RPA III™ Kit

(Part Number AM1414, AM1415)

Protocol

P/N 1414M Revision B **Revision Date: June 5, 2008**

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Literature Citation: When describing a procedure for publication using this product, please refer to it as the RPA III™ Kit.

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Patents and Licensing Notifications: The RPA III™ Kit is covered by US patent 5,422,241.

I. Introduction

A. Background

Procedure Overview

The Ribonuclease Protection Assay (RPA) is an extremely sensitive procedure for the detection and quantitation of RNA species (usually mRNA) in a complex sample mixture of total or Poly(A) RNA.

For the RPA, a labeled (nonisotopic or radioactive) RNA probe is synthesized that is complementary to part of the target RNA to be analyzed (Figure 1 on page 3). The labeled probe and sample RNA are incubated under conditions that favor hybridization of complementary sequences. After hybridization, the mixture is treated with ribonuclease to degrade unhybridized probe. Labeled probe that is hybridized to complementary RNA from the sample will be protected from ribonuclease digestion, and can be separated on a polyacrylamide gel and visualized either by autoradiography (radioactively-labeled probes) or by a secondary detection procedure (nonisotopically-labeled probes).

When the probe is present in molar excess over the target in the hybridization reaction, the intensity of the protected fragment will be directly proportional to the amount of target RNA in the sample mixture. Ribonuclease protection assays are thus analogous to S1 nuclease protection assays, but ribonuclease is generally acknowledged to be easier to fine-tune and less prone to degrade double-stranded nucleic acid than S1 nuclease (Molecular Cloning, 2001; and Friedberg, 1990).

The following sections include the experimental procedure as well as information on optimization, running the positive control reaction and troubleshooting. We encourage you to read the entire protocol if you are using the RPA III™ Kit for the first time.

Advantages of RPAs

High sensitivity

Compared to hybridization protocols that rely on RNA bound to a solid support (i.e. Northern blots), low abundance mRNAs are detected more readily and quantified more accurately by using a solution hybridization procedure such as the RPA (Frayn 1993, Lee and Costlow 1987).

Tolerant of partially degraded RNA

Since the probes used in the RPA are generally significantly shorter than the mRNA species being detected, the target RNA preparation can be less than completely intact. Breaks in the target RNA that occur outside the region that hybridizes to the probe will have no effect on the RPA, but would result in loss of signal on Northern blots.

Multiple target analysis

Of the methods employed for RNA quantitation, RPA analysis is the best choice for simultaneous detection of multiple targets in a given sample. Detection of multiple targets requires only that the probes protect RNA fragments that differ in size such that they can be separated on a denaturing polyacrylamide gel (Hobbs 1993).

Mapping studies

Due to the high resolution of the acrylamide gel system used to analyze the protected fragments, RPAs are well-suited for mapping positions of internal and external junctions in mRNA, for example transcription initiation and termination sites and intron/exon boundaries (Kekule 1990, Melton 1984, and Calzone 1987).

Versatility

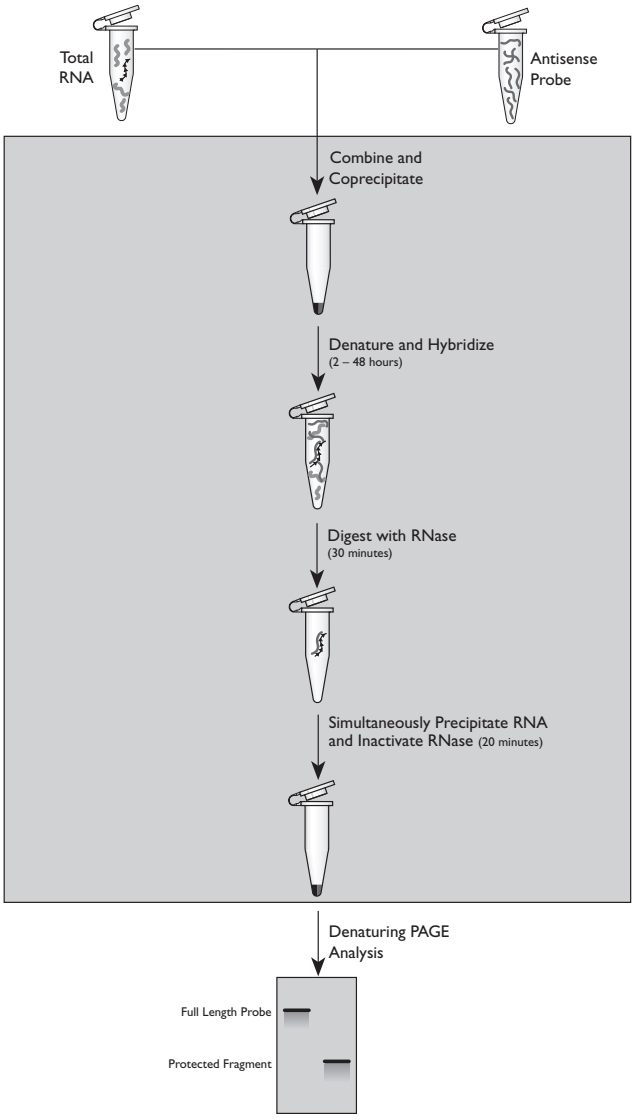
The RPA is versatile in that it can be used to discriminate between related targets or to simultaneously detect similar targets in a sample. To distinguish between related targets, probes are designed to span sub-regions of relatively higher sequence divergence (Brown 1993). In this way, RNase protection can be used to distinguish between different mRNAs coded by genes of multigene families which cross-hybridize to show a single band on Northern blots. Conversely, to minimize the effect of probe/target sequence differences, for example when using a rat probe to detect a heterologous mouse mRNA, the probe sequence and/or the RNase digestion conditions can be adjusted to minimize cleavage of mismatches.

The Ambion RPA III Kit simplifies RPAs

Ribonuclease protection assays have acquired a reputation for being difficult to set up and optimize. The Ambion RPA III Kit is designed to avoid many of the problems associated with ribonuclease protection assays, and to provide simplicity while still allowing flexibility for experimental optimization. The RPA III Kit differs from published procedures in several respects providing greater sensitivity, as well as being faster, easier to use, and less labor intensive. Figure 1 on page 3 provides an overview of the RPA III procedure.

Note that the proteinase K and phenol-chloroform steps of published procedures have been eliminated and have been replaced with a single precipitation and RNase inactivation step. This means that the entire procedure can be performed in a single tube. This assay is also completely compatible with nonisotopically-labeled probes, such as those generated by the Ambion Psoralen-Biotin Kit.

Figure 1. RPA III™ Procedure



B. Reagents Provided with the RPA III Kit and Storage

The kit contains reagents for 120 assays.

P/N AM1415	P/N AM1414	Components	Storage
100 µL	100 µL	Mouse Liver RNA: 0.5 mg/mL in 0.1 mM EDTA.	below –70°C
350 µL	1 mL	Hybridization III Buffer	–20°C
5.5 mL	17 mL	RNase Digestion III Buffer	–20°C
500 µL	500 µL	Yeast RNA: 5 mg/mL from torulla yeast, sheared to ~300 bases	–20°C
7.9 mL	22.5 mL	RNase Inactivation/Precipitation III Solution	–20°C
1.4 mL	1.4 mL	Gel Loading Buffer II: 1–2X gel loading solution	–20°C
10 µL	10 µL	pTRI-Actin-Mouse: 5 µg at 0.5 mg/mL*	–20°C
105 µL	300 µL	RNase A/RNase T1 Mix	–20°C
105 µL	300 µL	RNase T1: 5 U/µL	–20°C
1 mL	1 mL	5M Ammonium Acetate	–20°C
8 mL	8 mL	Probe Elution Buffer†	4°C

* Linearized pTRIPLEscript DNA in TE buffer; contains 250 bp of mouse β-actin in the antisense orientation relative to tandem SP6, T7, and T3 promoters.

† 0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS

C. Materials Not Provided with the Kit

- DNA template and reagents for preparing radiolabeled or nonisotopic RNA probe (see section IV.B for details of probe preparation)
- Constant temperature incubator or heat block (42–45°C and 85–95°C)
- RNAse-free polypropylene microfuge tubes
- Microcentrifuge capable of achieving at least 10,000 x g
- Adjustable pipettors and RNase-free tips
- Apparatus and reagents for preparing and running denaturing acrylamide gels (see section IV.G for gel preparation and electrophoresis)
- 100% ethanol (ACS grade)
- Trichloroacetic acid (molecular biology grade)
- Pasteur pipets and bulbs

D. Related Products Available from Applied Biosystems

MAXIscript® Kits P/N AM1308–AM1326	MAXIscript Kits are designed for synthesis of high specific-activity RNA probes with specific activities reaching 1 x 10^9 cpm/µg in just 10 minutes. MAXIscript Kits are available for DNA templates containing T7, T3, and SP6 promoters.
TURBO™ DNase P/N AM2238, AM2239	TURBO DNase (patent pending) is a hyperactive DNase that was developed using a protein engineering approach to improve wild-type DNase I. These changes markedly increase the affinity of the protein for DNA. The result is a versatile enzyme with a 6-fold lower K_m for DNA, and an ability to maintain at least 50% of peak activity in solutions approaching 200 mM monovalent salt, even when the DNA concentration is in the nanomolar (nM) range.
RNA Century™ Marker and RNA Century™-Plus Marker Templates P/N AM7780 & AM7782	Templates for the transcription of 100–500 and 100–1000 nt RNA molecular weight markers. Also available as pre-transcribed biotinylated RNAs (P/N AM7175, AM7180)
Antisense Internal Control Templates See web or print catalog for P/Ns	These templates for in vitro transcription consist of linearized pTRIPLEscript™ vectors that can be used to produce antisense RNA to messages that are expressed at relatively constant levels. They can be transcribed using any of the common RNA polymerases - SP6, T7, or T3. These templates can also be used in primer extension to make single-stranded DNA. Please see Ambion's catalog or our website (www.ambion.com) for a complete listing.
FirstChoice® Total and Poly(A) RNA See web or print catalog for P/Ns	Ambion provides high quality total and poly(A) RNA from a variety of human, mouse and rat tissues and from human cell lines. DNA is removed with a stringent DNase treatment. These RNAs are shown to be intact by denaturing agarose gel electrophoresis, Northern analysis, reverse transcription, and capillary electrophoresis using the Agilent 2100 bioanalyzer, and they are precisely quantitated. Please see our catalog or our website (www.ambion.com) for a complete listing.
BrightStar® BioDetect™ P/N AM1930	Low background, high sensitivity detection kit for biotinylated RNA and DNA probes. This nonisotopic detection system is compatible with Northern, Southern, dot blot and nuclease protection assays.
BrightStar®-Plus Membrane P/N AM10100–AM10104	Positively-charged nylon membranes recommended for use with Ambion BrightStar® nonisotopic labeling and detection products. These membranes are an excellent choice for Northerns and other blot hybridizations.
Electrophoresis Reagents See web or print catalog for P/Ns	Ambion offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. Please see our catalog or our website (www.ambion.com) for a complete listing as this product line is always growing.
RNAqueous® RNA Isolation Kits P/N AM1911, AM1912, AM1914, AM1920	RNAqueous Kits employ a simple and rapid procedure to purify total RNA from source material without using organic solvents (such as phenol). There are specialized RNAqueous Kits for several different applications. RNAqueous and RNAqueous®-Midi are designed for isolating RNA on a small or large scale respectively. RNAqueous®-4PCR is the kit of choice to isolate RNA that will be used in RT-PCR; it incorporates a DNase digestion and a novel reagent for rapid and safe removal of the DNase and divalent cations. RNAqueous®-96 and RNAqueous®-96 Automated bring the RNAqueous system to 96 well plate configuration.

