CHAPTER NINE

Detecting Ribosomal Association with the 5′ Leader of mRNAs by Ribosome Density Mapping (RDM)

Naama Eldad *and* Yoav Arava

Contents

Abstract

In eukaryotes, scanning of the 5′ leader by the small ribosomal subunit precedes recognition of the start codon. Thus, various sequence elements that are located within this region may affect ribosomes' progression and lead to significant effects on translation. Most notable are short ORFs located upstream of the start codon, which are known to regulate the translation of the main ORF in the transcript. The function of these elements is likely to correlate with altered ribosomal association with the 5′ leader of the mRNA. Currently, the only method to determine the ribosomal association of different regions of the mRNA *in vivo* is the Ribosome Density Mapping (RDM) procedure. This method entails cleavage of the target mRNA by specific oligodeoxynucleotides and RNase H and separation of the cleavage products by velocity sedimentation in a sucrose gradient. In this chapter, we provide a detailed protocol for this procedure and discuss its feasibility.

Department of Biology, Technion—Israel Institute of Technology, Haifa, Israel

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1. Introduction

In the eukaryotic translation initiation process, small ribosomal subunits scan the 5′ UTR in search of a start codon (AUG), which, once identified, serves as a start site for protein synthesis (Cigan and Donohue, 1987; Kozak, 1987). While, in most cases, this start codon is the first one encountered by the scanning subunit, an increasing number of mRNAs appear to have start codons upstream to the main ORF (Iacono *et al.*, 2005). Ribosomal subunits are likely to scan through these upstream AUGs (uAUG) and start translation at the bona fide start site (Kozak, 1987; Morris and Geballe, 2000). However, it was found for several mRNAs that the uAUG serves as a translational regulatory site at which ribosomes might initiate translation under certain environmental conditions (Morris and Geballe, 2000; Vilela and McCarthy, 2003).

Determining whether ribosomes indeed initiated translation at uAUGs is technically challenging. The common procedure involves mutating the uAUGs and inferring their functions from the levels of the encoded protein or a reporter protein. Alternatively, a toe printing assay might be used whereby the position of ribosomes is assayed using an *in vitro* transcribed mRNA (Sachs *et al.*, 2002). The Ribosome Density Mapping (RDM) procedure described later can also be applied for this purpose (Arava *et al.*, 2005). We have used it to show that ribosomes are associated with the 5′ leader of yeast GCN4 mRNA (Arava *et al.*, 2005). It allows the direct determination of ribosomal association *in vivo* and can be applied to many different mRNAs in various organisms. Moreover, it is not just restricted to the 5′ leader of the mRNA and can be applied to determine ribosomal association anywhere along the mRNA.

2. General Concept

RDM is based on the well-established procedure for separating mRNAs associated with ribosomes by velocity sedimentation in a sucrose gradient (Warner *et al.*, 1963). The main difference is that while routine procedures are based on separating the entire transcript in the gradient, in RDM, the transcript, while associated with ribosomes, is cleaved at site(s) of interest by RNase H and oligodeoxynucleotide (ODN) complementary to that site, and only then separated in a gradient. This leads to sedimentation of mRNA fragments according to their ribosomal association.

Four general steps are involved in the procedure (Fig. 9.1): (1) Cell lysis and separation of polysomes (Fig. 9.1, steps 1 and 2); (2) Collection of

