly terminate transcription by adding antibiotic to the medium. One variable is whether to run a transcription pulse assay or a transcription turn off assay. The transcription pulse assay has the advantage of generating a homogeneous population of transcripts which should decay in a homogeneous fashion, provided that the pulse length is much shorter than the half life of the mRNA (Loflin et al., 1999). Alternatively one can examine decay from steady-state by adding tetracycline to the medium of cells stably expressing the reporter gene. The major determining factor is the overall degree of reporter gene expression. The background signal increases when the amount of reporter mRNA reaches the lower limit of detection, a problem we encountered in using transcriptional pulsing. This is obviated by the higher starting levels of reporter mRNA present in experiments following decay from steady state. Because of its ubiquitous use as a reporter for studying mRNA decay we used human β-globin mRNA as the target for developing Invader probe sets and quantifying the impact of several instability elements on mRNA decay (Murray and Schoenberg, 2007).

Another experimental consideration when using reporter genes is whether to use transiently transfected cells or stably transfected cells. For experiments using tetracycline repressor control the starting cell line must stably express the tTA protein, but whether or not to construct stable cells that also express the reporter gene is another question. We have used transiently transfected cells and these typically give higher expression of the gene of interest; however, stably transfected cells show a more consistent level of expression and provide greater reproducibility. Also the use of stable lines makes it easier to analyze the effect of overexpression of a regulatory protein on decay, or to analyze the results of RNAi knockdown on decay.

Finally, there are some experimental considerations for setting up the assay itself. Every time the Invader RNA assay is run, there must be a standard curve for each RNA target using an in vitro transcript. This serves to establish the linear range of the assay, which can vary between experiments even for the same oligonucleotides and target. To facilitate statistical analysis of the data an Invader RNA assay should be run in triplicate or quadruplicate, and this becomes a greater issue when using transiently transfected cells since each experiment must also quantify changes in a cotransfected control and one should also examine an endogenous loading control. This is significantly easier with stable cell lines since the target and endogenous loading control (eg. β-actin, GAPDH) can be assayed at the same time by doing the Invader RNA assay in biplex format. Until more probes are available for commonly used cotransfected controls (eg. luciferase, β-galactosidase, GFP) these must be quantified by an alternative method, such as northern blotting or ribonuclease protection assay.

B. Methods: Invader RNA assay

At all times procedures should be performed in a manner that is consistent with preventing RNase contamination and protein degradation. If applicable, treat solutions with DEPC; wipe surfaces with RNaseAway (Invitrogen), wear gloves, use plasticware that is certified RNase-free, and perform all manipulations on ice. After RNA isolation, the Invader RNA assay itself can be run in one day.

RNA isolation—Only a small number of cells is needed for each point since the Invader RNA assay requires a small amount of input RNA. A 60 mm dish of cells yields enough RNA to run the Invader assay multiple times with enough RNA remaining to analyze by RPA or northern analysis to confirm the Invader results or run a cotransfection control. When preparing RNA