II. RPA III™ Kit Procedure

A. RNase precautions

Lab bench and pipettors

Before working with RNA, it is always a good idea to clean the lab bench, and pipettors with an RNase decontamination solution (e.g. Ambion RNase*Zap*® Solution).

Gloves and RNase-free technique

Wear laboratory gloves at all times during this procedure and change them frequently. They will protect you from the reagents, and they will protect the RNA from nucleases that are present on your skin.Use RNase-free pipette tips to handle the wash solutions and the Elution Solution, and avoid putting used tips into the kit reagents.

B. Hybridization of Probe and Sample RNA

Amount of sample RNA

The amount of sample RNA required will depend on the abundance of the mRNA being detected and on the specific activity of the probe. ***5–20 µg of total RNA is sufficient for most purposes***; there is no lower limit to how much RNA can be used in an RPA, but no more than ~100 µg should be used. Either total RNA or Poly(A) RNA can be used for RPA analysis.

IMPORTANT

We recommend using gel purified probe or probe that has been determined to consist mainly of full-length transcript as assessed by gel electrophoresis

For quantitative detection of mRNA, it is important that the labeled probe be present in molar excess over the target mRNA. Ideally, there should be a 3 to 10 fold molar excess of probe over target mRNA. If the abundance of the target mRNA is known, the amount of probe required to achieve 3 to 10 fold molar excess can be calculated.

For a relatively abundant target mRNA, such as GAPDH, that makes up ~0.1% of all mRNA and is 1.5 kb in length, a 4 fold molar excess of a 300 nucleotide probe with a specific activity of 3×10^8 cpm/µg would require 2.4 fmol or about 7×10^4 cpm, corresponding to about 240 pg. If the message is less abundant, or less sample RNA is used in the hybridization reaction, then fewer cpm of probe would be needed. If the probe is longer than 300 nucleotides, or has a specific activity greater than 3×10^8 cpm/µg, then more cpm of probe will be needed to achieve 4 fold molar excess. Further guidelines for optimizing amounts of probe and sample RNA are given in section III.B on page 14 (Also see section *IV.C. Calculating Yield and Specific Activity of Radiolabeled Transcription Reactions* starting on page 31).

Hybridization: Standard vs. Streamlined Procedures

There are two alternative procedures for hybridizing the probe and sample RNA.

Standard procedure: In the standard procedure, probe and sample RNAs are coprecipitated and resuspended in hybridization buffer. This method is most commonly used and recommended for new users; it provides maximum sensitivity.

Streamlined procedure: In the streamlined procedure, small volumes of the RNAs are added directly to hybridization buffer without an initial coprecipitation step. The streamlined procedure is best used for processing a large number of samples when maximum sensitivity is not critical, and when the combined volume of probe and sample RNA is less than 5 µL. In the streamlined procedure, the increased volume of the hybridization reaction will decrease the concentration of probe and sample, resulting in a decreased hybridization rate. The streamlined procedure saves about 30 minutes of hands-on time (and 30 minutes of precipitation/centrifugation time) but it may require longer hybridization times and it may be less sensitive.

Increasing the ratio of Probe Elution Buffer to sample RNA may actually speed up the apparent hybridization rates due to salt effects. Users may want to experiment with this variable.

C.I. Standard Hybridization Procedure

1. Mix sample RNA and labeled probe

For each experimental tube, mix labeled probe with sample RNA in a microfuge tube (see section IV.B on page 27 for information on probes). Use about 150–600 pg or 2–8 x 10^4 cpm per 10 µg total or 0.6 µg poly(A) sample RNA.

2. Set up 2 control tubes for each probe

For each different probe used, include two control tubes containing the same amount of labeled probe used for the experimental tubes in step 1, plus Yeast RNA equivalent to the highest amount of sample RNA. (Yeast RNA is ***not*** an appropriate control if the probe is expected to hybridize with sequences found in yeast RNA.) See section *III.B. Optimizing the RPA* starting on page 14 for further guidelines on setting up the initial experiment.

3. Coprecipitate the probes and sample RNAs

a. Add 1/10th volume of 5 M NH_4OAc supplied with the kit.

b. Add 2.5 volumes of ethanol, and mix thoroughly.

c. Allow RNA to precipitate at –20°C for at least 15 min.

STOPPING POINT

The experiment can be left at –20°C overnight at this point. Extended storage of radiolabeled probes will result in radiolysis, causing an increase in background on the final autoradiograph.

4. Centrifuge 15 min at maximum speed

Pellet the RNA by centrifuging at maximum speed in a microcentrifuge (≥10,000 x g) for 15 min, preferably at 4°C. To make it easier to locate the pellets, it is helpful to position all the tubes with the hinges of the lids facing away from the center of rotation; if this is done, the pellets will all form directly below the hinges.

5. Discard supernatant, air dry pellets 5 min

Remove the ethanol supernatant, taking care to avoid dislodging the pellets. It is advisable to spin tubes a second time for 5 sec to collect any supernatant that was clinging to the sides of the tube, and to remove this residual liquid with a very fine pipet tip. Alternatively, pellets can be washed with 70% ethanol. Let pellets dry for 5 min or so on the bench. Drying in a vacuum desiccator is not recommended because it may make resuspension difficult.

6. Resuspend pellets in 10 µL Hybridization Buffer III

After adding 10 µL of Hybridization Buffer III to each pellet, vortex each tube briefly, then microfuge for a few seconds to collect the liquid at the bottom of the tube.

7. Denature 3–4 min at 90–95°C

Heat samples to 90–95°C for 3–4 min to denature the RNA and facilitate its solubilization. Vortex tubes after the incubation and microfuge briefly to collect the contents in the bottom of the tube.

8. Hybridize overnight at 42°C

Incubate tubes at 42°C overnight to hybridize probe to its complement in the sample RNA. To minimize or eliminate condensation around the tops of the tubes during hybridization, they should be tightly capped and preferably incubated in a cabinet-type incubator. Alternatively, the tubes can be incubated in a water bath or in a water-filled heat block.

Hybridization times: For accurate quantitation the hybridization reaction must approach completion and the reaction incubations should be performed overnight. However, if accurate quantitation is not needed, hybridization times that yield very intense protected fragment signals may be reduced in subsequent experiments; the hybridization time can be as short as 2 hr for moderately abundant messages such as β-actin in mouse liver RNA.

Hybridization temperature: The temperature of hybridization can be optimized for certain RNAs. Higher hybridization temperatures are sometimes beneficial; some multiprobe RPA procedures suggest 56°C as the hybridization temperature. (If you are using Pharmingen's template sets, call our Technical Service Department and request our procedure for using these templates with MAXIscript and RPA III.)

9. Proceed to section II.D

Proceed to section II.D for nuclease digestion.

C.II. Streamlined Hybridization Procedure

If the probe and sample RNA are in a sufficiently small volume (5 µL), Hybridization III Buffer can be added directly to the sample RNA and probe, omitting the initial coprecipitation step. We recommend that the volume of probe in Probe Elution Buffer be ≤1 µL and that the volume of sample RNA be ≤4 µL, in order to keep the salt concentration sufficiently high in the hybridization reaction.

1. Mix sample RNA and labeled probe

For each experimental tube, mix labeled probe with sample RNA in a microfuge tube. Use about 150–600 pg or 2–8 x 10^4 cpm of probe per 10 µg total or 0.6 µg poly(A) sample RNA. Note that adding more probe than this will typically not enhance signal but may increase background.

2. Set up 2 control tubes for each probe

For each different probe used, include two control tubes containing the same amount of labeled probe used for the experimental tubes in step 1 plus Yeast RNA equivalent to the highest amount of sample RNA. (See section III.B on page 14 for further guidelines for setting up the initial experiment.)

3. Add 10 µL Hybridization III Buffer to each tube

After adding 10 µL Hybridization Buffer III to each tube, mix thoroughly by vortexing. Centrifuge tubes briefly to collect all liquid at the bottom of the tube.

4. Denature 3–4 min at 90–95° C

Heat samples to 90–95°C for 3–4 min to denature the RNA. Vortex tubes after the incubation and microfuge briefly to collect the contents in the bottom of the tube.

5. Hybridize overnight at 42°C

Incubate tubes at 42°C overnight to hybridize probe to its complement in the sample RNA. To minimize or eliminate condensation around the tops of the tubes during hybridization, they should be tightly capped and preferably incubated in a cabinet-type incubator. Alternatively, the tubes can be incubated in a water bath or in a water-filled heat block.

Hybridization times: For accurate quantitation the hybridization reaction must approach completion and the reaction incubations should be performed overnight. However, if accurate quantitation is not needed, hybridization times that yield very intense protected fragment signals may be reduced in subsequent experiments; the hybridization time can be as short as 2 hr for moderately abundant messages such as β-actin in mouse liver RNA.

Hybridization temperature: The temperature of hybridization can be optimized for certain RNAs. Higher hybridization temperatures are sometimes beneficial; some multiprobe RPA procedures suggest 56°C as

the hybridization temperature. (If you are using Pharmingen's template sets, call our Technical Service Department and request our procedure for using these templates with MAXIscript and RPA III.)

6. Proceed to section II.D

Proceed to section II.D for nuclease digestion.

D. RNase Digestion of Unhybridized RNA


NOTE

The RNase Digestion III Buffer contains GlycoBlue™ to facilitate precipitation of protected probes and aspiration of the precipitation solution.

1. Prepare a working dilution of RNase in RNase Digestion III Buffer

Thaw a bottle of RNase Digestion III Buffer, vortex well, and transfer 150 µL x the number of assay tubes to a fresh tube.

Vortex and spin the tube of RNase A/RNase T1 Mix briefly, and add the appropriate amount of RNase A/RNase T1 Mix to the RNase Digestion III Buffer. We recommend using a 1:100 dilution; however, the optimal concentration is somewhat template dependent and is best determined empirically. (Section III.B on page 14 and III.D on page 19 contain information on optimizing RNase concentrations.) Vortex and spin the RNase mixture briefly to assure even dispersion of the components.