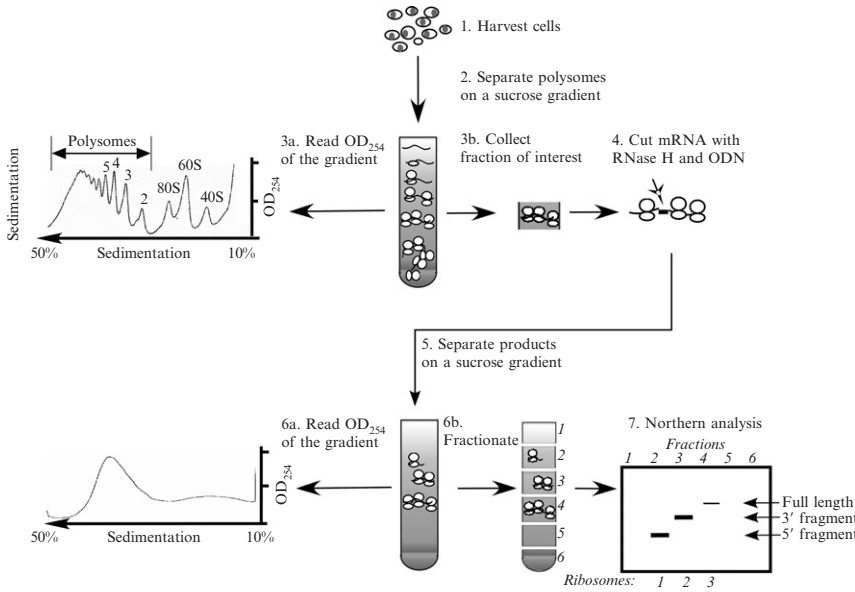

Figure 9.1 Schematic presentation of the RDM steps. See text for details.

a fraction of interest and RNase H cleavage (Fig. 9.1, steps 3 and 4); (3) Separation of the cleavage products according to their ribosomal association (Fig. 9.1, steps 5 and 6); and (4) Determination of the sedimentation position of the cleavage products by northern analysis (Fig. 9.1, step 7). Each of these steps is described later.

RDM provides the best results with mRNAs that are highly expressed, preferably from their own promoter. We observed a decrease in the quality of the results when using reporter mRNAs (e.g., GFP and luciferase) that are not native to the organism (yeast, in our case). The decrease in quality was apparent in the northern analyses, in which multiple bands or smears were observed. This is probably due to inefficient processing of these exogenous transcripts.

Each of the steps involved in RDM has been used separately in many organisms and for the study of various mRNAs. Thus, although the protocol presented herein is for the analysis of yeast cells grown at optimal conditions, it could be adapted easily to other experimental systems. It might also be applied for *in vitro* systems or extracts (e.g., reticulocytes lysate), yet we have not performed such experiments.

2.1. Reagents

Table 9.1 Solutions

Solutions	Stock	Ingredients	Storage	Remarks
Lysis buffer		20 m*M* Tris pH 7.4 140 m*M* KCl 1.5 m*M* $MgCl_2$ 0.5 m*M* DTT 1% Triton X-100 0.1 mg/ml CHX 1 mg/ml heparin	4°	Prepare one day before use
Gradient with heparin		11 ml 10–50% sucrose in a buffer: 20 m*M* Tris pH 7.4 140 m*M* KCl 5 m*M* $MgCl_2$ 0.5 m*M* DTT 0.1 mg/ml CHX 1 mg/ml heparin	4°	Prepare one day before use
Gradient without heparin		11 ml 10–50% sucrose in a buffer: 20 m*M* Tris pH 7.4 140 m*M* KCl 5 m*M* $MgCl_2$ 0.5 m*M* DTT 0.1 mg/ml CHX	4°	Prepare one day before use
RNase H buffer	5×	0.1 *M* Tris pH 7.4 0.5 *M* KCl 0.1 *M* $MgCl_2$ 0.5 m*M* DTT 2.5 mg/ml CHX	−20°	
Lysis Minus Detergent (LMD) buffer		Same as the lysis buffer but without Triton X-100	−20°	
MOPS buffer	10×	0.4 *M* MOPS 0.1 *M* Na Acetate 0.01 *M* EDTA Bring to pH 7.0 with acetic acid	RT	
RNA loading buffer	2×	For 15 ml stock, mix 10 ml of 100% formamide, 3 ml of 37% formaldehyde, 2 ml of 10× MOPS buffer and 25 μl of 10 mg/ml ethidium bromide	−20°	Highly toxic
Hybridization buffer	1×	0.4 *M* Na_2HPO_4 pH 7.2 6% SDS 1 m*M* EDTA	RT	Warm before using to dissolve any aggregates
Hybridization Wash 1	1×	40 m*M* Na_2HPO_4 pH 7.2 5% SDS 1 m*M* EDTA	RT	
Hybridization Wash 2	1×	40 m*M* Na_2HPO_4 pH 7.2 1% SDS 1 m*M* EDTA	RT	