for use in the Invader RNA assay, it may be important to separate cells into nuclear and cytoplasmic contents, as the assay will not differentiate between spliced and unspliced RNAs due to the small size of the target sequence. However, the kit includes a buffer for lysing cells directly on the plate for analysis of RNA without RNA isolation. To harvest the cells and prepare nuclear and cytoplasmic fractions, first remove the growth medium from the cells and wash the plate twice with PBS. Add 1 ml PBS to the plate and remove the cells by scraping. Gently mix the cells by pipetting and then transfer the cell suspension from the plate to a microcentrifuge tube. Pellet the cells by centrifuging at 1000×g, 4°C for 1 min. Remove the supernatant and resuspend the cell pellet in 200 ml cytoplasmic extraction buffer. (Cytoplasmic isolation buffer: 0.14 M NaCl, 1.5 mM $MgCl_2$, 0.01 M Tris-HCl pH 8.0, 0.0025% NP20, 0.01 M DTT (dithiothreitol), RNase OUT (Invitrogen), in RNase-free water to desired volume. DTT and RNase OUT should be added immediately before use. Store the buffer at 4°C.) Incubate the mixture on ice for 10 min then centrifuge at 12,000×g at 4°C for 5.5 min to pellet the nuclei. Remove the supernatant containing the cytoplasmic contents and transfer it to a clean microcentrifuge tube. The cytoplasmic contents and cell nuclei may be stored frozen at −80° C until further use at this point. If it is important to have a clean nuclear fraction, wash the nuclear pellet with sodium deoxycholate instead of freezing. To do this, following the removal of the supernatant containing the cytoplasmic fraction, resuspend the nuclear pellet in hypotonic buffer (10 mM NaCl, 10 mM Tris-HCl pH 7.4, 1.5 mM $MgCl_2$, protease inhibitor and RNaseOUT, with the last two items added immediately before use) and then add sodium deoxycholate to a concentration of 0.5%. Centrifuge the nuclei for 5.5 min at 12,000×g at 4° C, remove the supernatant and resuspend the pellet in TE. The nuclei may be frozen at −80°C for storage or processed immediately. RNA may be purified from the cytoplasmic or nuclear fractions using an RNA purification protocol of choice. For experiments involving quantitation of small mRNA decay products, the RNA purification method should recover small RNAs. Finally, quantify RNA yield by its absorbance at 260 nm in a spectrophotometer.

Invader RNA assay

1. Preparation of the standard curve: Samples for the standard curve are diluted and then aliquoted into an 8-tube strip for convenient loading into a 96-well dish later. A typical standard curve is composed of in vitro transcript at concentrations of 0, 0.01, 0.02, 0.04, 0.16, 0.64, 2.5, 5.0, 10.0 and 20.0 amol per well. Note for precise quantitation all dilutions and standard curves should be based on molar amounts of RNA, not micrograms. Because the volume of each diluted in vitro transcript added to each well is always 5 μl, the in vitro transcripts must be diluted to 1/5 of what will be in the reaction well. For example, to add 5.0 amol in vitro transcript, the in vitro transcript must be diluted to 1.0 amol/μl. 5 μl of this is 5.0 amol in the well. In vitro transcripts must be diluted in 20 ng/μl tRNA due to the very low concentration of the transcripts. Be sure to thoroughly vortex and centrifuge down each sample between dilutions. Each standard should be measured in triplicate or quadruplicate, so at least 15–20 μl of each standard concentration is required, plus extra volume for pipetting error, typically 10% volume. Transfer each standard to one tube in a 8-tube strip, and store on ice.

2. Preparation of samples: Sample RNAs are also diluted and then aliquoted into an 8-tube strip for convenient loading into a 96-well plate. Prepare samples by diluting RNA in water or in 20 ng/ml tRNA. RNA samples should be diluted in water if the expression if the target gene is expected to be low such that a large amount of 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 RNA will be added to each well (≤ 50 ng). It is not advisable to use more than 100 ng cellular RNA per well. The volume of sample RNA that will be added to each well is always 5 μl. 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/μl, such

that the addition of 5 μl to a well gives 30 ng of sample RNA in the well. Remember that each sample will be read in triplicate or quadruplicate, so prepare 15– 20 μl of each sample, plus 10% extra volume for pipetting error. Thoroughly vortex each sample, briefly centrifuge contents down and transfer each sample to a well of an 8-well tube strip, and store on ice.

3. Preparation of reaction mixes: It is convenient to prepare both primary and secondary reaction mixes prior to assembling the assay reactions and store them both on ice until needed. The reagents required for the preparation of the reaction mixes are provided in the Invader RNA assay generic kit (Third Wave Technologies, Inc.). Invader RNA assay kits for popular housekeeping genes such as GAPDH and β-actin are also available and include primary and secondary oligonucleotide mixes and in vitro transcripts for controls. For each reaction, prepare the primary reaction mix by combining 4 μl RNA Primary Buffer, 0.25 μl primary oligonucleotide mix for the mRNA of interest, 0.25 μl primary oligonucleotide mix for the housekeeping mRNA (optional as loading control, otherwise add Te), and 0.5 μl Cleavase per well. Cleavase is added last. The reaction mix should be vortexed, briefly spun down and stored on ice. The volume of primary buffer mix to prepare depends upon the number of wells being assayed plus extra for pipetting error. If preparing a small number of wells (≤20), use 20% extra volume. If preparing a larger number of wells it is sufficient to prepare enough extra volume for 4 wells. It is not usually necessary to prepare as large a volume as suggested in the assay manual.