2. Add 150 µL diluted RNase solution to each sample tube, and to one control tube

a. Briefly centrifuge tubes in case any condensation is present in the tubes from the hybridization incubation. Add 150 µL of the diluted RNase mixture prepared in the previous step to:
   - each tube containing sample RNA
   - and to one of the two Yeast RNA control tubes—this will be the ***no target control***. These tubes will serve as positive controls for the function of the RNases. They will also show if the probe is being protected in the absence of complementary sequence. Ideally there should be no signal at all in this lane of the gel.

b. Vortex and microfuge tubes briefly.

3. Add 150 µL RNase Digestion III Buffer *without* RNase to the remaining yeast RNA control tube(s)

The ***no-RNase control*** tube(s) will serve as a control for probe integrity. It will show the gel migration of the full-length probe. If there is any unexpected degradation of the probe, or persistent secondary structure, it will be seen in this control. This lane should show a single band migrating at the expected probe size.

4. Incubate 30 min at 37°C

Incubate for 30 min at 37°C. During this 30 min, 37°C incubation, unprotected single-stranded RNA will be digested. In rare cases, a lower incubation temperature may be desirable (more information in section III.D on page 19).

5. Add 225 µL RNase Inactivation Solution III

Add 225 µL RNase Inactivation/Precipitation III Solution, then vortex and centrifuge tubes briefly. While it is not necessary to add additional carrier during this precipitation, 1–2 µL of the Yeast RNA provided in the kit can be added to each sample just after the addition of the RNase Inactivation/Precipitation III Solution to increase the size and visibility of the final pellets.

IMPORTANT

Precipitation of very small fragments (less than 150 bases) can be improved by adding 75 µL of ethanol (for fragments of at least 100 bases) or 150 µL of ethanol (for fragments of at least 50 bases), in addition to 225 µL of RNase Inactivation/Precipitation III Solution.

6. Incubate 15 min at –20°C

Allow sample to precipitate at –20°C for at least 15 min.

STOPPING POINT

The experiment can be left at –20°C overnight or longer if desired at this point. Extended storage of radiolabeled probes will result in radiolysis, causing an increase in background on the final autoradiograph.

7. Centrifuge 15 min at maximum speed

Remove tubes from freezer and pellet the precipitated products of the RNase digestions for 15 min at maximum speed (≥10,000 x g), preferably at 4°C. To make it easier to locate the pellets, it is helpful to position all the tubes with the hinges of the lids facing away from the center of rotation; if this is done, the pellets will all form directly below the hinges.

8. Carefully remove all supernatant from each tube

RNA pellets do not adhere tightly to the walls of standard microcentrifuge tubes, remove the bulk of the supernatant by gentle aspiration or by carefully pouring the solution out of the tubes from the side opposite the RNA pellet. To remove the last traces of supernatant, recentrifuge the tubes for about 5 sec and withdraw the residual supernatant with a very fine pipet tip. Alternatively, pellets can be washed with 70% ethanol.

IMPORTANT

Do not remove the residual fluid by vacuum-drying, because the salts present in RNase Inactivation/Precipitation III Solution will cause aberrant migration of the protected fragment during electrophoresis.

E. Separation and Detection of Protected Fragments

1. Prepare a denaturing polyacrylamide gel

The gel size and acrylamide concentration will be dictated by the experiment; specifically, the number and sizes of probes, and their relation to each other. A 5% acrylamide gel will effectively resolve fragments of about 50–1000 nucleotides (See section IV.G on page 39 for gel recipes). Section IV.A on page 26 contains more information on multiple probes.

2. Resuspend pellets in Gel Loading Buffer II

The volume of Gel Loading Buffer II used is not critical, but the best resolution is obtained when the gel loading buffer forms a 2–3 mm layer in the well; this is usually 4–10 µL. Vortex vigorously and microfuge briefly.

3. Incubate 3 min at 90–95°C

Incubate samples for 3 min at 90–95°C to completely solubilize the RNA and denature it. After the incubation, vortex and microfuge again briefly. Store the tubes on ice before loading them on the gel.

4. Load the samples and run the gel

Rinse the urea out of the wells of the gel, and immediately load each sample. Load only 10 to 20% of the no-RNase control relative to the experimental samples to avoid obscuring the signal from adjacent lanes (the no-RNase control is expected to contain many more counts than the experimental samples).
It is helpful to have size markers on the gel, single-stranded RNA markers are the most accurate, but double-stranded DNA markers can be used if it is not critical to know the exact size of the products.

Most gels should be run at ~250 volts constant voltage or 25–30 mAmps constant current. Run the gels until the leading dye band (bromophenol blue) is near the bottom of the gel.

5. Visualize labeled probe

Detection of radiolabeled probes

Transfer the gel to filter paper, mark the origins and orientation of lanes, cover with plastic wrap, and expose to x-ray film for an appropriate length of time. We usually expose from overnight to several days using single-side coated x-ray film (e.g. Kodak XRP) without an intensifying screen, or for several hours to overnight with an intensifying screen. Expose at –80 or –20°C. The gel can be re-exposed several times if necessary after allowing it to warm up to room temperature and wiping off condensation. The gel should be stored at –80 or –20°C if not re-exposed immediately. Users may prefer to dry gels onto chromatography paper; we do not find it necessary to dry standard 0.75 mm thick gels. If probes were labeled with ^{35}S instead of ^{32}P, gels should be dried and exposed directly to x-ray film (without plastic wrap).

Detection of nonisotopic probes

Transfer the gel to a positively-charged nylon membrane by electroblotting, crosslink nucleic acids to the membrane, and follow the protocol of an appropriate detection procedure for visualization of the nonisotopic probe (e.g. the Ambion BrightStar BioDetect Kit).

III. Optimization and Troubleshooting

A. Running the Positive Control Reaction

The positive control reaction included with the RPA III Kit consists of Mouse Liver sample RNA and pTRI-Actin-Mouse: a DNA template for transcription of an antisense mouse β-actin RNA probe.

1. Probe Preparation

To synthesize a radiolabeled positive control probe, use 1 µL of pTRI-Actin-Mouse DNA template in a 10 µL or 20 µL in vitro transcription reaction containing at least 3 µM [α-^{32}P]UTP or [α-^{32}P]CTP (50 µCi of 800 Ci/mmol 10 µCi/ µL) and up to 5 µM unlabeled nucleotide corresponding to the ^{32}P-NTP used. (The lower the concentration of unlabeled nucleotide, the higher the specific activity of the transcript will be and, thus, the greater the sensitivity of the assay. For maximum sensitivity, do not add any unlabeled form of the limiting nucleotide.)

Any of the three common phage polymerases can be used in the transcription reaction because pTRI-Actin-Mouse has tandem SP6, T7, and T3 promoters. The sizes of the transcripts are 334 bases, 304 bases, and 276 bases when pTRI-Actin-Mouse is transcribed with SP6, T7, and T3 polymerases, respectively. Each of these probes will protect 245 bases of the mouse β-actin mRNA. It is recommended that the probe be treated with DNase to remove template, and that it be gel purified for use in the control RPA.

2. RPA setup

Use roughly 4×10^4 cpm of β-actin probe with several different amounts of Mouse Liver RNA, for example 2.5 µL, 5 µL, 10 µL, and 20 µL (corresponding to 1.25 µg, 2.5 µg, 5 µg, and 10 µg). Include two yeast RNA control hybridizations with the same amount of probe and 2 µL Yeast RNA. Follow the RPA procedure outlined in the previous sections; hybridize overnight at 42°C, and use RNase A/RNase T1 Mix at a 1:100 dilution.

3. Expected results

Protected fragments of 245 bases should be seen in the reactions containing Mouse Liver RNA, and the protected fragments should be more intense with increasing amounts of Mouse Liver RNA. The no-target/+RNase lane should have no signal, and the no-target/no-RNase lane should show a band corresponding to full-length probe. An example of an RPA III experiment using the control RNA and the control probe is shown in Table 1 on page 15.

B. Optimizing the RPA

1. Pilot experiment setup

The following experiment is to help determine the appropriate amounts of sample RNA and probe to use when the abundance of the target mRNA is unknown. The RPA should be tried with a range of input sample RNA, using a constant amount of medium-high to high specific activity probe. Also, to optimize conditions for the RNase digestion, vary the concentration of the RNase mixture and determine whether using RNase T1 alone offers any advantages. If the sample RNA is in short supply, these experiments can be scaled back to test a smaller subset of conditions. The details of the pilot experiment, are shown in Table 1 on page 15. Table 2 on page 15 outlines the procedure for making the RNase dilutions. Figure 2 on page 16 shows the results of a pilot experiment done according to these instructions.

a. Experimental probe

The example below is for a 300 nucleotide transcript, labeled to a specific activity of 4.4 x 10^8 cpm/µg. If the probe is longer or shorter than 300 nucleotides, more or less cpm, respectively, will be required to achieve 2 fmol.

To make a probe with a specific activity of ~4 x 10^8 cpm/µg, use 4 µL of [α-^{32}P]UTP or [α-^{32}P]CTP (800 Ci/mmol, 10 mCi/mL) in a 20 µL transcription reaction that also contains 5 µM unlabeled UTP or CTP.

b. Mouse β-actin control probe

The probe for the control reaction is made by transcribing pTRI-Actin-Mouse as described in section *IV.B. Preparation and Purification of RNA Probes* on page 27. For more detailed information on probe synthesis and purification, see section *IV.C. Calculating Yield and Specific Activity of Radiolabeled Transcription Reactions* on page 31.

c. Molecular size markers

End-label *Sau*3A restriction fragments of plasmid pUC19 with [γ-^{32}P]ATP, using polynucleotide kinase, or transcribe the Ambion Century™ Marker Template (P/N AM7780) or Century™ Marker Template Plus (P/N AM7782) to produce labeled RNA size markers.

2. General instructions for the pilot experiment

Follow the procedure in section *II. RPA III™ Kit Procedure* on page 6. Run only 10–20% of the no-target/no-RNase controls (tubes 12 and 18), to prevent overexposure of the autoradiograph. Expose the gel overnight with an intensifying screen at –80°C. The gel may be re-exposed several times if necessary; but let it warm up to room temp, and wipe off any condensation.

Table 1. Suggested RPA Pilot Experiment.

Tube	Sample RNA	Amount Probe	RNase Dilution*	Variable Tested
1	1.25 µg sample RNA	8.7 x 10^4 cpm = 2 fmol	1:100	Sensitivity/linearity of assay
2	2.5 µg sample RNA		1:100	
3	5 µg sample RNA		1:100	
4	10 µg sample RNA		1:100	
5	10 µg sample RNA		1:50	RNase concentration
6†	10 µg sample RNA		1:100	
7	10 µg sample RNA		1:300	
8	10 µg sample RNA		1:1000	
9	10 µg sample RNA		1:50 RNase T1 only	T1 only
10	2 µL Yeast RNA		1:100	Nonspecific background
11	2 µL Yeast RNA		1:1000	
12	2 µL Yeast RNA		No RNase‡	Probe intactness
13	20 µL ML RNA**	6 x 10^4 cpm β-actin	1:100	Positive control reaction to check sensitivity and linearity
14	10 µL ML RNA		1:100	
15	5 µL ML RNA		1:100	
16	2.5 µL ML RNA		1:100	
17	2 µL Yeast RNA		1:100	
18	2 µL Yeast RNA		No RNase	

* See Table 2 for dilution instructions.
† Note that this sample can be used as a replicate to confirm the results of Tube #4.
‡ Use RNase Digestion III Buffer alone.
**Mouse Liver RNA supplied with the kit.

