*mir*Vana™ miRNA Detection Kit

(Part Number AM1552)

Instruction Manual

Manual 1552M Revision B **Revision Date: April 15, 2008**

For research use only. Not for use in diagnostic procedures.

Literature Citation: When describing a procedure for publication using this product, please refer to it as the *mir*Vana™ miRNA Detection Kit.

If a paper that cites one of Ambion's products is published in a research journal, the author(s) may receive a free Ambion T-shirt by sending in the completed form at the back of this instruction manual, along with a copy of the paper.

Warranty and Liability: Applied Biosystems is committed to delivering superior product quality and performance, supported by industry-leading global service and technical support teams. Warranty information for the accompanying consumable product is available at www.ambion.com/info/warranty in "Limited Warranty for Consumables," which is subject to the exclusions, conditions, exceptions, and limitations set forth under the caption "EXCLUSIONS, CONDITIONS, EXCEPTIONS, AND LIMITATIONS" in the full warranty statement. Please contact Applied Biosystems if you have any questions about our warranties or would like information about post-warranty support.

Patents and Licensing Notifications: The *mir*Vana™ miRNA Detection Kit is covered by US patent 5422241.

Trademarks: Applied Biosystems, AB (Design), Ambion, FirstChoice, MAXIscript and RNase*Zap* are registered trademarks, and *mir*Vana, Decade and GlycoBlue are trademarks of Applera Corporation or its subsidiaries in the US and/or certain other countries. All other trademarks are the sole property of their respective owners.

I. Introduction

A. Background and Product Description

Procedure Overview

The *mir*Vana™ miRNA Detection procedure is fast and takes place in a single microfuge tube. Sample RNA containing the target RNA(s) of interest is simply mixed with one or several radiolabeled antisense RNA probes and hybridization buffer. After heat denaturation, the mixture is incubated at 42°C to hybridize the probes to the complementary RNA molecules in the experimental RNA samples. After hybridization, unhybridized RNA species (from the sample) and excess RNA probes are removed by a rapid ribonuclease digestion step. Finally, radiolabeled probe protected from RNase digestion by hybridization to the target RNA is recovered using Ambion® patented single step technology for simultaneous ribonuclease inactivation and nucleic acid precipitation. Radiolabeled protected RNA probe fragments are then analyzed on a denaturing polyacrylamide gel. Because no transfer to solid support is required and because the hybridization is performed in solution, the procedure ensures a sensitive and linear detection signal after autoradiography.

The *mir*Vana miRNA Detection Kit includes a control dsDNA template and Probe Elution Buffer to transcribe and gel purify a 32 nt antisense RNA probe. When used in a *mir*Vana miRNA Detection reaction with the provided control Mouse Kidney Total RNA, this probe generates a 22 bp protected fragment specific for miR-16 miRNA (Lagos-Quintana et al. 2001).

High sensitivity

The kit contains a 2X Hybridization Buffer that was developed with short antisense probes (typically 19–35 target-specific bases) to provide optimal sensitivity and specificity for detection of short RNA molecules such as siRNA or miRNA. Compared to hybridization protocols that rely on RNA bound to a solid support (i.e. traditional Northern blots), small RNA molecules are detected more readily and quantitated more accurately using a solution hybridization procedure. Quantitative analyses can be performed in solution with as little as 10–50 ng of total RNA to detect attomole (10^{-18} mole) amounts of target RNA.

Versatility

Several radiolabeled probes have been successfully used with the *mir*Vana miRNA Detection Kit to analyze the expression of miRNA, siRNA, small nuclear RNA (snRNA), and messenger RNA (mRNA). Another advantage of the assay over Northern blotting is the potential to simultaneously detect several tiny RNAs of the same size or both tiny RNA and longer RNA species (e.g. siRNA and target messenger RNA) in the same experimental sample.

Simplicity

The Ambion *mir*Vana miRNA Detection Kit is designed to avoid some of the problems associated with ribonuclease protection assays, and to offer simplicity while still allowing flexibility for experimental optimization. The procedure differs from standard protocols in several respects providing greater sensitivity and specificity, as well as being faster and easier to use. The provided 2X Hybridization Buffer streamlines the procedure as the probe and sample RNA do not need to be co-precipitated or dried down prior to resuspension in hybridization buffer. Also, no proteinase K digestion or phenol-chloroform extraction steps are required. This means that the entire procedure can be conducted in a single tube, reducing hands-on time and variability between experimental samples.

B. Reagents Provided with the Kit and Storage

The *mir*Vana miRNA Detection Kit should be stored at –20°C in a non-frost-free freezer.

The *mir*Vana miRNA Detection Kit provides reagents for 100 assays. The kit also contains control dsDNA template for 10 transcription reactions, and Probe Elution Buffer for up to 40 gel purifications.

Amount	Component	Storage
1 mL	2X Hybridization Buffer	–20°C
300 µL	RNase A/T1 Solution	–20°C
500 µL	Yeast RNA (5 mg/mL)	–20°C
17 mL	RNase Digestion Buffer	–20°C
22.5 mL	RNase Inactivation/PPT Solution	–20°C
1.4 mL	Gel Loading Buffer II	–20°C
20 µL	Mouse Kidney Total RNA (0.5 mg/mL)	–20°C
10 µL	Control Template (10 µM)	–20°C
8 mL	Probe Elution Buffer	–20°C
1.75 mL	Nuclease-free Water	any temp*

* Store at –20°C, 4°C or room temp

C. Materials Not Provided with the Kit

Radiolabeled RNA probe

DNA template and reagents for preparing radiolabeled RNA probe—the Ambion *mir*Vana™ miRNA Probe Construction Kit was developed specifically for production of RNA probes for use in miRNA and siRNA studies (see section IV.A starting on page 26 for instructions on probe preparation).