To prepare the secondary reaction mix for one reaction, combine 2 μl RNA Secondary Buffer, 0.75 μl secondary oligonucleotide mix (ie. arrestor) for the mRNA of interest, 0.75 μl FRET-labeled probe oligonucleotide mix (ie. the FRET-labeled probe plus SRT) and for reactions run in biplex mode, 1.5 μl of a secondary oligonucleotide mix for the housekeeping gene. The latter is a packaged mixture from Third Wave Technologies and contains all three oligonucleotides. If one is not assaying a housekeeping gene in the same reaction this should be substituted with 1.5 μl of Te per well so that the total volume of the reaction is 5 μl. The secondary reaction mix should be vortexed, spun down, aliquoted into an 8-tube strip and stored on ice. The secondary reaction mix can be prepared before the assay or during the first incubation.

4. Perform assay: The reactions should be assembled on ice in a 96-well microtiter plate with round-bottomed wells. First, 5 μl primary reaction mix is added to each well using an electronic repeat pipettor for speed and accuracy. Then, 5 μl in vitro transcript standard or 5 μl sample RNA is added to the plate with an 8-channel micropipettor. Finally, each reaction is overlaid with 10 μl Chill-Out wax using an electronic repeat pipettor. The plate should be covered with adhesive film and incubated at 60°C for 90 min either in a thermocycler with a 96-well plate format heating element or in a hybridization oven (particularly useful for multiple plates). After 90 min, the plate is removed (if a hybridization oven is used), the adhesive film is removed and 5 μl secondary reaction mix is added to each sample with a 8-channel micropipettor. The adhesive film is replaced and the plate is further incubated at 60°C for 60 min to 90 min, depending on the desired signal strength, as longer incubation times give a stronger signal. Note that this is a particular advantage of the Invader assay because the signal amplification afforded by increased reaction time is not affected by issues of target amplification common to PCR. The plate is then removed from the thermocycler or hybridization oven, the adhesive film removed and stop buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA) can be added. The addition of stop buffer is optional. The plate should be read immediately in a fluorescence plate reader. We use a Tecan Genios instrument with Magellan II software. Some preliminary settings to try include setting “gain” to 50, “# flashes” to 10, “lag time” to 0 μsec, “integration time” to 50 μsec, and “plate definition file” as NUN96ft.pdf. The gain should be adjusted so that the “0 amol/well” sample of the standard curve has a value of approximately 100.

Data analysis, standard curves—The first step in analyzing the data from the Invader RNA assay is to draw the standard curves. This will serve as a control the sensitivity of the assay as well as demonstrate the linear range of the assay, which is important for data analysis. First, the data should be exported to Microsoft Excel. For each set of triplicates or quadruplicates points on the standard curve, the mean, standard deviation and percentage coefficient of variation (= [(standard deviation)/(average raw signal)]×100) should be calculated. The value of the percent coefficient of variation, which is a measure of the variation between replicates, should be under 10%, and preferably under 5%. The presence of an outlier can cause this value to be very high (≥10%). To generate net values for each of the standard samples >0 amol in vitro transcript (IVT)/well, subtract the mean value for the 0 amol IVT/well from each of the other standard curve mean values. For all standard samples >0 amol IVT/well, the FOLD>zero is calculated by dividing the average gross signal for any particular sample by the average gross signal for the 0 amol IVT/well sample. The SD>zero for all standard samples >0 amol IVT/well is calculated by dividing the net signal for any particular sample by the standard deviation of the 0 amol IVT/well sample. For all standard samples > 0 amol IVT/well, a Student's T Test is calculated comparing that sample to zero. This gives the confidence that the value is different than zero. The student T Test function in Excel can be used, including all 4 zero signal values and all 4 sample signal values, and selecting 2 tails, type 1. For all standard samples, 95% confidence values should be calculated using the confidence function in Excel. When the confidence function in Excel is used, "alpha" should be chosen as = 0.05, "size" should be chosen as 4 (quadruplicates) or 3 (triplicates), and also choose the worksheet cell containing the standard deviation of the sample which is being analyzed. For all samples >0.01 amol a Student's T Test should be calculated to compare it to the next lowest standard sample. The TTest function in Excel can be used as above, but the 4 signal values for the sample being analyzed should be selected, as well as the 4 signal values for the sample below it. Other selections may remain the same.