Table 9.2 Enzymes and solutions

Product name	Concentration	Cat. #	Remarks
Heparin	10 mg/ml	Sigma H3393	Store at −20°. Dissolve in water.
Cyclohexamide (CHX)	10 mg/ml	Sigma C7698	Store at −20°. Dissolve in water. Vortex well. CHX is highly toxic.
RNAse Inhibitor **or** rRNAsin	40 U/μl **or** 40 U/μl	Takara 2311A **or** Promega N2515	
RNase H	5 U/μl	New England Biolabs M0297	
Antisense ODN	10 pmol/μl		~20 bases ~50%GC. Dissolve in water.
Guanidinium HCl	8 *M*	Sigma G4505	Stir and warm until solution becomes clear. Dissolve in water.
RNA Marker	1 mg/ml	Ambion #7145	RNA Century Plus

3. Methods

3.1. Cell lysis and separation of polysomes

The first step in the RDM protocol is cell lysis and separation of polysomes through sucrose gradient (Fig. 9.1, steps 1 and 2), followed by the collection of a specific fraction that contains the mRNA of interest associated with ribosomes for RNase H cleavage. There are two reasons for performing RNase H cleavage on only a selected fraction and not on the entire extract: (1) Isolation of a subset of mRNAs with a specific number of bound ribosomes simplifies interpretation of the results; (2) Large amounts of heparin are used during the lysis to inhibit RNase activity. This heparin does not sediment into the gradient, and therefore the isolated fraction is clean of heparin and can be used in the following enzymatic step.

1. Grow 50 ml of yeast cells to OD_{600} 0.5 to 0.8 in YPD (1% Yeast extract, 2% Peptone, 2% Dextrose).

   The amount of cells taken for analysis is important, since too many cells will yield an overload of RNases in later steps. The growth conditions are also important since certain conditions might be enriched in RNases. Highly expressed genes (at the levels of "housekeeping genes") are likely to yield good results in RDM, but the analysis of low abundance mRNAs might be problematic. Increasing the amount of cells by increasing the volume of culture may help as long as the density remains low (OD_{600} 0.5–0.8).
2. Add cyclohexamide (CHX) to a final concentration of 0.1 mg/ml, transfer to an ice-cold 50-ml conical tube, and immediately spin down the cells at 4000 rpm (3220*g*) for 4 min at 4°.

   At these concentrations, CHX inhibits the 60S subunit translocation and retains ribosomal association with mRNA.
3. Discard the supernatant, resuspend the cells' pellet in 4 ml of ice-cold lysis buffer, and spin cells again as in step 2.

   The quick spin-down and washes are important to remove leftovers of dextrose found in the rich medium. This probably helps in blocking initiation events.
4. Wash again as in step 3.
5. Resuspend the cells' pellet with 350 μl of lysis buffer, transfer to a micro tube with a screw cap, and add ice-cold glass beads (diameter 0.4–0.6 mm) to cover the cells and lysis buffer.
6. Break the cells in a bead beater by two pulses of 1.5 min at maximum level.
7. Transfer the cells' lysate into a new tube. This can be done by a quick spin-down of the glass beads and collecting the supernatant. Alternatively, it is possible to pierce the bottom of the tube with a heated needle, place it on top of a 15-ml tube, and spin at 4000 rpm for 1 min at 4° (the lysate will drip into the 15-ml tube and the glass beads will be

retained in the pierced tube). We use the cylinder of a 5-ml syringe as an adaptor between the pierced tube and the 15-ml tube.