General laboratory equipment and supplies

- RNase-free 1.5 mL or 0.5 mL polypropylene microfuge tubes, adjustable pipettors and RNase-free tips
- ACS grade 100% ethanol
- Constant temperature incubator (42°C) and heat block (95–100°C)
- Microcentrifuge capable of at least 10,000 X g

Apparatus and reagents for preparing and running denaturing acrylamide gels (high quality urea, acrylamide and bis-acrylamide, Tris-borate-EDTA buffer, ammonium persulfate, TEMED)

D. Related Products Available from Applied Biosystems

miRNA Certified FirstChoice® Total RNA See web or print catalog for P/Ns	All of Ambion's high quality total RNA from normal human, mouse, and rat tissue is prepared by methods that quantitatively recover microRNAs. The entire line of FirstChoice Total RNAs are free of DNA and shown to be intact by stringent quality control standards.
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.
*mir*Vana™ miRNA Probe Construction Kit P/N AM1550	This kit is designed to produce short (<100 nt) labeled RNA transcripts for use in hybridization assays to detect small RNAs, including miRNA and siRNA. The kit supplies reagents for both transcription template preparation and RNA probe synthesis. Radiolabeled probes made with the kit are ideal for use with the *mir*Vana miRNA Detection Kit.
*mir*Vana™ Probe & Marker Kit P/N AM1554	The *mir*Vana Probe & Marker Kit is an end labeling kit designed for making short radiolabeled probes, and low molecular weight markers for studies involving microRNAs. It can be used with synthetic RNA or DNA oligonucleotides to prepare labeled probes, and the kit also provides reagents to prepare small radiolabeled RNA size markers (Decade™ Markers), and single-nucleotide RNA ladders. Rapid cleanup reagents are included to prepare the reaction products for various downstream application.
*mir*Vana™ miRNA Isolation Kit P/N AM1560	The *mir*Vana miRNA Isolation Kit (patent pending) is designed especially for the isolation of small RNAs, such as microRNA (miRNA), small interfering RNA (siRNA), and small nuclear RNA (snRNA), from tissues and cells. The kit uses a fast and efficient glass fiber filter (GFF) based procedure to isolate total RNA ranging in size from kilobases down to 10-mers. It also includes a procedure to enrich the population of RNAs that are 200 bases and smaller, which enhances the sensitivity of small RNA detection by solution hybridization and Northern blot analysis.
Decade™ Markers P/N AM7778	The Decade Marker System is a set of reagents to prepare radiolabeled low molecular weight RNA markers: from 10–100 nt in 10 nt increments. The user supplies only [γ-^{32}P]ATP to end label a single, gel purified RNA transcript which is then cleaved into the 10 molecular weight markers in a simple 5 minute reaction.

RNase-free Tubes & Tips See web or print catalog for P/Ns	Ambion RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. See our latest catalog or our website (www.ambion.com/prod/tubes) for specific information.
RNase*Zap*® Solution P/N AM9780, AM9782, AM9784	RNase*Zap* RNase Decontamination Solution is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap* Solution.

II. *mir*Vana™ miRNA Detection Kit Instructions

A. Sample RNA

Standard RNA isolation procedures involving RNA-binding glass fiber filters ***must not*** be used to prepare the experimental RNA samples because these methods will not quantitatively recover RNA smaller than 200 nt. We recommend using the Ambion® *mir*Vana™ miRNA Isolation Kit to purify representative total RNA populations or to isolate control fractions specifically enriched or depleted for small RNA species. When working with total RNA purchased from commercial suppliers, be sure to inquire about the method used to isolate the RNA. Many of Ambion FirstChoice® Total RNA products from mouse, rat and human tissues have been validated for miRNA research. A list is available on our web site, or by calling our Technical Services Department.

www.ambion.com/techlib/resources/miRNA/index.html

B. Radiolabeled RNA Probe Preparation and Amount

Only the probe is visualized at the end of the *mir*Vana miRNA Detection procedure; thus it is critically important that very high quality probes are used in the procedure. We recommend using high specific-activity ^{32}P-labeled antisense RNA probes prepared by in vitro transcription, or 5' labeling.

In vitro transcribed RNA

The *mir*Vana miRNA Probe Construction Kit is ideal for this application—it was developed specifically to quickly prepare short, high specific activity, antisense RNA probes of any sequence. RNA probes made by in vitro transcription should be gel purified to remove transcripts that are shorter than the full-length probe, because these shorter products may cause "background" smears and/or spurious protected bands in the assay.

5' end-labeled RNA oligonucleotides

Alternatively gel- or HPLC-purified chemically synthesized RNA oligonucleotides can be 5' end labeled and purified with the *mir*Vana Probe & Marker Kit. Because they include only a single radiolabeled nucleotide per molecule, probes generated with this procedure are typically of lower specific activity than in vitro transcribed probes, but they will not require gel purification after the labeling reaction. More information about probe design and preparation can be found in section *IV. Additional Procedures* on page 26.

Use a 3–10 fold molar excess of probe over target

For quantitative detection of the target RNA, it is important that the labeled probe be present in a 3–10 fold molar excess over the target RNA in the hybridization reaction. In most cases 1–5 x 10^4 cpm of high specific activity probe will meet this requirement. See section III.B on page 17 for instructions on optimizing the *mir*Vana miRNA Detection assay.