After the statistical analysis of the data for the standard curve is finished, plot the standard curve as net counts vs. amol IVT/well using a linear scale (not logarithmic). Low points that have either TTest scores above 0.05 or FOLD>zero scores less than 1.15 should be discarded. The lowest value that can be plotted is the limit of detection (LOD) of the assay. High points that have plateaued should also be discarded. The linear range of the assay is the range of values between the lowest and highest plotted points. The data points should be fitted with a linear trendline and the R^2 value should be calculated.

Data analysis, mRNA decay—The standard curve should serve as a guideline for which RNA samples to include for analysis. That is, only signal values that fall within the linear range of the standard curves run simultaneously to the experiment should be used. To calculate net signal values for all experimental values, subtract the signal value from the 0 amol IVT/well sample on the standard curve. For biplexed assays, the signal values for the mRNA of interest can be normalized for loading by dividing them by the control signal values. For each set of quadruplicates or triplicates, the mean and standard deviation should be calculated. For mRNA decay experiments where transcription is inhibited at time=0 (by the addition of a transcriptional inhibitor), calculate the percent remaining by using time=0 hours as 100% (Percent remaining= [(signal of sample at time=x)/(signal of sample at time=0)]×100). Finally, plot the data as percent remaining (y axis, logarithmic) vs. time (x axis, linear), fit the line and calculate the half life for the mRNA. For experiments studying the polarity of mRNA decay (Fig. 3) the decay curves for each probe set should be plotted on the same graph. By quantifying changes in the absolute amount of each portion of the target mRNA as a function of time this graph will directly indicate the polarity of the decay process, since that portion that decays most rapidly will have a steeper slope than more slowly decaying parts of the mRNA.