Table 2. Preparation of RNase Dilutions for RPA Pilot Experiments.

RNase(s)	Dilution	Total Volume Needed*	Volume RNase(s)	Volume RNase Digestion III Buffer
RNase A/RNase T1 Mix	1:50	160 µL	3.2 µL	156.8 µL
RNase A/RNase T1 Mix	1:100	1900 µL	19 µL	1881 µL
RNase A/RNase T1 Mix	1:300	160 µL	53.3 µL 1:100 dilution	107 µL
RNase A/RNase T1 Mix	1:1000	320 µL	32 µL 1:100 dilution	288 µL
RNase T1	1:50	160 µL	3.2 µL	156.8 µL

* Calculated volumes include ~10% overage for pipetting error.

3. Interpreting the pilot experiment results

The lane numbers in the figure 2 below correspond to the tube numbers in Table 1 on page 15. The interpretation of these results is discussed below, grouped by the variables tested.

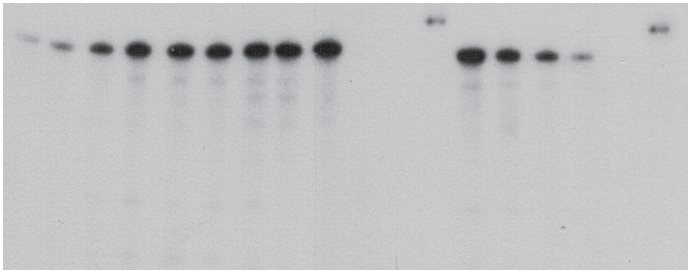


Figure 2. RPA III Optimization Experiment.

A probe to β-actin was synthesized from the RPA III control template. Mouse kidney RNA was used in tubes 1–9 for optimization and the positive control RNA from the kit was used in tubes 13–16. The gel was exposed to film for 1 hr at –80°C using a single intensifying screen. The signal from the no-RNase controls are substantially less because only 1/10th of the sample was loaded on the gel.

a. Kit Control (lanes 13–18)

This part of the experiment is to verify that the RPA III Kit is functioning properly, and to rule out technical difficulties in performing the assay. There should be an easily visible protected fragment band of 245 nucleotides in lanes 13–16. The intensity of the protected band should correlate with the amount of Mouse Liver RNA used. The no-target/no-RNase control lane (18) should show a band corresponding to full-length transcript (size depends on which RNA polymerase was used for transcription), and the no-target/+RNase control lane (17) should be completely empty.

b. Experimental no-target controls (lanes 10–12)

The no-target/no-RNase control lane (12) should show a band corresponding to the full length probe. Since probe molecules that have been protected by RNA in the sample will produce the data in an RPA, it is essential that this lane shows a single band of the expected size. If the autoradiograph shows severe degradation of probe in the yeast RNA control lane without RNase, then ribonuclease contamination of your tubes, pipette tips, or a component of the kit should be suspected.

The two no-target/+RNase control lanes (11 and 12) should be empty. If lane 10 that was digested with RNase at 1:100 has full-length probe left, it is probable that leftover DNA template is protecting the probe, see section *III.E. Full-Length Probe Is Seen in all Lanes* starting on page 21 for troubleshooting suggestions.

Lane 11 was digested with the lowest amount of RNase used in the experiment. This reaction will show whether the 1:1000 dilution of RNase used in tube 8 is sufficient to degrade all of the probe.

c. Increasing sample RNA (lanes 1–4)

The autoradiograph should show a band of the expected protected fragment size from the samples in Tubes 1 to 4. Comparison of the signals from the samples in tubes 4 and 6 (which are from identical reactions) will indicate whether reproducibility in the assay is a problem.

If the intensity of the major protected fragment in the RPA autoradiograph increases with increasing input RNA, then the probe was present in molar excess over the mRNA in all the reactions. This result indicates that the mRNA is abundant, and that an acceptable signal can be obtained with less input target RNA or with a probe of lower specific activity.

If the intensity of the major protected fragment fails to increase at some point in the series of increasing target reactions, at that point the probe was no longer in molar excess. For accurate quantitation of RNA levels, it is essential that the probe is present in molar excess compared to the target.

If the signal is very weak or absent, even in the reaction with the highest amount of input RNA, the message may be very rare—see *III.C. Increase the sensitivity of the assay* on page 18.

d. Different concentrations of RNase (lanes 5–9)

In the second set of pilot reactions (Tubes 5–9) the ribonuclease was varied to optimize the signal-to-noise ratio in the RPA. Most probe/target mRNA combinations will give acceptable, even identical, results when digested with RNase concentrations that vary over at least a 10 fold range. Some templates, however, may require fine-tuning of RNase digestion conditions to adjust the signal-to-noise ratio. The results of Tubes 5 to 8 should give an indication of whether "high-end" RNase concentrations will suppress unwanted background without causing an unacceptable level of degradation of the protected fragment, or whether conversely, "low-end" RNase concentrations result in reduced background.

In some cases, such as with A-U rich probes, digestion with RNase T1 alone (lane 9) is optimal.

C. Troubleshooting Faint or Absent Protected Fragment Bands

In the event that the RPA autoradiograph does not show the expected results (i.e. no band of the predicted size is present), but the probe is intact (as assessed by the no-target/no-RNase control), consider the following for troubleshooting the procedure.

The target is not present in the sample

It is possible that absence of signal is a legitimate result, and that the gene of interest is not expressed or is very rapidly degraded in the tissue or organism tested. To confirm this possibility, the RPA should be repeated along with an assay of a separate sample known to contain detectable levels of the mRNA. If this is not possible, in vitro synthesized sense-strand RNA can be added to the RNA samples to serve as a positive control. Spike the sample RNA with increasing amounts of sense-strand transcript and repeat the RPA. This will also show the limit of sensitivity of the assay for a particular probe. See section *IV.E. Quantitation of mRNA* on page 37 or refer to Technical Bulletin #165—available on our website at www.ambion.com—for more information on synthesis of sense-strand probes.

Increase the sensitivity of the assay

a. Use a probe with a higher specific activity

The specific activity of a probe is determined by the specific activity of the radiolabeled nucleotide, and its proportion to the corresponding unlabeled nucleotide in the transcription reaction. The highest specific activity is obtained by using only radiolabeled nucleotide in the transcription reaction and none of the unlabeled form. See section IV.B on page 27 for more information on probe synthesis; section IV.C on page 31 shows how to calculate specific activity.

The specific activity of nonisotopically labeled probes can be decreased only, because using more than the recommended ratio of modified to unmodified nucleotide will impede synthesis and/or hybridization of probes due to steric interference.

b. Use more target RNA

Another way to increase the sensitivity of the assay is to use more sample RNA—up to about 50 µg per hybridization reaction. Poly(A) selected RNA can also be used instead of total RNA to increase the amount of mRNA target.

c. Use a longer probe

Longer probes will contain more labeled molecules and will make the RPA more sensitive. It is not recommended to use probes longer than 1000 bases because they will be difficult to separate on polyacrylamide gels. See section IV.B on page 27, and IV.C on page 31, for a discussion of probe size and synthesis.

Check the probe template

a. Synthesis of a sense probe instead of antisense
Accidental transcription of the wrong strand from the template DNA in the probe synthesis reaction will produce a probe that cannot hybridize to mRNA. In order to make an antisense probe (mRNA-complementary) from cloned sequences, the template should be linearized on the side of the insert that encodes the amino-terminal side of the protein, i.e. on the 5' side of the gene. PCR templates should be designed with the phage polymerase promoter on the 3' or carboxy terminal side of the desired probe sequence.

b. Subcloning artifacts
Another potential source of probe-related problems is aberrations generated during subcloning. Make sure that the template used to make the probe has not undergone deletions or rearrangements during subcloning. (This can be checked by sequencing).

Suboptimal hybridization temperature

In some cases it is better to hybridize at higher temperatures. This has specifically been seen in multiple probe RPAs, and may serve to prevent probe:probe interactions.

Overdigestion with RNase

Overdigestion with RNase is rarely seen using the RNase(s) at a 1:100 dilution. Evidence of overdigestion would be smearing of the signal below the expected position of the protected fragment in an experiment where the no-target/no-RNase control lane did not show smearing.

D. Protected Fragments that are Smeared or Consist of a Ladder of Bands

No-target/no-RNase control lane is not smeared

a. Excess probe
Decreasing the probe amount or its specific activity may improve the signal-to-noise ratio and help to eliminate unwanted background. While quantitative results require that the probe be present in molar excess over the target mRNA, a large excess of probe can result in high background.

b. Overloaded gel
Overloading gel wells reduces the resolution of the bands. Use wider gel wells: Wells that are 0.5–1.0 cm wide work well for loading the 4–8 µL samples resulting from the RPA procedure.

c. Heterogeneity in probe length
This is usually due to premature termination of transcription during the probe synthesis reaction, or to radiolytic, enzymatic, or nonspecific degradation of the probe. Since these probe fragments can still hybridize to their complements in the sample RNA, protection of fragments that are shorter than expected will be seen (Zinn 1983). To prevent this type of background we strongly recommend that the probe be gel-purified, stored at –80°C or –20°C, and used within a

few days of synthesis for best results. The lower the specific activity of the probe, the less prone it will be to radiolytic decay, and the longer it can be stored. Probes with specific activities of 1–3 x 10^8 cpm/µg can generally be used within five days with good results (see Table 3 on page 32 to estimate the specific activity of your probe). As an assessment of probe quality, the no-target control lane without RNase should show a band representing mainly full-length probe. (See section IV.B on page 27 for the procedure for gel purifying the probe.)

d. Suboptimal RNase digestion conditions

Vary the concentration of RNase A/RNase T1 Mix in the RNase digestion step over a range of about 50 to 100 fold, for example by using RNase A/RNase T1 Mix diluted 1:50, 1:150, 1:500, 1:1000, 1:2000. Background smearing may be reduced by fine tuning of the RNase digestion.

IMPORTANT

When the RNase concentration is decreased, the yeast RNA control reaction should be digested with the lowest concentration (highest dilution) of RNase used, to assure validity of the experimental results.

Underdigestion will usually leave leftover full-length probe in the no-target/+ RNase control lane. The experimental samples will show smearing or a ladder of bands in between the full-length probe and the protected fragment.