C. Hybridization of Probe and Sample RNA

Experimental setup

Use a master mix when possible

In this procedure, sample RNA and RNA probe are hybridized in a final volume of 20 µL; 10 µL of which is 2X Hybridization Buffer. For consistent results, we strongly recommend that you set up a hybridization master mix containing the "constant" components of the experiment. Typically a single master mix containing the 2X Hybridization Buffer, water, and one or several of the other components is used for all of the control and experimental tubes. For some experiments, however, different master mixes will be needed for each probe or each type of target RNA sample. Comprehensive examples of experimental setup are presented in section II.F starting on page 11.

Control reactions

In addition to the experimental samples, prepare two important ***no target*** controls for each probe in the experiment:

Control		Purpose
no target/no RNase	+ probe no sample RNA no RNase digestion (step D.2)	To assess probe quality and recovery.
no target	+ probe no sample RNA + RNase digestion (step D.2)	To assess nonspecific background signal and verify that probe digestion was complete.

Amount of sample RNA

The mass amount of sample RNA required will depend on the abundance of the target RNA being detected and on the specific activity of the probe. ***Typically, 0.5–5 µg of sample RNA is used.*** There is no lower limit to how much RNA can be used, but the upper limit is ~50 µg. The ***sample RNA must be in 9 µL or less of a dilute aqueous solution*** such as water or TE.

Normalization of RNA samples with Yeast RNA

The total amount of RNA in each sample and control tube should be roughly equal (+/– 10%) to ensure that the RNase digestion conditions will be similar in each. If the amounts of sample RNA in different exper-

imental samples varies, use the Yeast RNA (provided with the kit) to adjust the total amount of RNA in each hybridization reaction, including controls, up to 4–5 µg.

1. Assemble the hybridization reactions at room temp

a. Thaw the following kit components and reagents at room temp

- 2X Hybridization Buffer
- Yeast RNA
- Nuclease-free Water
- Experimental sample RNA(s)
- Radiolabeled probe(s)

Briefly vortex each tube; keep the Yeast RNA and experimental sample RNA on ice. If probes are in Probe Elution Buffer keep at room temp (if probes are in dilute aqueous solution keep them on ice).

b. In an RNase-free microcentrifuge tube prepare a master mix for hybridization based on the following final component amounts per tube, and mix thoroughly:

Amount	Component
10 µL	2X Hybridization Buffer
1–5 x 10^4 cpm	Labeled RNA probe
0.5–5 µg	Sample RNA
to 5 µg	Yeast RNA
to 20 µL	Nuclease-free Water

IMPORTANT

The 2X Hybridization Buffer is very dense, so be sure to blend the master mix thoroughly once all the components are assembled. After dispensing the master mix into each tube containing the variable components, mix thoroughly as well. Incomplete mixing often results in aberrant or irreproducible results.

For optimal mixing of the hybridization reactions, we recommend that you dispense the variable component(s) that are not in the master mix into each tube first. Then add the master mix to each tube and mix thoroughly by pipetting the mixture up and down several time or by vortexing 5–10 sec. Centrifuge the tube briefly to collect the reaction mixture at the bottom of the tube.

c. Set up two *no target* control tubes for each probe

For each different probe used, include two ***no target*** control tubes containing the following:

- the same amount of labeled probe used for the experimental tubes in step 1.b
- Yeast RNA equal to the total amount of RNA (sample RNA + Yeast RNA) in each experimental tube
- 10 µL of 2X Hybridization Buffer
- Nuclease-free Water to 20 µL

2. Incubate 3 min at 95–100°C

This 3 min heat treatment at 95–100°C denatures the RNA to reduce the effect of secondary structure on hybridization in the next step. Include all the experimental and control samples in this step and the next step.

3. Incubate 2 hr to overnight at 42°C

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

The ***hybridization time*** can be as short as 1 hr for relatively abundant target RNA such as miR-16 in mouse kidney (see Figure 4 on page 15). Hybridization times that yield very intense protected fragment signals may be reduced in subsequent experiments. For accurate quantitation, however, hybridization reactions must go essentially to completion.

IMPORTANT

Keep the reactions at hybridization temperature until adding the RNase mixture in step D.2 below.

The ***temperature of hybridization*** can also be optimized for certain RNAs. Hybridization temperatures up to 52°C are sometimes beneficial for highly structured target RNA or reactions containing multiple probes.

D. RNase Digestion of Hybridized Probe and Sample RNA

NOTE

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

1. Prepare a working dilution of RNase A/T1 Solution in RNase Digestion Buffer

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

b. Gently vortex the RNase A/RNase T1 Solution, spin briefly, and add RNase A/RNase T1 Solution to the RNase Digestion Buffer.
We recommend using a 1:100 dilution of RNase A/RNase T1 in RNase Digestion Buffer for initial experiments; however, the RNase concentration may need to be adjusted according to the target RNA and/or the amount of sample RNA used. (Section III on page 16 contains optimization and troubleshooting information. Also see Figure 4 on page 15.)

c. Gently vortex and spin briefly to mix the components thoroughly.

2. Add 150 µL diluted RNase to each sample RNA tube, and to one of each pair of no target controls

a. After hybridization, remove the tubes from the incubator or heat block and centrifuge briefly if any condensation is present on the sides or top of the tubes. ***Immediately*** add 150 µL of the diluted RNase mixture prepared in step 1 to:
   - each tube containing sample RNA,
   - and to one of the two ***no target*** control tubes that have been prepared for each probe in the experiment.
     This control will serve as a positive control for the RNase digestion step and no signal should be detected in this lane of the gel.

b. Mix thoroughly by pipetting the mixture up and down several time or by vortexing 5–10 sec. Centrifuge the tube briefly to collect the reaction mixture at the bottom of the tube.

3. Add 150 µL RNase Digestion Buffer *without* RNase to the remaining no target control tube(s)

The ***no target/no RNase*** control tube(s) will serve as a control for probe integrity. It shows the gel migration of the full-length probe, visible as a single band migrating at the expected probe size. Any unexpected degradation of the probe, or persistent secondary structure will be seen in this control.