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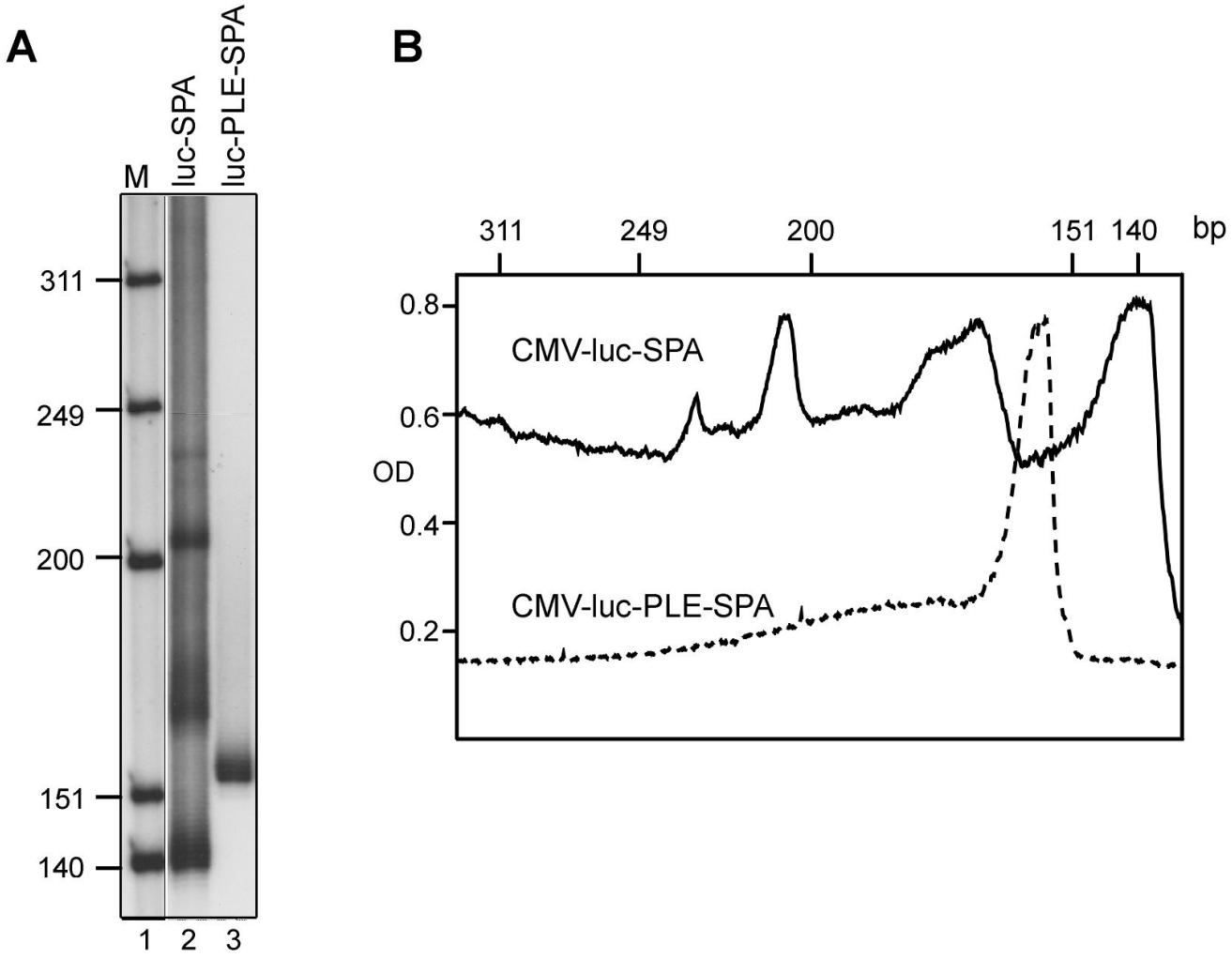

Fig. 1. RT-PCR analysis of poly(A) tail length

RNA was recovered from cells expressing luciferase mRNA with a synthetic polyadenylation element (luc-SPA) or luciferase mRNA with a poly(A) limiting element (luc-PLE-SPA) that restricts the length of the poly(A) tail to <20 nucleotides. The length of the poly(A) tail on each mRNA was assayed by RT-PCR using an upstream radiolabeled primer within luciferase mRNA and the oligo(dT) primer adapter and the resulting phosphorimager analysis is shown in **A. B** shows a graphical view of the data in **A** obtained with the graphing function within ImageQuant. This assay shows the heterogeneous poly(A) tail on the control mRNA with peaks corresponding to the 25 nucleotide spacing for poly(A)-binding protein and the discrete poly (A) tail on PLE-containing mRNA.