Overdigestion by RNase will be evident as a smear or ladder of bands below the expected size of the protected fragment. Using the RNase(s) at a 1:100 dilution rarely results in overdigestion of the hybrids.

If the double-stranded RNA hybrid formed by the probe and sample RNA is A-U rich, "breathing" (local denaturation) may occur, which results in cleavage. (The 3' untranslated regions of cDNAs are frequently very A-U rich.) This produces a smear or ladder of bands below the expected size of the protected fragment. If this problem is suspected, RNase T1 alone should be used for the RNase digestion because it cleaves 3' to guanosine residues only, whereas RNase A cleaves 3' to cytosine and uridine residues. Substitute RNase T1 for RNase A/RNase T1 Mix in the RNase digestion, using various concentrations ranging from about a 1:50 dilution to a 1:250 dilution. Alternatively, the incubation time of the RNase digestion can be reduced or the incubation temperature can be lowered to room temperature, 15°C, or 4°C.

e. Mismatches between probe and target RNA

See *III.H. There are mismatches between probe and mRNA transcript* on page 25.

f. Degradation of target RNA

As discussed earlier, the RPA assay is relatively insensitive to mRNA degradation, especially if the probe spans a short (~300 nucleotides) subregion of the mRNA. However, a badly degraded RNA sample will contain species capable of protecting only part of the probe. The longer the probe, the less degradation of the target mRNA can be tolerated. If degradation of mRNA is suspected to be a problem, the integrity of the sample RNA can be assessed by electrophoresis on a 1% denaturing agarose gel (smearing of the ribosomal RNA bands indicates degradation), or by running the sample on a bioanalyzer (Agilent).

Smear or ladder in the no-target/no-RNase control lane

Poor results seen in the context of a degraded probe indicate problems with probe synthesis, purification, or stability. Check the recommendations for probe synthesis and purification in section IV.B on page 27.

a. Degradation of probe

Smeared signal that starts at the expected position of protected fragment and continues down can be caused by degradation of the probe. This is most often from radiolysis, but it can also be due to RNase contamination of the probe solution, your tubes, or pipette tips.

The probe can be gel purified a second time to isolate full length molecules or it can be resynthesized. In nuclease protection assays, the data can only be as good as the probe.

b. Overloaded gel

Overloading gel wells reduces the resolution of the bands. Use wider gel wells: Wells that are 0.5–1.0 cm wide work well for loading the 4–8 µL samples resulting from the RPA procedure.

A ladder of bands in the no-target/+RNase control lane

Self-protection of probe

The probe may be protecting itself by intramolecular hybridization, resulting in smaller than expected, fairly intense bands in all lanes of the autoradiograph. Analysis of the nucleotide sequence of the probe for regions capable of forming secondary structure (i.e. hairpin loops) can sometimes confirm this explanation. Change the size of the probe or use a different probe sequence to overcome this problem.

E. Full-Length Probe Is Seen in all Lanes

There are several possible causes for this result. In each case, full-length probe is seen in each experimental sample lane and also in the no-target/+RNase control lane. The relative intensity of the full-length probe band compared to the protected fragment band, as well as the presence or absence of smearing between the two, will provide clues to which of the following problems is occurring.

RNase(s) were completely inactive or were omitted

The full-length probe band would be very strong in this case, and there would be no band at the expected position of the protected fragment. The RNase(s) can be checked for activity by comparing on a gel RNA treated with RNase, with RNA incubated in digestion buffer without nuclease. The RNase dilution and the amount of RNA used should mimic the experimental conditions. If all of the sample RNA is not degraded to very low molecular weight pieces, there could be a problem with the nucleases.

Too much probe was added to the reaction

If too much probe is used, there may be a faint band of undigested probe left. If this is the case, the protected fragment band will often be more intense than the full-length probe band, and both bands will be distinct, with no smearing in between the two. In general, no more than 2–8 x 10^4 cpm of high specific activity probe should be used for up to 10 µg of RNA; this is enough probe to provide 4 fold molar excess for a moderately abundant message like β-actin. A pilot experiment using different amounts of input RNA can be performed to determine the amount of probe necessary for probe excess.

Residual DNA is protecting the probe from digestion

If the probe is protected by residual DNA in the samples there may be a faint full-length probe band in all lanes (including the no-target/+RNase control lane), and a protected fragment band in the experimental sample lanes, with little if any smearing between the bands. Below are three strategies that can be used to avoid this problem.

a. If T7 RNA polymerase was used to transcribe the probe, use less template

The yield of full-length transcripts from transcription reactions with T7 RNA polymerase will not be affected when the template amount is dropped to ~100 ng of linearized plasmid or ~10 ng of PCR product (or other DNA template that does not contain non-transcribed sequence).

b. Treat RNA probe prep with DNase I

It has been reported that gel purification of transcription reactions without DNase pretreatment is not always sufficient to remove all of the DNA template (Krieg 1991). If the transcription reaction was not treated with DNase I, this simple step may eliminate the problem. Gel purification without DNase I pretreatment is often sufficient to remove all DNA template from transcription reactions where linearized plasmid was used as a template.

Checking DNase I activity:

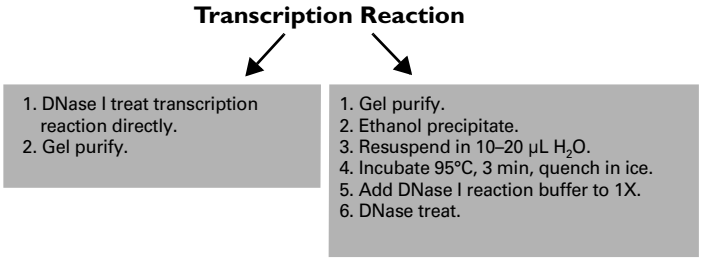
Treat an aliquot of the DNA template with DNase I; use the amount of template that was used in the transcription reaction to prepare the RNA probe. The reaction conditions (i.e. buffer, temperature, and time) should mimic the conditions used to treat the probe preparation. Run the DNase-treated template on an ethidium bromide

stained agarose gel with an equal amount of non-DNase-treated template and determine if all of the template has been digested. If there is no DNA left, the enzyme activity is probably not the problem.

c. Heat denature gel purified RNA transcripts before DNase I treatment

Sometimes, a small amount of template DNA is protected from the DNase by hybridization with the newly transcribed RNA. One way to avoid this phenomenon, is to denature the nucleic acids to give the DNase better access to the DNA template. It is important to heat denature ***after*** gel purification because heat treatment in the presence of divalent cations (present in most transcription buffers) can cause degradation of RNA; in addition, any RNase that was bound to ribonuclease inhibitor may be released during the heat treatment.

The following flow chart outlines the two options for DNase I treatment and gel purification of transcription reactions.



DNase I digestion

1. Use 2 U DNase I per µg DNA template in the transcription rxn.
2. Reaction buffer as per manufacturer's recommendation, or use the Ambion MAXIscript Transcription Buffer.
3. Incubate 37°C, 15 min.
4. Inactivate the DNase I by adding EDTA to 20 mM.

The RNase did not completely degrade unprotected probe

Incomplete RNase digestion is indicated if there is a full-length template band, followed by a smear of material that ends where the protected fragment is expected to migrate. This rarely occurs when the RNase A/RNase T1 Mix is used at 1:100, but if it does, the dilution of the enzyme mix can be adjusted.

Transcription of antisense and sense-strand in probe synthesis reaction

If the DNA template used to prepare the probe is linearized with a restriction enzyme that leaves a 3' protruding end (e.g. *Kpn* I or *Pst* I), low levels of promoter-independent transcription may originate from the ends of the template (Schenborn and Mierendorf 1985). These transcription products could be copurified with the probe during gel purification and protect full-length probe from RNase digestion.

A small amount of plasmid that was not linearized may also result in synthesis of sense-strand transcript if it contains opposable promoters. Linearizing the template with two different restriction enzymes that cleave on the 5' side of the insert may help ensure complete linearization of a refractory template.

F. Aberrant, Pointed or Smeared Bands

If the supernatant from the final precipitation step is not completely removed, salts from the precipitation mixture will be concentrated and may lead to aberrant migration ("tunneling") of the protected fragment. It is recommended that the final pellet be briefly respun after the supernatant is removed, and that the residual supernatant be thoroughly aspirated using a very fine-tipped pipet.

G. Radioactive Material that Fails to Migrate into the Gel Matrix

It is unclear what causes this problem; it may be the result of several different factors. Probes that have not been gel purified experience this "hang up in the well" phenomenon more frequently than gel purified probes. Phenol/chloroform extraction of non-gel purified probes tends to diminish the amount of material left in the wells. Another contributor seems to be residues left in microfuge tubes from the manufacturing process. Autoclaving of siliconized tubes appears to exacerbate this problem.

H. Reasons for Legitimate but Unexpected Bands on Autoradiographs

Due to the extreme sensitivity of ribonuclease protection assays, probe-related sequences in the sample RNA that might go undetected by other methods may be seen. Typically, these extra bands are smaller and less intense than the primary protected fragment, and they may permit additional valid conclusions to be made concerning the nature of the expression or the processing of the gene(s) being studied. Extra bands, however, can also be artifactual, thus interpretation of the data requires caution and knowledge of the various factors that can contribute to the pattern of fragments seen in RPAs.

Probe hybridizes to transcripts with differing, but related sequences

If the probe is complementary to a gene that is a member of a family of related genes, and more than one member of the family is present in the sample RNA, then the probe may cross-hybridize to the mRNA from the related gene(s). Usually the related mRNA will have multiple mismatches with the probe, resulting in protected fragments that are shorter than those seen with the completely homologous RNA. This can be advantageous for distinguishing between messages from multigene families which run as a single band on Northern blots. Using higher concentrations of RNase can maximize recognition of small differences between the probe and sample RNA, while using lower concentrations of RNase or use of RNase T1 alone can minimize probe cleavage at mismatched positions.

Probe detects heterogeneity in initiation, termination, or processing of mRNA transcripts

Probes that include the site of initiation or termination of transcription, or intron/exon splicing sites, may be capable of protecting fragments of different sizes that result from heterogeneity in the mRNA. Such heterogeneity could result from differential use of transcription initiation sites (e.g., multiple promoters) (Kekule 1990), from differential transcription termination sites or even from differences in the amount of polyadenylation at the 3' end of the mRNA (Whittemore and Maniatis 1989). Differentially spliced messages and splicing intermediates are also sometimes detected in RNAs.

There are mismatches between probe and mRNA transcript

If the probe is not completely complementary to the sample RNA, total or partial cleavage of the probe-mRNA hybrid may occur at the position of the mismatch. Such a situation might occur if there are allelic differences between the genes used for the probe and those expressed in the sample RNA. An even greater potential for mismatch occurs when the probe and sample RNA are derived from different species. Mismatches due to differences between the probe and target RNA may result in cleavage of the protected fragment into two or more discrete bands. The extent of RNase cleavage at mismatch positions depends on the nature of the mismatch (e.g. A-C mismatches are cleaved more frequently than are G-U mismatches), and on the RNase digestion conditions. While ribonuclease protection assays are capable of detecting some single-base mismatches, other mismatches are refractory to RNase digestion (Myers 1985). If the mismatches consist of more than single-nucleotide differences, they will be cleaved much more efficiently. Cleavage at mismatch positions can often be minimized by decreasing the RNase concentration and/or the incubation temperature during RNase digestion, or by using RNase T1 instead of the RNase A/RNase T1 Mix.