4. Incubate 30–45 min at 37°C

During this incubation, unprotected single-stranded RNA is digested. We recommend a 30 min incubation at 37°C for initial experiments; however, optimization experiments (see section III.B on page 17) may indicate that a shorter or longer incubation time is optimal.

5. Add 225 µL RNase Inactivation/PPT Solution followed by 225 µL 100% ethanol

This step simultaneously inactivates RNases and precipitates protected RNA fragments. Add the following to each tube in the order shown, then mix thoroughly by vortexing or inverting the tubes several times:
- 225 µL RNase Inactivation/Precipitation Solution
- 225 µL 100% ethanol (ACS grade or better)

6. Place tubes at –20°C for 30 min or longer

Transfer tubes to –20°C freezer for at least 30 min.

NOTE

The experiment can be left at –20°C overnight or longer if desired at this step.

7. Spin 15 min at top speed in a microcentrifuge

Centrifuge the tubes for 15 min at 4°C at maximum speed (at least 10,000 X g).

8. Carefully remove all supernatant from each tube

Aspirate supernatant from the tubes with a very fine pipet tip or a drawn-out Pasteur pipette connected to a vacuum pump, or by carefully pouring the solution out of the tubes from the side opposite the RNA pellet. RNA pellets may not adhere tightly to the walls of standard polypropylene microcentrifuge tubes, so be careful when removing the supernantant.

NOTE

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

To remove the last traces of supernatant, recentrifuge the tubes for about 5 sec (room temp is okay) and withdraw the residual supernatant. If all of the supernatant is removed, including residual drops on the side of the tubes, an ethanol wash is not required, otherwise wash the pellets once with cold 75% ethanol.

E. Separation and Detection of Protected Fragments

NOTE

For best resolution and shorter run time, we recommend letting the gel polymerize for 1 hr and pre-running the gel for at least 1 hr at constant current prior to loading. We typically prepare the gel just before starting the RNase digestion (step II.D.1) and start the pre-run while precipitating the protected fragments at –20°C (step II.D.6).

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. Typically 15% polyacrylamide gels 0.75 mm thick, 15 cm wide x 12 cm long, with 20 wells that are about 4 mm in width are used (see recipe on page 35).

2. Air dry the protected fragments for 5–10 min

Air dry protected fragments by leaving the tubes open on the bench for 5–10 min at room temp. Do not let the pellets dry completely as this will make them difficult to resuspend. Do not dry under vacuum.

3. Resuspend pellets in Gel Loading Buffer II (GLB II)

Experimental samples and no target control: The volume of GLB 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. We typically resuspend RNA pellets in 5 µL GLB II if the entire reaction will be loaded on the gel or in 10 µL if only a fraction of the reaction will be loaded.

No target/no RNase control: This control is expected to contain many more counts than the experimental samples, so we recommend resuspending it in 10 µL GLB II and subsequently diluting a 0.5–1 µL sample into 5 µL final volume of GLB II to make a 1:20 or 1:10 dilution, respectively.

Vortex vigorously for 10–15 sec to dissolve the pellets, and microcentrifuge briefly to collect samples at the bottom of the tubes.

4. Incubate 3 min at 95–100°C

This incubation serves to completely solubilize and denature the protected fragments.

5. Load the samples and run the gel

Rinse the urea out of the wells of the gel, and ***immediately*** load each sample. Load equal volumes of sample and controls onto the gel; after dilution an equal volume of the ***no target/no RNase*** control will represent ~5–10% of the counts in the experimental samples so that it doesn't obscure the signal in adjacent lanes.

It is helpful to have size markers on the gel; single-stranded RNA markers such as Ambion Decade 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.

12 x 15 cm gels can be run at 10–30 mAmp constant current. The position of bromophenol blue and xylene cyanol in 15% gels corresponds to approximately 10 and 30 nt, respectively. Run gels until the leading dye band (bromophenol blue) is near the middle of the gel (~6 cm). The run time is about 30 min, although it will vary depending on the length of the gel and the percentage of acrylamide.

6. Detect radiolabeled protected fragments by autoradiography

Remove at least one glass plate and cover the gel with plastic wrap. Expose the gel face to X-ray film for 1 hr to overnight with an intensifying screen at –80°C. The gel can be re-exposed several times if necessary after allowing it to warm up to room temp and wiping off condensation moisture. The gel should be stored at –80° or –20°C if not re-exposed immediately. It is not necessary to dry standard 0.75 mm thick gels for autoradiography.

F. Experimental Setup Examples

Below are shown four representative experiments performed with the *mir*Vana miRNA Detection Kit. This information provides examples of experimental setups and actual data obtained with the kit.

1. Multiple probes with a single experimental RNA sample

In this experiment four different probes are tested with 1 µg Mouse Kidney Total RNA. To bring the RNA amount to 4 µg, 3 µg of Yeast RNA is added to each experimental sample. For each of the four probes, a ***no target*** control and a ***no target/no RNase*** control are set up containing 4 µg of Yeast RNA (for a total of 8 control reactions).

To set up this experiment two master mixes (experimental and no target control) containing all of the components except the probes are mixed thoroughly. 10% extra is included to account for pipetting error.

	No target Master Mix		Experimental Master Mix	
Component	Per tube	For 8 controls	Per tube	For 4 samples
2X Hybridization Buffer	10 µL	88 µL	10 µL	44 µL
Yeast RNA (5 mg/mL)	0.8 µL	7 µL	0.6 µL	2.6 µL
Mouse Kidney RNA (0.5 mg/mL)	– –	– –	2 µL	8.8 µL
Nuclease-free Water	to 18 µL	63.4 µL	to 18 µL	23.8 µL

a. The appropriate probes are placed in each microcentrifuge tube (2 µL containing 5 x 10^4 cpm), 18 µL of the appropriate master mix is then added, and the tube contents are mixed thoroughly.