8. Transfer the resulting lysate from the 15-ml tube to a new ice-cold micro tube. Usually, there is a small pellet that should be resuspended with the supernatant and also transferred to the micro tube.
9. Centrifuge for 5 min at 9500 rpm at 4° and transfer the supernatant to a new ice-cold micro tube.
10. Bring to a final volume of 1 ml with lysis buffer and carefully load the lysate on 10 to 50% sucrose gradient *without* heparin.
    For gradient preparation instructions, see Arava (2003).
11. Separate complexes by ultra-centrifugation using a SW41 rotor at 35,000 rpm for 2:25 h at 4°.
    The separation times may be adjusted according to the desired resolution. Many technical aspects of velocity sedimentation analysis are discussed in Rickwood (1992).

3.2. Fraction collection and RNase H cleavage

Following centrifugation, the fraction of interest is collected for cleavage by RNase H and ODN (Fig. 9.1, steps 3 and 4). This is usually the fraction that contains the majority of transcripts of the gene of interest, but could be any other fraction that contains sufficient amounts of the mRNA of interest. Prior knowledge of this fraction is therefore necessary. Such knowledge is usually achieved by performing a preliminary polysomal separation experiment in which the cells' extract is separated into fractions that are analyzed by northern blot for the mRNA of interest. In the case of yeast, a previous genome-wide analysis that characterized the ribosomal association of thousands of mRNAs could be of assistance (Arava *et al.*, 2003). For genes that are expected to be regulated through ribosomes associated with their 5′ leader, this fraction is likely to be the monosome fraction (Arava *et al.*, 2003; Kuhn *et al.*, 2001; Tzamarias *et al.*, 1989).

Collecting the correct fraction is greatly facilitated by the use of a continuous ultraviolet (UV) detector, such as the ISCO UA-6 system. The OD_{254} reading allows accurate determination of the sedimentation of various complexes (e.g., 40S, 80S, and various polysomal complexes; Fig. 9.1, step 3a). This information can be used to correct for small variations in sedimentation. Importantly, in many cases, it enables the determination of the number of ribosomes on the mRNA or on the resulting fragments, thereby allowing more accurate conclusions.

1. Collect the fraction of interest (620 μl) into an ice-cold micro tube.
   The collected polysomal fraction also contains a significant amount of RNases. An overload of RNases cannot be completed by the commonly used RNase Inhibitors (rRNasin, RNase Inhibitor, etc.) and will therefore lead to massive degradation. It is therefore recommended not to collect more than the amount indicated here.

2. Immediately add 70 μl of 0.1 *M* DTT (final concentration 10 m*M*) and 7 μl of 40 u/μl RNase inhibitor (final concentration 0.4 u/μl).
3. Transfer 600 μl of this mixture to another micro tube containing 10 μl of antisense ODN (10 pmole/μl) complementary to the cleavage site on the mRNA. The remaining 70 to 100 μl serves as a control sample (''uncut'') and will be subjected to the following incubations (steps 3 to 6).

   We used many ODNs ranging in length from 18 to 25 nts and with GC content from 40 to 60%. These ODNs varied in efficiency, yet we are unable to directly link the differences in efficiency to their length or GC content. This is probably because many additional factors, such as structured cleavage site or presence of a ribosome, affect cleavage efficiency. In the case of cleavage at the translation start site region, target sequences should be selected from ~20 nts upstream to the start codon in order to avoid any inhibition by initiating ribosomes. It is recommended to examine the cleavage efficiency in a test reaction that includes polysomal RNA and the ODNs. We perform the test reactions on an isolated polysomal fraction and not on clean RNA (e.g., RNA isolated by the hot phenol or Tri-reagent methods), since many ODNs perform differently among these populations.

   Multiple ODNs complementary either to the same transcript or to different transcripts can be used in the same reaction. If several ODNs to the same transcript are used, partial products are also expected (i.e., cleavage by only one ODN; Fig. 9.2). Thus, the cleavage plan should be such that the full cleavage products and the various partial cleavage products are of distinct sizes. In cases where there is no good separation in size, it is possible to design probes that will recognize only some of the products in the northern analysis.
4. Put the sample in a beaker containing water at 37° and let it gradually cool to room temperature for 20 min.
   This supposedly allows annealing of the ODN to its target sequence.
5. Add 150 μl of 5× RNase H buffer and 10 units of RNase H.
6. Incubate the samples at 37° for 20 min.