IV. Additional Procedures

A. Tips for Multi-Probe RPAs and Use of Internal Controls

1. Templates

Quantitation of mRNA by nuclease protection assay requires that signal resulting from undigested full-length probe not interfere with signal from protected fragments. Additionally each of the full-length fragments should be spaced sufficiently so that their signals will not obscure those from other protected fragments. When only one or two probes are being used, this is typically not a problem; but when a large number of probes are used simultaneously, probe design becomes very important. If possible, probes should be designed in such a manner that the size of the expected protected fragments will be at least 10% different from any other full-length probe or protected fragment.

Additionally, there is the potential problem of probe interactions. There has been one report of a probe for 18S RNA, which was present at relatively high levels in the hybridization, forming an RNase resistant complex with one of the other probes in a multi-probe RPA. This occurred via hybridization of a 20 bp complementary region that was transcribed from the probe templates' multiple cloning sites (Ginter 1994).

2. Transcription reactions in small volumes

Probe synthesis tends to be one of the most costly aspects of multi-probe analysis. Probe synthesis costs can be reduced by performing transcription reactions in smaller volumes. A 2–5 µL transcription will usually provide enough gel purified probe to perform dozens of nuclease protection assays. Several small transcription reactions can be conveniently assembled by generating a master mix comprising transcription buffer, NTPs, and polymerase and aliquoting appropriate volumes of master mix into individual tubes containing the various templates to be transcribed.

3. Reduce radiolytic probe decay

Radiolabeled transcripts degrade relatively quickly due to radiolytic decay. High background and even aberrant bands can result from using radiolabeled probes that are several days old. For the best results, probes should be gel-purified, eluted, and assembled in hybridization reactions on the same day.

4. Equalize protected fragment intensities

a. Radiolabeled Probes

When targets that vary widely in abundance are assayed simultaneously, it is important to equalize the intensities of the protected fragment bands by adjusting the specific activities of the probes. This is done by adding unlabeled NTP to dilute the corresponding labeled NTP in the transcription reaction, thus lowering probe specific activity. For instance, when transcribing probes for two messages that differ 200-fold in abundance, unlabeled UTP should be

added to the transcription reaction for the more abundant mRNA at a final molar concentration that is 200-fold greater than the labeled UTP. The table below provides a starting point for reducing specific activities of probes to common internal controls, further optimization of probe specific activity may be required to achieve ideal results. For each probe, add the same number of cpm to the hybridization reactions so that all of the full-length probes will be visible in the no target/no RNase control lane.

Internal Controls	labeled:unlabeled nucleotide	[α-^{32}P]UTP	UTP	specific activity
Ribosomal RNAs (18S and 28S)	1:160,000	0.01 µL	2 µL	2.5 x 10^4 cpm/µg
Moderate Abundance Targets (β-actin and GAPDH)	1:200	4 µL	1 µL	2 x 10^7 cpm/µg
Low Abundance Targets (cyclophilin)	1:20	4 µL	0.1 µL	2 x 10^8 cpm/µg

b. Nonisotopically labeled probes

When non-isotopic labeling is used, the specific activities of the probes should be adjusted by titrating non-isotopically labeled with unlabeled probe at ratios of 1:160,000 for ribosomal RNAs, 1:200 for GAPDH and β-actin, and 1:20 for cyclophilin.

5. Decrease probe interactions by raising the hybridization temperature

Occasionally, cross-hybridization between probes (observed as a distinct band in the no target/+ RNase control lane as well as in the sample RNA lanes) or between a probe and non-target RNA can affect detection or quantitation. Often, increasing hybridization stringency by raising the hybridization temperature to 56°C or 68°C can eliminate the cross-hybridization without affecting probe:target interactions.

B. Preparation and Purification of RNA Probes

In ribonuclease protection assays, only the probe is visualized at the end of the experiment; thus it is critically important that the probe is of very high quality. Since the success of the experiment is entirely dependent on the probe, we offer the following advice on its preparation and purification.

1. Probe size

Although probe lengths ranging from 50 nucleotides to over 1000 nucleotides have been used successfully in RPAs, 200–500 nucleotides is the optimum probe size. The probe size must be within the effective separation range of the gel that will be used to visualize the results. Minimizing probe size helps to maximize synthesis of full length transcripts. Also, the shorter the probe, the more tolerant the assay is of partially degraded sample RNA. On the other hand, the shorter the probe, the less sensitive the assay will be. For example, a

probe for a 150 nt fragment will only be one-third as sensitive as a probe for a 450 nt fragment, since fewer labeled residues will be protected by the shorter probe. With probes shorter than 100 nucleotides a larger volume of Inactivation/Precipitation Solution must be used to efficiently precipitate them.

2. Non-homologous probe sequence

It is important that the probe be somewhat longer (in the range of 10%–20%) than the protected fragment so that an obvious difference in size is seen between the full-length undigested probe and the protected fragment after RNase digestion. The shift in size from full length probe to a smaller protected fragment helps to validate that RNA from the sample is protecting the probe and is not an artifact such as leftover transcription template.

3. Making radiolabeled RNA probes

General recommendations

Standard protocols for in vitro transcription from bacteriophage promoters may be found in most manuals of molecular biology methods (*Current Protocols in Molecular Biology*, 1987, Molecular Cloning, A Laboratory Manual, 1989, and Melton 1984). These protocols are basically similar, but vary in the recommended concentrations of nucleotide triphosphates, time and temperature of reaction, reaction volume, and method of purification of the labeled transcript. In vitro transcription kits for probe synthesis are available from Ambion (MAXIscript® kits, P/N AM1308–AM1326). Our standard radiolabeling synthesis reaction includes 3 unlabeled ribonucleotides, each at a concentration of 500 µM, and a fourth radioactive ribonucleotide added to ~3 µM. Typically 5 µL aqueous, buffered [α-^{32}P]UTP or [α-^{32}P]CTP (800 Ci/mmol, 10 mCi/mL) is used to achieve 3 µM limiting nucleotide in a 20 µL reaction.

Longer probes

Transcripts longer than about 400 nucleotides may require that more limiting nucleotide (5–25 µM) is present in the transcription reaction. This is done by adding more radiolabeled nucleotide, by using a more concentrated stock (e.g. 20 µCi/µL), or by supplementing the reaction with an appropriate amount of unlabeled UTP or CTP. The increased sensitivity from long probe will offset the decrease in the specific activity caused by adding unlabeled nucleotide to the transcription reaction. We recommend keeping the limiting nucleotide concentration low and gel purifying the full-length transcript, even if it is only a fraction of the total in vitro transcripts. The specific activity of the probe will then be high enough to provide maximum sensitivity. A 10–20% yield of full-length gel-purified transcript is usually more than enough for most experiments. (We typically end up throwing away about 90% of a high yield probe preparation, because the shelf life of high specific activity

radiolabeled probe is only a few days). Probes that are over 1000 bases will not be well separated on 5% polyacrylamide gels, so probes longer than this are generally not recommended for RPAs.

4. Nonisotopically labeled RNA probes

Nonisotopically labeled RNA probes are usually synthesized under conditions where there is no limiting NTP, however, there is a maximum level of substitution (e.g. 40% biotin-CTP:60% CTP) above which probe synthesis and/or hybridization is compromised. Follow the manufacturer's recommendation for ratio of modified to unmodified nucleotide to use in transcription reactions. Ambion has tested many of the commonly available nonisotopically modified nucleotides and we offer Technical Bulletin #173 which gives our recommendations for their use in in vitro transcription reactions.

5. After the transcription reaction

a. DNase treat

It is important that the DNA template be destroyed after the RNA transcription reaction. Use 1–2 units DNase I per transcription reaction, and incubate for 15 min at 37°C. If the transcript will not be gel purified, inactivate the DNase I by adding EDTA to 20 mM.

b. Gel purify

We generally recommend that probe be gel purified to remove transcripts that are shorter than full-length which will cause "background" smears and bands in the RPA. Gel purification is recommended over other purification methods because it separates full-length from shorter transcription products, as well as from unincorporated label.

i. Running the gel

To gel purify probe, add an equal volume of Gel Loading Buffer II to the DNase-treated transcription reaction, heat 3 min at 95°C, and then load all or part of the transcription reaction on a 0.75 mm thick 8 M urea 5% acrylamide gel and run for about 20 min to 1 hr at 100–300 volts. For this application, it is useful to have a "preparative scale" comb with wide (about 1 cm) teeth that will form wells capable of holding large volumes.

ii. Recovering full length radiolabeled probes

After electrophoresis the gel is covered with plastic wrap and exposed to x-ray film for 30 sec to several min; the exposure time will depend on the specific activity of the probe. The goal is to get an autoradiograph with a faint or "grey" probe signal so that a small discrete gel fragment can be localized. Fluorescent stickers are the easiest way to orient the film with the gel to cut out the band. After exposure, the film is developed and the full-length labeled transcript is identified; it is usually the most slowly migrating, most intense band on the autoradiograph. The film is placed on top of the gel and plastic wrap and aligned with the gel

via the glow in the dark stickers, then it is lightly taped in place. Next, the gel and film are inverted so that the position of the probe band on the film can be circled with a felt-tip pen on the back of the glass plate. When the gel is turned back over and the film removed, the area of the gel indicated by the circled region on the glass plate is excised with a razor blade or scalpel, transferred with clean forceps to a microfuge tube, and submerged in about 350 µL of Probe Elution Buffer (provided in the kit). It is always a good idea to re-expose the gel after cutting out the probe band to be sure that the probe was recovered.

iii. Recovering full length nonisotopically labeled probes

Nonisotopically-labeled probes can be visualized by UV shadowing. UV shadowing works best if the gel is removed from both glass plates and enclosed in thin plastic wrap. The wrapped gel should then be laid on a Fluor-coated TLC plate, (P/N AM10110) or on a new intensifying screen. To see the RNA, short wave UV light is directed onto the gel surface in the dark. There must be at least ~ 0.4 µg of RNA present in the band to use UV shadowing. The RNA will appear as a dark purple or black band. The xylene cyanol and bromophenol blue bands will also be visible when the gel is illuminated with UV light; if the band of interest comigrates with one of these bands, it may be difficult to distinguish between RNA and dye (consider running a lane of Loading Buffer alone in order to differentiate the dyes from the band of interest). The full length transcript is usually the most slowly migrating, most intense band on the gel; it should be excised with a clean scalpel and transferred to an RNase-free microfuge tube containing about 350 µL of Probe Elution Buffer (provided in the kit).

iv. Elution of RNA from acrylamide gel slices

To elute the labeled probe, chop up the gel fragment to increase surface area and incubate the tube at 37°C. After about 2 hr enough probe will usually have eluted to set up many hybridization reactions; however, for the sake of convenience or to maximize recovery of probe from the gel, the incubation can be continued overnight. After overnight incubation, about 95% of the label will typically be in the Probe Elution Buffer. Longer probes elute more slowly.