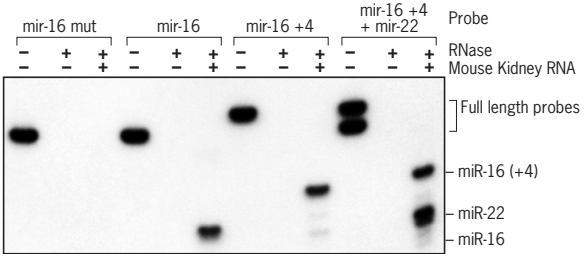

Figure 1. miR-16 and miR-22 miRNA (22 nt) Detection in 1 µg Total RNA from Mouse Kidney.

mir-16, mir-16 mut, and mir-22 probes are 32 nt long and produce a 22 nt long protected fragment; the mir-16 mut probe contains 3 mismatch mutations (ACG to CGA) corresponding to nucleotides 9–11 of the miR-16 miRNA sequence; the mir-16 +4 probe (36 nt) carries 4 additional A residues between the 22 nt sequence specific for miR-16 and the 10 nt leader sequence, producing a 26 nt long protected fragment. All of the probes were prepared with the *mir*Vana™ miRNA Probe Construction Kit. RNA was resolved on a denaturing 15% polyacrylamide gel. The gel was exposed for 2 hr at –80°C with an amplifying screen.

2. Multiple probes with multiple experimental RNA samples

The mir-16 probe is now used to detect *mir-16* expression in mouse thymus. In the same experiment *mir-22* and *mir-16* expression is compared in mouse lung by multitarget analysis. Because the amounts of experimental sample RNA in the experiment are small (25–500 ng), 4 µg of Yeast RNA is used in all of the assays.

a. To set up this experiment two master mixes (thymus and lung) containing all of the components except the sample RNA are mixed thoroughly. 10% extra is included to account for pipetting error.

	Thymus Master Mix		Lung Master Mix	
Component	Per tube	For 6 assays	Per tube	For 6 assays
2X Hybridization Buffer	10 µL	66 µL	10 µL	66 µL
Yeast RNA (5 mg/mL)	0.8 µL	5.3 µL	0.8 µL	5.3 µL
mir-16 (5 x 10^4 cpm/µL) probe	1 µL	6.6 µL	– –	– –
mir-16 + 4 (5 x 10^4 cpm/µL) probe	– –	– –	1 µL	6.6 µL
mir-22 (5 x 10^4 cpm/µL) probe	– –	– –	1 µL	6.6 µL
Nuclease-free Water	to 19 µL	47.5 µL	to 19 µL	40.9 µL

b. 1 µL of RNase-free water (for the ***no target/no RNase*** control) or 1 µL of the appropriate total RNA diluted to contain 25, 50, 100, 250, or 500 ng RNA are placed in each microcentrifuge tube. Then 19 µL of the Thymus or Lung Master Mix is added, and the tube contents are mixed thoroughly.

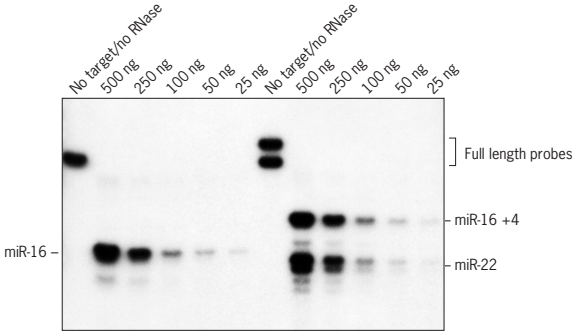

Figure 2. Analysis of miR-16 or miR-22 miRNA Expression in Mouse Thymus & Lung

miR-16 and miR-22 miRNA were detected in 500, 250, 100, 50, or 25 ng of Ambion® FirstChoice® Total RNA from mouse thymus (mir-16 probe) or mouse lung (mir-16 +4 and mir-22 probes). The gel was exposed for 3.5 hr.

3. A single probe with multiple experimental RNA samples

In this experiment mir-16 probe is used to analyze *mir-16* expression in 10 different mouse tissues. 4 µg of Yeast RNA is used for each of the two ***no target*** controls (not shown in the figure) and to complement the 1 µg of sample RNA in the experimental reactions.

a. To set up this experiment one master mix containing all of the components except the experimental sample RNA is mixed thoroughly. One Master Mix for 13 tubes is needed for this experiment (12 hybridization reactions plus ~10% for pipetting error).

	Master Mix	
Component	Per tube	For 13 assays
2X Hybridization Buffer	10 µL	130 µL
Yeast RNA (5 mg/mL)	0.8 µL	10.4 µL
mir-16 (5 x 10^4 cpm/µL) probe	1 µL	13 µL
Nuclease-free Water	to 19 µL	93.6 µL

b. 1 µL of RNase-free water (for the ***no target*** controls) or 1 µL of the appropriate total RNA diluted to contain 1 µg RNA are placed in each microcentrifuge tube. Then 19 µL of Master Mix is added, and the tube contents are mixed thoroughly.