   Longer incubation times may improve RNase H cleavage but may also result in partial degradation.
7. Stop the reaction by bringing the sample volume to 1 ml with Lysis Minus Detergent (LMD) buffer.

   The LMD contains heparin that inhibits RNase H activity. The LMD buffer will also dilute the sucrose in the sample to allow overlaying on a 10 to 50% sucrose gradient.
8. Set aside 1/10 of the reaction as a pregradient control (''cut'').

3.3. Separation of cleavage product

Following cleavage, the products are separated on a sucrose gradient according to their ribosomal association (Fig. 9.1, steps 5 and 6). Since the sample includes only complexes at a fixed size (the size that was collected initially), the resulting OD profile usually has only one peak (Fig. 9.1, step 6a). It is

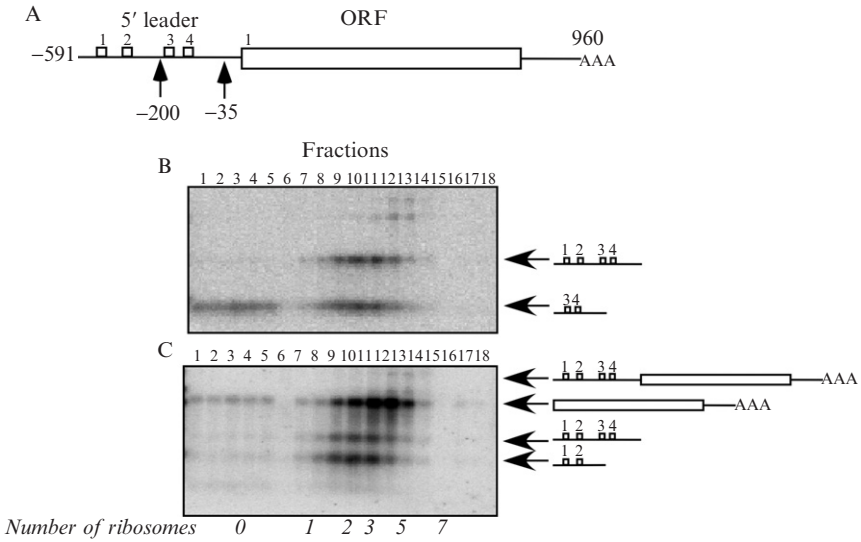

Figure 9.2 RDM analysis of GCN4 mRNA. (A) Schematic presentation of GCN4 mRNA with the four uORFs located in its 5′ leader. These uORFs play an important role in regulating the translation of GCN4 ORF under different growth conditions. They exert their role by affecting the efficiency of ribosomes' scanning of the 5′ leader (Hinnebusch, 2005). (B and C) To demonstrate ribosomal association with the 5′ leader, yeast cells were grown under conditions that induce the GCN4 translation. Polysomal fraction containing the majority of GCN4 mRNA (with about 5–6 ribosomes) was isolated, and the GCN4 mRNA was cleaved at two positions simultaneously by adding two ODNs complementary to positions −35 and −200 upstream to the AUG of GCN4 protein (indicated by arrows in A). Cleavage products were then separated by velocity sedimentation in a sucrose gradient and 18 fractions were collected. The sedimentation position of the double-cleaved product (−200 to −35) was determined by northern analysis using a labeled oligonucleotide complementary to that region (B). The blot was then hybridized with a probe recognizing the entire transcript to determine the sedimentation position of the rest of the cleavage products (C). The identity of each cleavage product was determined according to its length and is indicated schematically to the right of each blot. The number of ribosomes associated with complexes sedimenting at each fraction is indicated below the blots. This analysis shows that under these growth conditions, the 5′ leader of GCN4 sediments as associated with ribosomes. The band corresponds in size to the entire 5′ leader sediments as associated with ~2 ribosomes. The 5′-most region, which contains only uORFs 1 and 2, appears to be highly associated with ribosomes since practically all of the cleavage products that correspond to it sediment in fractions 9 to 11 and almost none are in fractions 1 to 6. On the other hand, a substantial amount of the fragment that contains uORFs 3 and 4 appears to sediment in fractions 1 to 6 as free of ribosomes. These results are in agreement with the model that some of the ribosomes that scan uORFs 1 or 2 dissociate from the mRNA prior to approaching uORF3 (Abastado *et al.*, 1991).