Alternatively, the elution can be done for 1–2 hr at 65°C with intermittent vortexing. This method is not very efficient, but enough probe for several experiements will usually be eluted.

The amount of radioactive label in a small aliquot of the eluted probe (e.g 1–2 µL) should determined by counting in a scintillation counter. The probe is typically stored at –20°C in Probe Elution Buffer (it is not necessary to remove the gel slice).

c. When can gel purification be skipped?

We recommend that you gel purify probe the first time you use the kit and with new probes or probes which are giving high background. However, if ~90% of the transcript is present as a single band of the expected size (often true for nonisotopically-labeled probes and low-specific activity probes), as assessed on a gel, it is probably not necessary to gel purify the probe.

When using non-gel purified probe, either remove unincorporated nucleotides before measuring the final cpm of the probe or determine what proportion of radiolabel was incorporated into transcript by trichloroacetic acid (TCA) precipitation. The appropriate amount of transcription reaction (after DNase treatment and enzyme inactivation) can then be used directly in the RPA. Unincorporated nucleotides will generally not cause problems on an RPA, since they will not hybridize to the target mRNA and will run off the bottom of the gel at the end of the assay.

C. Calculating Yield and Specific Activity of Radiolabeled Transcription Reactions

Specific activity is measured in cpm/µg; it reflects the degree to which a molecule is labeled with radioactive nucleotides. The specific activity of RNA transcripts is determined solely by the ratio of ^{32}P labeled NTP to unlabeled NTP present in the reaction and is, therefore, independent of the mass yield of RNA. High specific activity probes are more sensitive than lower specific activity probes.

Specific activity can be calculated in either of two ways. The chart shown below was created by calculating specific activity based on the amounts of labeled and unlabeled nucleotides in the transcription reaction, and the specific activity and concentration of the radiolabeled nucleotide used. Since no data from the actual transcription reaction is needed to do this calculation, it is referred to as "theoretical specific activity." The other way to calculate specific activity also uses the specific activity and concentration of the radiolabeled nucleotide, but instead of ***calculating*** the amount of radiolabel in the reaction, it is measured directly by scintillation counting. This is referred to as "experimental specific activity" and it is explained in the form of an example in the next section. The results from the two calculations are nearly identical; the slight difference that may be seen between the results is usually due to pipetting error.

1. Calculating theoretical yield and specific activity

Table 3 shows the specific activity of transcription products that would result from using the listed amounts of some of the commonly available radiolabeled nucleotides. In preparing this chart, it was assumed that each of the 4 nucleotides were incorporated in equimolar amounts, so

that the final fraction of the limiting nucleotide (e.g. "U") in the transcript is 0.25. If it is known that the composition of the RNA product differs significantly from a 1:4 ratio, a correction factor should be applied to reflect the actual proportion of labeled nucleotide. This chart was prepared using a calculation similar to that shown in the next section, and we have provided it so that specific activity and mass yield can be determined without going through the whole calculation.

Mass yield

Actual mass yield can be calculated using Table 3 by multiplying the theoretical maximum yield by the percent radiolabel incorporated. Alternatively, mass yield can be determined by dividing the theoretical specific activity shown in the table by the empirically determined total cpm of the purified product.

Table 3. Theoretical Yield and Specific Activity from Radiolabeled Transcription Reactions

Radiolabeled Nucleotide (e.g. ^{32}P-UTP)				unlabeled counterpart (e.g. UTP)	theoretical maximum RNA yield	theoretical specific activity of reaction products (cpm/µg)
sp. activity (Ci/mmol)	concentration (mCi/mL)	volume used	final conc. (in a 20 µL rxn)	concentration (µM)		
800	10	5 µL	3.125 µM	0	80.1 ng	1.4×10^9
800	10	5 µL	3.125 µM	5	208.3 ng	5.3×10^8
800	10	5 µL	3.125 µM	10	336.5 ng	3.3×10^8
800	10	5 µL	3.125 µM	100	2.6 µg	4.2×10^7
800	10	1 µL	625 nM	500	12.8 µg	1.7×10^6
800	20	5 µL	6.25 µM	0	160.3 ng	1.4×10^9
800	20	5 µL	6.25 µM	5	288.5 ng	7.6×10^8
800	20	5 µL	6.25 µM	10	416.7 ng	5.3×10^8
800	20	5 µL	6.25 µM	100	2.7 µg	8.1×10^7
800	20	1 µL	1.25 µM	500	12.9 µg	3.4×10^6
3000	10	5 µL	825 nM	0	21.2 ng	5.2×10^9
3000	10	5 µL	825 nM	3	98.1 ng	1.1×10^9
3000	10	1 µL	165 nM	0	4.2 ng	5.2×10^9
3000	10	1 µL	165 nM	3	81.2 ng	2.7×10^8
6000	40	5 µL	1.68 µM	0	43 ng	1.0×10^{10}
6000	40	5 µL	1.68 µM	3	119.9 ng	3.7×10^9
6000	40	1 µL	335 nM	0	8.6 ng	1.0×10^{10}
6000	40	1 µL	335 nM	3	85.5 ng	1.0×10^9

2. Calculating experimental yield and specific activity

To calculate the actual yield of RNA, first establish how much radiolabel (e.g. [α-^{32}P]UTP) was incorporated into transcript product—the ratio of labeled to unlabeled nucleotide (e.g. [α^{32}-P]UTP to UTP)

incorporated is assumed to reflect the ratio of labeled to unlabeled nucleotide available in the reaction. The amount of radiolabel incorporated into RNA can be determined by trichloroacetic acid (TCA) precipitation and scintillation counting. Alternatively, an aliquot of product that has been separated from unincorporated nucleotides can be counted directly. Separation of the probe transcript from unincorporated nucleotides can be accomplished with a spin column, precipitation with ammonium acetate and ethanol, or lithium chloride; or gel purification. (Note that yield based on product that has been gel purified only takes into account full length probe whereas the other methods will include any prematurely terminated transcripts.) In the following example, TCA precipitation will be used to determine the amount of radiolabel incorporated.

Consider the following example:

20 µL in vitro transcription reaction with:

- 5 µL of [α^{32}-P]UTP (800 Ci/mmol, 10 mCi/mL)
- 2 µL of 50 µM unlabeled ("cold") UTP

At the end of the synthesis reaction, 1 µL of DNase I is added; after incubation, 21 µL of gel loading buffer is added; the final volume is now 42 µL.

A 2 µL aliquot of the final reaction is removed and diluted into 198 µL of TE containing 100 µg of carrier RNA;

- 100 µL of this dilution is counted directly in a scintillation counter and found to contain 2.6×10^6 cpm.
- The remaining 100 µL of the diluted reaction is TCA precipitated and the precipitate is captured on a filter. The filter is counted and found to have 1.3×10^6 cpm. This represents the amount of ^{32}P incorporated into RNA
- The counting efficiency of the ^{32}P isotope in liquid scintillation cocktail is assumed to be 100%. The specific activity of the RNA probe is calculated as follows:

a. What proportion of the UTP was incorporated into RNA (i.e. TCA precipitable material)?

$$\frac{1.3 \times 10^6 \text{ cpm TCA}}{2.6 \times 10^6 \text{ cpm total}} = 50\%$$

(the proportion of [α^{32}-P]UTP incorporated is assumed to reflect the proportion of total [labeled and unlabeled] UTP incorporated)

b. How many moles of [α^{32}-P]UTP were in the reaction?

This is calculated by converting the volume of [α^{32}-P]UTP added (5 µL) to the number of mCi of ^{32}P added, and then converting the amount of [α^{32}-P]UTP in mCi to a molar amount using the known specific activity and concentration of the [α^{32}-P]UTP (800 Ci/mmol, 10 mCi/mL).

$$\text{\# mCi [}\alpha^{32}\text{-P]UTP in reaction} = \frac{0.005 \text{ mL} \times 10 \text{ mCi}}{\text{mL}} = 0.05 \text{ mCi}$$

$$\text{\# mmol [}\alpha^{32}\text{-P]UTP} = \frac{0.05 \text{ mCi} \times 1 \text{ mmol}}{800 \text{ Ci}} \times \frac{1 \text{ Ci}}{1000 \text{ mCi}}$$

$$= 6.25 \times 10^{-8} \text{mmol} = 62.5 \text{ pmoles in rxn}$$

c. How many moles of unlabeled UTP were in the reaction?

$$2 \text{ µL} \times \frac{50 \text{ µmol}}{1000 \text{ mL}} \times \frac{1 \text{ mL}}{1000 \text{ µL}} = 1 \times 10^{-4} \text{ µmol} = 100 \text{ pmol}$$

d. How much total UTP was in the reaction?

62.5 pmol [α^{32}-P]UTP + 100 pmol unlabeled UTP = 162.5 pmol total UTP

$$\frac{162.5 \text{ pmol}}{20 \text{ µL}} = 8.13 \text{ µM}$$

e. How much total UTP was incorporated into RNA?

162.5 pmol in reaction x 50% incorp'n = 81.3 pmol incorporated

f. What mass amount of RNA was synthesized?

If we assume that the RNA synthesized contained equal molar amounts of all 4 ribonucleotides (ATP, CTP, GTP, and UTP), and it is known that the amount of total UTP in the reaction is limiting, then the amount of the other 3 nucleotides incorporated will equal the amount of UTP incorporated. Therefore, 81.3 pmol of each ribonucleotide was incorporated. The sum of the molecular weights of the 4 ribonucleotides is about 1282 g/mol. (The average molecular weight of a nucleotide in RNA is 320.5 g/mol.)

(If the nuceotide composition of the RNA product is known to contain non-equimolar amounts of all 4 ribonucleotides, a correction factor can be applied to more accurately reflect the amount of product synthesized.)

$$\text{ng synthesized} = \frac{1282 \times 10^{12} \text{ pg}}{10^{12} \text{ pmol}} \times 81.3 \text{ pmol} = 1.04 \times 10^{5} \text{ pg} = 104 \text{ ng}$$

g. How many cpm were incorporated into the RNA product?

The final reaction volume from which the 2 µL sample was removed to determine label incorporation was 42 µL.

1 µL of the sample was TCA precipitated and found to contain 1.3×10^6 cpm.