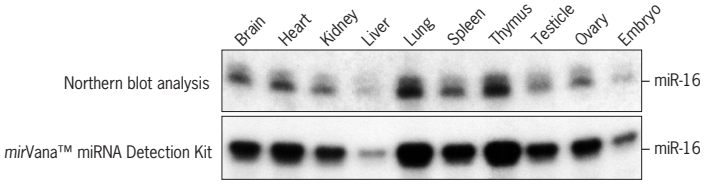

Figure 3. miR-16 miRNA Expression Across Mouse Tissues

miR-16 miRNA was detected in 1 µg of Ambion® FirstChoice® Total RNA from 10 different mouse tissues using the *mir*Vana™ miRNA Detection Kit (bottom panel). The gel was exposed for 2 hr. The same variation of *mir-16* expression across mouse tissues was demonstrated by Northern blot analysis using the same high specific activity mir-16 probe (top panel, 2 day exposure).

4. A single probe and sample RNA, different reaction parameters

The mir-16 probe and Mouse Kidney Total RNA are used to test different parameters of the *mir*Vana miRNA Detection procedure.

Testing reaction conditions

In the first part of the experiment, nine variations of hybridization time and RNase digestion conditions are tested with 1 µg of sample RNA. Thus a master mix containing all of the components is prepared (plus 10% for pipetting error), and 20 µL is dispensed into each tube.

	Reaction Condition Master Mix	
Component	Per tube	For 9 samples
2X Hybridization Buffer	10 µL	100 µL
Mouse Kidney Total RNA (0.5 mg/mL)	2 µL	20 µL
mir-16 probe (5 x 10^4 cpm/µL)	1 µL	10 µL
Yeast RNA (5 mg/mL)	0.6 µL	6 µL
Nuclease-free Water	to 20 µL	64 µL

Testing sample RNA quantity

The second part of the experiment compares different amounts of sample RNA, and includes the two ***no target*** controls.

4 µg of RNA consisting of either Yeast RNA, Mouse Kidney Total RNA, or a mixture of the two is dispensed into 6 tubes; the RNA volume is 8 µL. Then 12 µL of the "RNA Quantity Master Mix" containing water, 2X Hybridization Buffer and probe (plus 10% for pipetting error) is added.

Component	RNA Quantity Master Mix	
	Per tube	For 6 assays
mir-16 probe (5 x 10^4 cpm/µL)	1 µL	6.6 µL
Nuclease-free Water	to 12 µL	6.6 µL

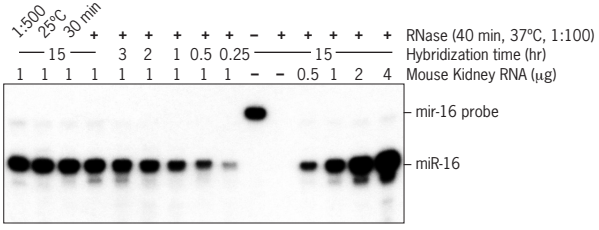

Figure 4. miR-16 miRNA Detection in Total RNA from Mouse Kidney

In this experiment, various parameters of the *mir*Vana™ miRNA Detection Kit procedure were tested: amount of sample RNA (0.5, 1, 2 or 4 µg) time of hybridization (15 min, 30 min, 1, 2, 3, or 15 hr) and RNase digestion conditions (time, temperature, dilution). The gel was exposed for 2 hr. Note that the same signal is obtained in lanes 4 and 13 (same reaction but from two different experimental setups) showing the reproducibility of the assay.

III. Troubleshooting

A. Running the Positive Control Reaction

The positive control reaction included with the *mir*Vana miRNA Detection Kit consists of Mouse Kidney Total RNA and a DNA template (Control Template) for transcription of an antisense RNA probe specific for miR-16 miRNA.

1. Probe preparation

To synthesize a radiolabeled positive control probe, use 1 µL of the Control Template in a 20 µL in vitro T7 RNA Polymerase transcription reaction containing at least 3 µM [α-^{32}P]UTP (5 µL of 800 Ci/mmol, 10 mCi/mL) and up to 5 µM unlabeled UTP. The lower the concentration of unlabeled nucleotide, the higher the specific activity of the transcript, and thus the greater the sensitivity of the assay. For maximum sensitivity, do not add any unlabeled form of the limiting nucleotide. Only T7 RNA polymerase can be used in the transcription reaction with this template. Treat the control probe with DNase to remove the DNA template, and gel purify it (section IV.B on page 29) for use in the control *mir*Vana miRNA Detection reaction.

The size of the antisense mir-16 RNA probe from the Control Template is 32 nt, and the protected mir-16 fragment is 22 nt.

NOTE

The control probe can also be used in Northern blot experiments (see Figure 3 on page 14).

2. Assay setup

Use about 5 x 10^4 cpm of probe with different amounts of Mouse Kidney RNA, e.g. 2, 4, and 8 µL corresponding to 1, 2, and 4 µg, respectively. Bring the final amount of RNA in each tube to 4 µg by adding Yeast RNA. Include two ***no target*** control hybridization reactions containing the same amount of probe and 4 µg Yeast RNA. Follow the protocol outlined in section *II. mirVana™ miRNA Detection Kit Instructions*; hybridize overnight at 42°C, use RNase A/RNase T1 Mix at a 1:100 dilution for 30 min at 37°C and analyze on a denaturing 15% polyacrylamide gel, loading only 10% of the ***no target/no RNase*** control.

3. Expected results

Protected fragments of 22 nt should be seen in the reactions containing Mouse Kidney RNA, and the protected fragments should be more intense with increasing amounts of Mouse Kidney RNA. The ***no target*** control lane should have no signal, and the ***no target/no RNase*** lane should show a band corresponding to full length probe (32 nt). An example of a control experiment using the provided Mouse Kidney Total RNA and probe made from the Control Template is shown in Figure 4 on page 15.