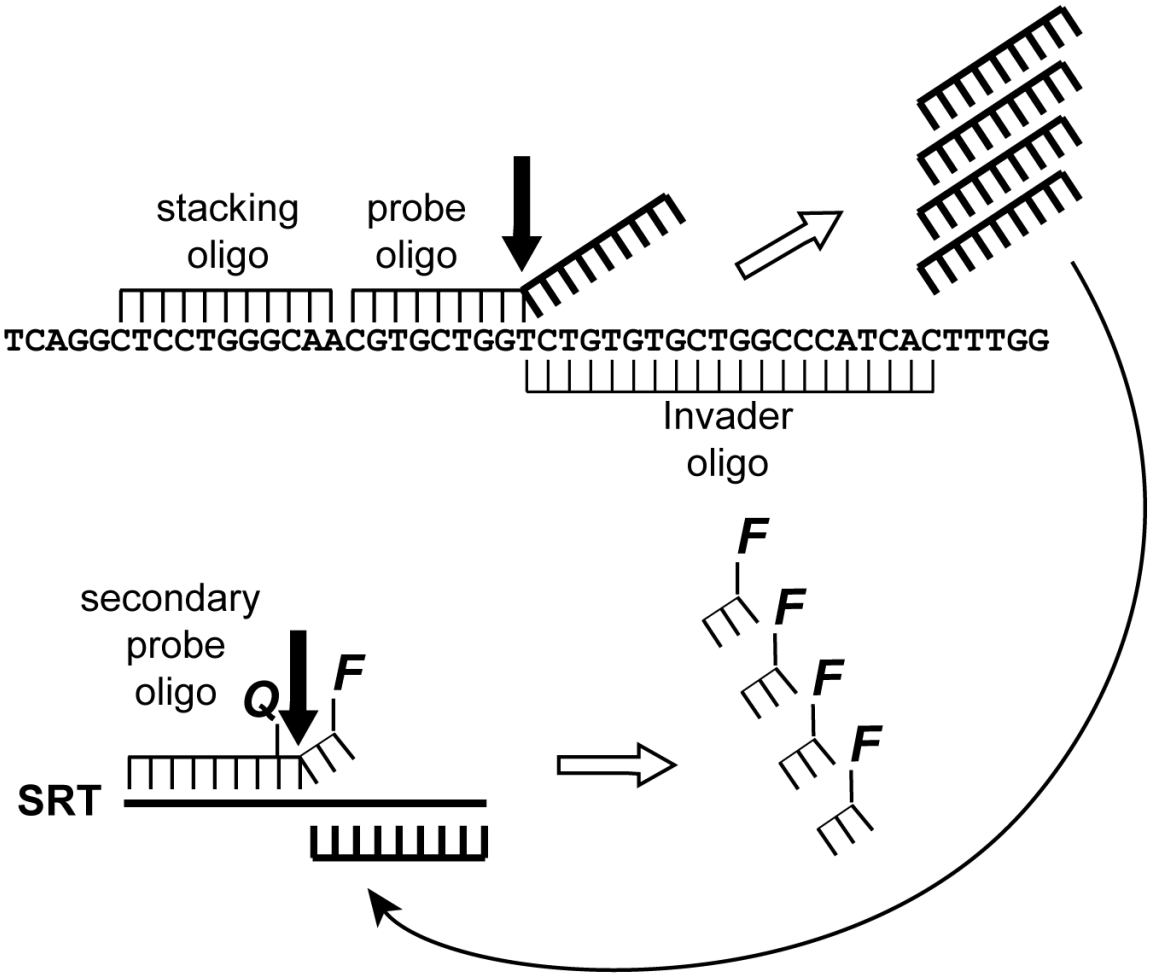

Fig. 2. Basic features of the Invader RNA assay

The Invader RNA assay uses 2 isothermal reactions. In the first reaction the target mRNA is bound by a stacking oligonucleotide that serves to stabilize the binding of a probe oligonucleotide, and an invasive oligonucleotide whose Tm is greater than that of the primary probe bound immediately downstream. This has a 1 nucleotide overlap that distorts the duplex between the primary probe and the target, creating a site for cleavage by the Cleavase enzyme (Cleavase, filled arrow). Cleavage generates a generic flap sequence (shown in bold), which serves as the invasive oligonucleotide in a second reaction in which a FRET-labeled probe oligonucleotide is bound to a secondary reaction template (SRT). Cleavage at the overlap in this complex generates a fluorescent signal. Shown are the β-globin exon 3 probe sets from Murray and Schoenberg (2007).