So the whole reaction contained the following amount of TCA precipitable material or RNA:

$$42\ \mu L \times 1.3 \times 10^6\ cpm/\mu L = 55 \times 10^6\ cpm = 5.5 \times 10^7\ cpm$$

h. Specific activity of the product

The specific activity of the transcript will be the product of the total counts incorporated (*g*) divided by the mass amount of RNA produced (*f*).

$$\frac{5.5 \times 10^7\ cpm}{104\ ng} = 5.29 \times 10^5\ cpm/ng = 5.29 \times 10^8\ cpm/\mu g$$

D. Calculating Yield of Nonistopically Labeled Transcription Reactions

Comparison to standards

The rate of incorporation of a given modified nucleotide can be indirectly monitored by doing spot detection assays of probes prepared at different times, or by comparison to known standards. Yield can also be inferred by comparison of signal generated from standards and newly-labeled probe. Detection methods for nonisotopic labels are specific to the label used.

Measuring yield by absorbance at 260 nm

IMPORTANT

Some nonisotopic labels such as digoxigenin interfere with quantitation by absorbance at 260 nm. Transcripts with these labels should be quantitated by comparison to known standards, or by ethidium bromide spot assay.

After removing unincorporated nucleotides from the reaction, the RNA transcript can be quantitated by measuring A_{260} units in the following manner:

1. Zero spectrophotometer at 260 nm with a quartz cuvette filled with 500 µL water.
2. Add 5 µL or 25% of the transcription reaction to the cuvette, cover top with parafilm, and mix by inverting the cuvette several times.
3. Multiply reading by the dilution factor of 100 to give A_{260}, and then by 40 to give µg/mL.
   In general, the following constants can be used to convert A_{260} units to µg:

oligonucleotides (between 10 and 40 bases)	30 µg/mL/A_{260} unit
single-stranded nucleic acids > 40 bases	40 µg/mL/A_{260} unit
double-stranded nucleic acids	50 µg/mL/A_{260} unit

To convert µg to pmol use the following formula:

$$\frac{(\mu g \times 10^{-6})(1 \times 10^{12}\ pmol/mol)}{(330\ g/mol)(\#\ of\ bases)} = pmol\ of\ nucleic\ acid$$

Example: How many pmol of a 300 nucleotide long RNA transcript were produced in a 20 µL MAXIscript reaction? Five microliters was diluted to 500 µL in water, and the A_{260} read 0.1.

$$(100 - dilution\ factor)(40\ \mu g/mL/A_{260})(0.1\ A_{260}) = 400\ \mu g/mL$$

$$(400\ \mu g/mL)(0.02\ mL) = 8\ \mu g$$

$$\frac{(8 \times 10^{-6}\ g)(1 \times 10^{12}\ pmol/mol)}{(330\ g/mol)(300\ nt)} = 80.8\ pmol$$

Some practical examples:

Nucleic Acid	A_{260} units	µg/mL	pmol/mL
20-mer oligonucleotide	1	30	4545
tRNA (76 bases)	1	40	1600
PSTV RNA (359 bases)	1	40	338
STNV RNA (1300 bases)	1	40	93
pUC19 (2686 base pairs)	1	50	28
pBR322 (4363 base pairs)	1	50	17
M13 (6407 bases)	1	40	19

Ethidium bromide spot assay

Another technique that can be used to quantitate RNA samples is an ethidium bromide spot assay. Simply make a standard curve of various dilutions of an RNA of known concentration and compare the ethidium fluorescence of the unknown sample to that from the standard curve.

Make a standard curve with several 2-fold dilutions of an RNA solution of known concentration. Using 2 µg/mL ethidium bromide as the diluent, start at about 80 ng/µL RNA, and make several 2-fold dilutions, ending about 1.25 ng/µL RNA. Make a few dilutions of the unknown RNA as well. The final concentration of ethidium bromide in all the samples should be 1 µg/mL. Spot 2 µL of the RNA standards and the unknown RNA dilutions onto plastic wrap placed on a UV transilluminator. Compare the fluorescence of the RNAs to estimate the concentration of the unknown RNA sample. Make sure that the unknown sample dilutions are in the linear range of ethidium bromide fluorescence. This assay will detect as little as 5 ng of RNA with an error of about 2-fold.

E. Quantitation of mRNA

Several different procedures exist for determining the abundance of a particular mRNA in a heterogeneous sample RNA mixture. The preferred approach is based on construction of a standard curve, using known amounts of in vitro synthesized sense-strand RNA hybridized with an excess of labeled antisense probe. Hybridization reactions containing various amounts of the sample RNA mixture are analyzed in conjunction with the reactions used to generate the standard curve. The intensity of probe fragments protected by the different amounts of sample RNA is compared to the standard curve and used to define the absolute amount of the protecting RNA species in the sample RNA.

The sense-strand transcripts used for calibration of the assay can be made by linearizing the DNA template used for antisense probe synthesis on the other side of the probe insert, and synthesizing transcript from the opposite strand, provided the insert is flanked by opposable promoters. While sense strand can be synthesized completely unlabeled, quantitation of the sense-strand transcript is most easily accomplished by including a trace amount of radiolabeled NTP; for example 0.1 µL [^{32}P]UTP, in the presence of a high concentration (500 µM) of unlabeled UTP. Following synthesis, the specific activity and yield of ^{32}P-transcripts are determined by counting an aliquot of the reaction in a scintillation counter. Since the specific activity is known, the mass amount and molar amount of sense strand synthesized can be calculated based on the size of the in vitro transcript. If the protected fragment in the sample RNA is the same size as the sense strand transcript used to generate the standard curve, equivalent intensities between the probe fragment protected by synthetic sense-strand and the probe fragment protected by the complementary mRNA in the experimental lane indicate equimolar amounts of protecting RNA (mRNA or sense strand transcript). If the sizes of the protected fragments differ, the molar amounts will differ in proportion to the sizes of the protected fragments. Also, the intensities of the different-sized protected bands will differ proportionally. The assay can be quantitated using a posphorimager, by densitometric scanning of the autoradiograph, or by excising and scintillation counting the regions of the gel that contain the protected fragments (Roller 1989 and Hershey and Krause, 1989). Alternatively, the standard curve can be constructed by TCA precipitating and counting the protected species (Lee and Costlow, 1987). For some purposes, precise quantitation may not be necessary, especially when the amount of ^{32}P-sense strand in the calibration reactions is varied in small increments. Conversion of molar or mass amounts of protecting mRNA in

the total RNA sample into number of transcripts per cell requires knowledge of the number of cells used to prepare the sample RNA. Detailed description for the quantitation of cell number can be found in the following references: Lee and Costlow (1987), and in Rymaszewski (1990).

F. Mapping mRNA

Because of its high sensitivity and resolution, the RPA procedure is well suited for mapping internal and external boundaries in mRNA. The basic requirement for mapping using RPAs is that the probe span the region to be mapped. This usually means that the probe is derived from a genomic clone, as opposed to a cDNA clone. For example, in order to map the transcription initiation site for a given mRNA, a probe is prepared by subcloning and transcribing a genomic fragment that extends from upstream of the gene of interest to some point in the first exon. Probe synthesis, purification, hybridization, and RNase digestion are carried out using the standard RPA III protocol. The transcription start site is mapped by comparing the size of the protected fragment to the size of the undigested probe; for example, if the protected fragment is 75 nucleotides shorter than the genomic DNA-complementary part of the probe, the transcription start site would lie 75 nucleotides upstream from the 3' end of the target DNA-complementary portion of the probe. For exact determination of the size of the protected fragment (necessary to map the transcription start site to the single nucleotide level), the protected fragment may be resolved on a sequencing gel in conjunction with a DNA "sequencing ladder" reaction. [The relative mobilities of RNA and DNA fragments in 7 M urea acrylamide gels differ by approximately 5–10%, depending on the particular electrophoresis parameters chosen (Molecular Cloning, a Laboratory Manual, 1989), with RNA fragments running somewhat slower than their DNA counterparts.] If such a high degree of resolution is not required, adequate molecular size markers may be prepared by end-labeling DNA fragments generated by digestion of a plasmid with a frequent-cutting restriction enzyme or by using the Ambion Century™ Marker Template Set (P/N AM7780) to generate RNA markers of 100, 200, 300, 400, and 500 bases, or the Decade™ Markers to generate 10–100 base labeled RNA markers. More detailed information on using RPAs for mapping may be found in the following references: Melton 1984, Calzone 1987, Takahashi 1989, and Zinn 1983.

G. Recipes

1. 10X TBE

TBE is generally used at 1X final concentration for preparing gels and/or for gel running buffer.

IMPORTANT

Do not treat TBE with diethylpyrocarbonate (DEPC).

Concentration	Component	for 1 L
0.9 M	Tris base	109 g
0.9 M	Boric Acid	55 g
20 mM	0.5 M EDTA solution	40 mL

Dissolve with stirring in about 850 mL nuclease-free water. Adjust the final volume to 1 L.

Alternatively, Ambion offers nuclease-free solutions of 10X TBE (P/N AM9863, AM9865) and ready-to-resuspend powdered 10X TBE packets (P/N AM9864). Both are made from of ultrapure molecular biology grade reagents.

2. Denaturing acrylamide gel mix

5% acrylamide /8M urea gel

15 mL is enough gel solution for one 13 x 15 cm x 0.75 mm gel

for 15ml	Component
7.2 g	Urea (high quality)
1.5 mL	10X TBE
1.9 mL	40% Acrylamide (acryl: bis-acryl = 19:1)
to 15 mL	dH_2O

Stir at room temperature until the urea is completely dissolved, then add:

120 µL	10% ammonium persulfate
16 µL	TEMED

Mix briefly after adding the last two ingredients, which will catalyze polymerization, then pour gel immediately. (It is not necessary to treat the gel mixture with diethylpyrocarbonate)

Gel set up

- Follow the manufacturers instructions for the details of attaching gels to the running apparatus.
- Use 1X TBE as the gel running buffer.
- It is very important to rinse the wells of urea-containing gels immediately before loading the samples.

Electrophoresis conditions

Gels should be run at about 20 V/cm gel length; for 13 cm long gel this will be about 250 V. Alternatively, denaturing acrylamide gels of this size can be run at ~25 mAmp, constant current.

3. RNase-free water

a. Add DEPC to 0.05% to double-distilled, deionized water (i.e. add 0.5 mL per liter of water).

b. Stir well, incubate several hours to overnight at 37°C or 42°C.

c. Autoclave 2 L or smaller volumes for at least 45 min. The scent of DEPC should be either not detectable or only very slightly detectable.

V. Appendix

A. References

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B. Safety Information

The MSDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day.

IMPORTANT

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address: www.ambion.com/techlib/msds
- Alternatively, e-mail your request to: MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.

- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

C. Quality Control

Functional Evaluation

All components are tested in a functional RPA as described in this protocol using 2.5, 5, and 20 µg of mouse liver RNA and a β-actin probe. A single protected fragment 245 bp in length is obtained when analyzed on a 5% denaturing PAGE gel.

Two controls are also run:

- The probe + yeast RNA is digested to completion with RNase leaving little or no background signal.
- The probe + yeast RNA without any RNase added shows a single band at 300 bp.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.