B. Pilot Experiment for Assay Optimization

1. Pilot experiment setup

The following experiment is suggested to help new users determine the appropriate amounts of sample RNA and probe to use when the abundance of the target RNA is unknown. Carry out the assay with a range of input sample RNA, using a constant amount of high specific activity probe. Also, to optimize conditions for the RNase digestion, vary the dilution of the RNase A/T1 Solution in RNase Digestion Buffer. You may also want to test additional variables such as the hybridization temperature (25–52°C), the hybridization time (1–24 hr), the probe amount (1–10 x 10^4 cpm), the RNase digestion incubation time (15–45 min), or the RNase digestion incubation temperature (room temp to 37°C). If sample RNA is in short supply, these experiments can be scaled back to conserve sample RNA. The details of the pilot experiment are shown in Table 1, and instructions for making the RNase Digestion Buffers are shown in Table 2 on page 18.

a. Probe

For maximum sensitivity, prepare high specific activity experimental and control probes, i.e. labeling reactions should not contain any unlabeled limiting nucleotide. For more detailed information on probe synthesis and purification, see section IV.A, IV.B, and IV.C.

b. Molecular size markers

To assess the size of the probes and protected fragments we recommend using 10–50 nt molecular size markers. Ambion Decade™ Markers (included in the *mir*Vana Probe & Marker Kit) will generate a 10 nt ladder using polynucleotide kinase and [γ–^{32}P]ATP. The kit also contains reagents to prepare a single nucleotide ladder from a 5' end radiolabeled RNA probe.

c. General instructions for the pilot experiment

Follow the protocol in section *II. mirVana™ miRNA Detection Kit Instructions* on page 5. Prepare two Master Mixes containing either your experimental probe or the mir–16 probe made from the Control Template, Hybridization Buffer, and Nuclease-free Water. Dispense the RNA into your assay tubes, and then add the appropriate Master Mix. Run only 10% of the ***no target/no RNase*** controls (tubes 11 and 16), to prevent overexposure of the autoradiograph. Expose the gel initially for 3 hr with an intensifying screen at –80°C.

Table 1. Suggested Pilot Experiment

Lane	Sample RNA	Yeast RNA	Amount Probe	RNase Dilution
1	0.5 µg	3.5 µg	5 x 10^4 cpm (expt'l)	1:100
2	1 µg	3 µg		1:100
3	2 µg	2 µg		1:100
4	4 µg	– –		1:100
5	4 µg	– –		1:50
6	4 µg	– –		1:200
7	4 µg	– –		1:500
8	4 µg	– –		1:1000
9	– –	4 µg		1:100
10	– –	4 µg		1:1000
11	– –	4 µg		No RNase*
12	1 µg MK RNA†	3 µg	5 x 10^4 cpm (mir-16)	1:100
13	2 µg MK RNA	2 µg		1:100
14	4 µg MK RNA	– –		1:100
15	– –	4 µg		1:100
16	– –	4 µg		No RNase*

* Use RNase Digestion Buffer alone.

† Mouse Kidney RNA supplied with the kit (0.5 mg/mL).

Table 2. Preparation of RNase Digestion Buffer

RNases	Dilution	Volume Needed	RNase A/T1 Solution	RNase Digestion Buffer
RNase A/RNase T1 Soln	1:50	160 µL	3.2 µL	156.8 µL
RNase A/RNase T1 Soln	1:100	1600 µL	16 µL	1584 µL
RNase A/RNase T1 Soln	1:200	160 µL	80 µL 1:100 dilution	80 µL
RNase A/RNase T1 Soln	1:500	160 µL	32 µL 1:100 dilution	128 µL
RNase A/RNase T1 Soln	1:1000	320 µL	32 µL 1:100 dilution	288 µL

2. Interpretation of pilot experiment results

a. Kit Control (lanes 12–16)

This part of the experiment is to verify that the *mir*Vana miRNA Detection procedure is functioning properly, and to rule out technical difficulties in performing the assay. There should be an easily visible protected fragment band of 22 bp in lanes 12–14. The intensity of the protected band should correlate with the amount of Mouse Kidney RNA used. The ***no target/no RNase*** control lane (16) should show a band corresponding to full length transcript, and the ***no target*** control lane (15) should be completely empty. A similar experiment is shown in Figure 4 on page 15.

b. Experimental no target controls (lanes 9–11)

The ***no target/no RNase*** control lane (11) 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 this lane, then ribonuclease contamination of your tubes, pipette tips, or a component of the kit should be suspected.

The two ***no target*** control lanes (9 & 10) should be empty. Lane 10 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. If the intensity of the major protected fragment in the autoradiograph increases with increasing input RNA, then the probe was present in molar excess over the target RNA in all the reactions. For accurate quantitation of RNA levels, it is essential that the probe is present in molar excess compared to the target and that hybridization goes to completion.

If the signal is very weak or absent, even in the reaction with the highest amount of input RNA, the target RNA may be very rare; see section III.C.2 on page 20 for suggestions.

d. Different concentrations of RNase (lanes 5–8)

The ribonuclease concentration was varied to optimize the signal-to-noise ratio in the assay. Most probe/target RNA 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 for optimal results.

C. Troubleshooting Faint or Absent Protected Fragment Bands

If the *mir*Vana miRNA Detection assay autoradiograph does not show the expected results (i.e. no band of the predicted size representing the major protected fragment is present), but the probe is intact (as assessed by the ***no target/no RNase*** control), consider the following suggestions for troubleshooting the procedure.