impossible to deduce the ribosomal association of the cleavage products from this single peak. There are two options to overcome this limitation: (1) Use of an external profile—since the sedimentation profiles are highly reproducible, it is possible to infer the number of ribosomes sedimenting at a certain position from a similar gradient that was centrifuged under similar conditions but with an entire cell extract (Fig. 9.1); (2) Spike-in extract—another option is to set aside a sample of the whole-cell extract (~1/10 of the extract) before loading on the first gradient (Fig. 9.1, step 2) and spike it into the RNase H cleavage sample before loading on the second gradient. The spiked aliquot will result in an OD_{254} profile that contains multiple peaks (as in the first profile), which can be used to determine the sedimentation position of the various complexes.

1. Carefully load the sample on a sucrose gradient containing heparin and centrifuge as in Section 3.2.
2. After centrifugation, collect multiple fractions from the gradient. The number of fractions to collect depends on the desired resolution; a reasonable number of fractions to start with is 18 (fraction vol. ~0.6 ml).
3. Collect fractions into 13-ml tubes (e.g., 95 × 16.8 mm polypropylene from Sarstedt cat no. 55.518) containing 1.5 volume of 8 *M* GuHCl (final concentration of ~5.5 *M*).
4. Add 2.5 sample volumes of 100% ethanol. Mix well and incubate at −20° for at least 1 h.
5. Centrifuge samples for 20 min at 12,000 rpm at 4° using a SM 24 rotor. Carefully discard the supernatant.

   The advantage of the SM 24 rotor is that it has enough room for all 18 tubes. Any other equivalent rotor will be good also.
6. Wash with 500 μl of ice-cold 80% ethanol, centrifuge as in step 5, and carefully discard the supernatant. Note that the pellet might be unstable.
7. Resuspend with 400 μl of TE, transfer to a micro tube, and precipitate again by adding 40 μl of 3 *M* NaAce, pH 5.3, and 1 ml of 100% ethanol. Mix well and incubate at −20° for at least 1 h.
8. Centrifuge samples for 20 min at 14,000 rpm at 4°. Discard the supernatant.
9. Wash by adding 100 μl of ice-cold 80% EtOH. Centrifuge as in step 8 and discard the supernatant.
10. Dry the pellet and resuspend in 10 μl of sterile, RNAse-free water. Keep at −20°. Take about half of the sample to northern analysis.

3.4. Determination of sedimentation position

The sedimentation of RDM cleavage products is determined by standard northern analysis (Fig. 9.3, step 7). Any northern protocol can be used for this purpose. The protocol presented here is widely used

for the analysis of yeast mRNAs and is based on buffers described in Church and Gilbert (1984). It is relatively simple and provides reliable results when analyzing abundant mRNAs (>5 copies/cell [Wang *et al.*, 2002]) that yield cleavage fragments exceeding ~200 nts. For shorter fragments, it is possible to separate fragments on polyacrylamide gel and then transfer to a nylon membrane for northern blot analysis (Muhlrad and Parker, 1992).

Performing the analysis by RT-PCR in order to detect low-abundance mRNAs might be problematic. This is because in every RDM experiment there is a significant amount of primary transcript that was not cleaved and therefore sediments in the gradient with its full load of ribosomes. RT-PCR with any set of primers will not distinguish between signals derived from the cleavage products and signals from the full-length transcript.