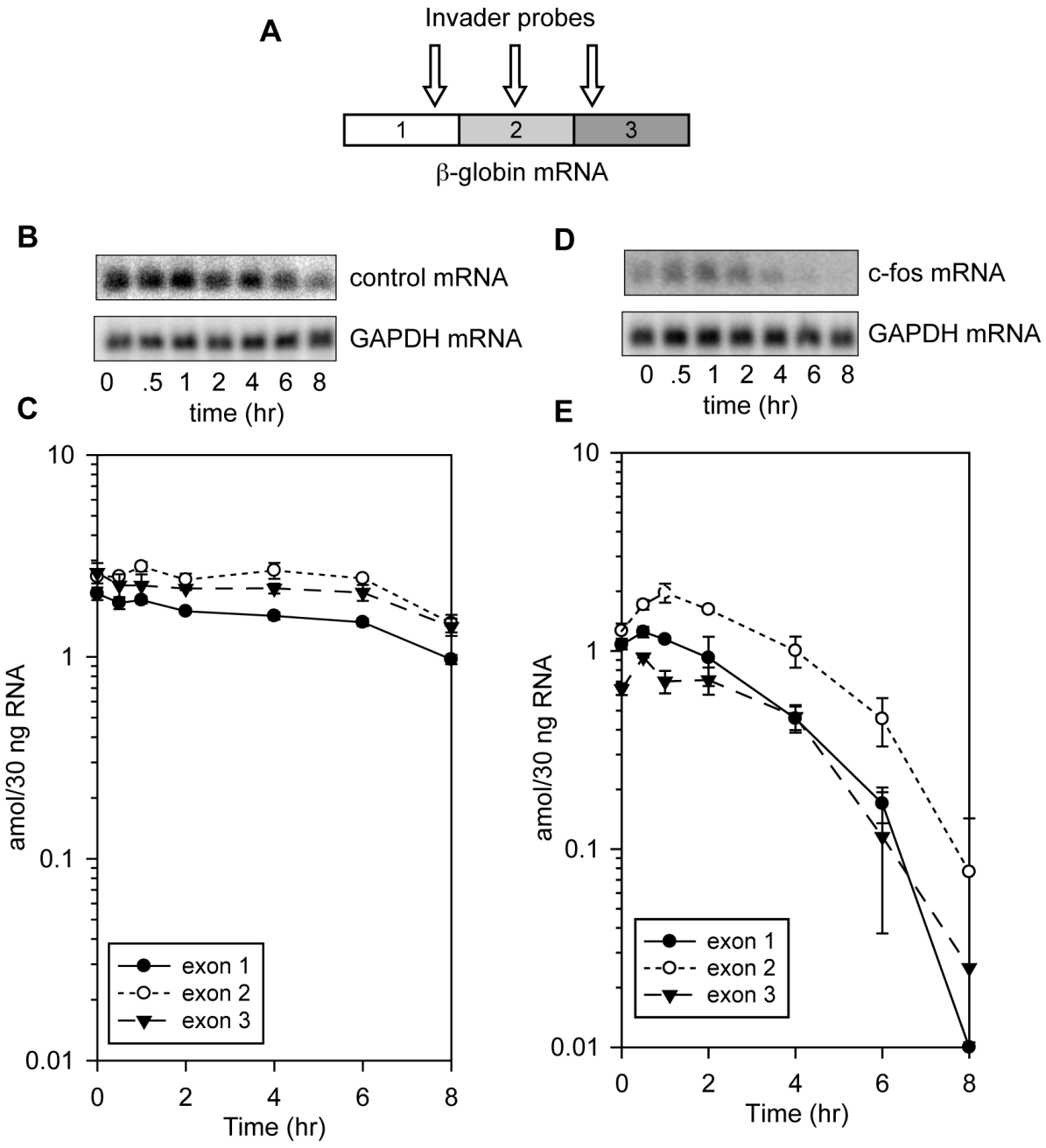

Fig. 3. Analysis of mRNA decay polarity using an Invader RNA based assay. A

Invader probe sets were prepared to each of the 3 exons of human β-globin mRNA. Tetracycline was added at time = 0 to a murine fibroblast cell line that stably expresses the human β-globin gene (**B** and **C** or the human β-globin gene with the c-fos ARE in the 3′-UTR (**D** and **E**) under tetracycline repressor control. RNA from triplicate cultures was isolated at the indicated times and pooled samples were assayed by northern blotting (**B** and **D**), and individual samples were isolated by Invader RNA assay. The results show that stable mRNA decays with no evidence for polarity whereas mRNA with the c-fos ARE decays faster from both ends than the middle. These data are abstracted from Murray and Schoenberg (2007).