1. Prepare 1.2 to 2.5% agarose gel (depending on the expected sizes of fragments) in 1× MOPS and formaldehyde (1.3% final concentration). Dissolve the agarose first in water, let cool to 65°, and then add the 10× MOPS and formaldehyde.
   The gel should be poured in a hood because of the use of formaldehyde.
2. Mix 5 μl of each RNA sample with 7.5 μl of RNA loading buffer and 2.5 μl of 6× loading dye.
3. Incubate at 55° for 10 min to open RNA secondary structures.
4. Load the sample on the gel. Also load an RNA size marker, the "uncut" and "cut" controls, and a sample (2–10 μg) of untreated RNA. Run the gel in 1× MOPS buffer to obtain the best resolution of the expected cleavage products.
   Running an RNA size marker is critical for the correct identification of the cleavage products. Resuspend the RNA marker in the same buffer as the samples, yet exclude any dyes that may obscure some of its bands. The uncut and cut samples are also important because they can indicate the efficiency of cleavage as well as the nonspecific bands.
   MOPS buffer has a weak buffering capacity. A long running time tends to increase pH in the upper chamber and might lead to degradation of the RNA. It is therefore advised to circulate the buffer between chambers during the running time.
5. Blot the RNA from the gel to a nylon membrane.
6. Cross link the RNA to the membrane using an UV cross linker or a dry oven.
7. The quality of RNA and transfer can be evaluated by methylene blue staining. This is done by immersing the membrane in 5% acetic acid for 5 min and then in 0.1% methylene blue in 5% acetic acid for 5 min. The membrane is then washed in water, and distinct bands of the 25S (3400 nts), 18S (1800 nts) rRNA, and RNA marker bands should appear. Mark the positions of these with a pencil because the methylene blue staining will disappear during the hybridization.
8. Prepare a radioactive probe to hybridize with the membrane.

When identification of all cleavage products is desired, the PCR product corresponding to the entire mRNA is labeled by the "random priming" method (Sambrook and Russell, 2001). When detection of only some of the fragments is desired, or when the mRNAs have high homology with unwanted sequences, specific oligonucleotides (~30 nts) are labeled by T4 kinase and radioactive γ^{32}P-ATP (Sambrook and Russell, 2001). The former labeling method yields probes with higher specific activity and is more efficient because it detects all products in one experiment. The following protocol is performed with such a probe, yet it can be modified to an oligonucleotide probe simply by lowering the temperatures of hybridization and washings.

9. Hybridize the probe with the membrane at 57° for at least 6 h in the hybridization buffer.
10. Following hybridization, wash the membrane 2× 15 min in Hybridization Wash 1 and 2× 15 min in Hybridization Wash 2 at 57°.
11. Expose to a phosphor-imager screen or a film. Three bands should appear: two that are similar in size to the cleavage products (5′ and 3′ to the RNase H cleavage site), and a longer band similar in size to the full-length transcript that represents the remainder of an uncut mRNA (Figs. 9.1 and 9.2).

    The most common problem is the appearance of multiple bands and nonspecific signals. If there are other mRNAs that cross-hybridize with the gene of interest, a possible solution is to use a different probe that is more specific to the target sequence (e.g., oligonucleotide probe). Alternatively, increasing the hybridization or washing temperature may remove some of these nonspecific signals. Another unwanted result is the appearance of smears or degradation products that hinder the detection of the specific bands. Degradation usually occurs at the RNase H cleavage step because the fraction collected may contain an overload of RNases. In such a case, it is recommended to decrease the amount of cell extract that is loaded on the first gradient and add some more RNase inhibitor. Note that prior to loading on the gradient, the cells are in a lysis buffer that contains heparin, and after the RNase H reaction, an LMD buffer is added that also includes heparin. These steps are therefore less likely to lead to degradation. We also noticed increased degradation levels when overexpressing reporter genes. A possible solution to this is to express the reporter gene from a weaker promoter and to fuse to it UTRs from the organism into which it is inserted.

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