1. The target is not present in the sample

It is possible that absence of signal is a legitimate result, and that the RNA of interest is not expressed or was not efficiently recovered during RNA sample preparation. To confirm this possibility, repeat the assay along with an assay of a separate sample RNA prep known to contain detectable levels of the target RNA. If this is not possible, increasing amounts of chemically synthesized or in vitro transcribed sense strand RNA can be added to RNA samples to serve as a positive control. This

strategy will also show the limit of sensitivity of the assay for a particular probe. For more information on using synthetic sense strand RNA in solution hybridization, see section *IV.D. Quantitation of Target RNA* on page 32 or refer to Technical Bulletin #165—available at:
www.ambion.com/techlib/tb/tb_165.html

2. Increase the sensitivity of the assay

a. Use a probe with a higher specific activity.
Probe specific activity is determined by the specific activity of the radiolabeled nucleotide, and its proportion to the corresponding unlabeled nucleotide in the labeling 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.A.3. Making radiolabeled RNA probes* on page 27 for more information on probe synthesis.

b. Use more target RNA.
Another way to increase the sensitivity of the assay is to use more sample RNA – as much as about 50 µg per hybridization reaction can be used. Fractions enriched in small RNA species with the *mir*Vana miRNA Isolation Kit procedure can also be used instead of total RNA to increase the amount of small RNA target (e.g. miRNA or siRNA).

3. Check the probe design

Transcription of the "wrong strand" from the template DNA in the probe synthesis reaction will produce a probe that cannot hybridize to the target RNA. In order to make an antisense probe (target-complementary) from cloned sequences, plasmid template should be linearized on the 5' side of the gene or portion of gene. PCR templates should be designed with the phage polymerase promoter on the 3' side of the desired probe sequence. If using the *mir*Vana miRNA Probe Construction Kit, the DNA oligonucleotide template must have the same sequence as the sense target RNA except that U residues are replaced with T's. The 8 nt sequence `5'-CCTGTCTC-3'`, complementary to the T7 Promoter Primer provided with the kit, must also be added to the 3' end of the DNA oligonucleotide sequence.

4. Suboptimal hybridization temperature

In some cases it is better to hybridize at temperatures higher than 42°C. This can be helpful in multiple probe assays, and may serve to prevent probe:probe interactions.

5. Overdigestion with RNase

Overdigestion with RNase is rarely seen using a 1:100 dilution of RNase A/T1 Solution. Evidence for 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

1. 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 RNA, a large excess of probe can result in high background.

b. Gel quality and sample volume

For best resolution, we recommend letting the gel polymerize for at least 1 hr and pre-running the gel for at least 1 hr at constant current prior to loading. It is also critical to rinse the urea out of the wells immediately before loading. Finally, overloading gel wells reduces product resolution and may affect data quality. Wells that are 0.4–0.6 cm wide work well for loading the 5–10 µL samples from this procedure. The best resolution is obtained when the gel loading buffer forms a 2–3 mm layer in the well.

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 may be seen (Zinn, et al. 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 used with satisfactory results. As an assessment of probe quality, the ***no target/no RNase*** control lane should show a band representing mainly full-length probe.

d. Suboptimal RNase digestion conditions

Vary the concentration of RNase A/RNase T1 Solution in the RNase digestion step over a range of about 50 to 100 fold, for example by using RNase A/RNase T1 Solution 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*** control lane (no target/+RNase). The experimental samples will show smearing or a ladder of bands 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 A/RNase T1 Solution at a 1:100 dilution rarely results in overdigestion of the hybrids.

e. Local "breathing"

Depending on the sequence of the double-stranded RNA hybrid formed by the probe and target RNA, "breathing" (local denaturation) may occur, which can result in partial cleavage of the protected probe:RNA target hybrid. With internally labeled probe (i.e. prepared by in vitro transcription) this will produce a shorter discrete band(s) or a ladder of bands shorter than the expected size of the protected fragment but will not affect the overall interpretation of the data. With 5' end-labeled probes however, breathing may result in a complete loss of signal. If this problem is suspected, the incubation temperature of the RNase digestion reaction can be lowered to room temp or 16°C.

f. Mismatches between probe and target RNA

See *III.G.3. There are mismatches between probe and target RNA* on page 25.

g. Degradation of target RNA

The *mir*Vana miRNA Detection assay is relatively insensitive to RNA degradation. However, a badly degraded sample RNA preparation may contain species capable of protecting only part of the probe. The longer the probe, the less target RNA degradation can be tolerated. If degradation of target RNA is suspected to be a problem, the integrity of the sample RNA can be assessed by electrophoresis on a 1% denaturing formaldehyde or glyoxal based agarose gel (smearing of the ribosomal RNA bands indicates degradation). Alternatively, the sample RNA can be analyzed by capillary electrophoresis using an Agilent 2100 bioanalyzer. A 28S:18S rRNA peak ratio at or near 2:1 indicates that the RNA is intact. Another way to check the integrity of the sample RNA is to use the provided control probe to detect miR-16 miRNA in your RNA sample. This probe has been successfully used to detect miR-16 in total RNA from various cell lines, and from human, rat, and mouse tissues.

There is one report suggesting that autoclaving of siliconized tubes resulted in a high pH residue which degrades RNA.

2. Smear or ladder is also seen 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.A and IV.B.

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, pipette tips, or a component of the kit.

E. Full Length Probe Is Seen in All Lanes

There are several possible causes for this result. In each case, full-length probe, or band(s) slightly shorter than the probe, are seen in the experimental sample lane and also in the ***no target*** control lane (no target/+RNase).

Nonspecific protected bands may be observed in some cases along with the expected target-specific protected fragment. These bands are of low intensity, and may vary in size, but generally are easily distinguishable from the expected band on a gel. They will not affect analysis of the results, and can essentially be ignored.

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.

1. RNases were completely inactive or were not added

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 RNases can be checked for activity by comparing on a gel RNase-treated RNA with RNA incubated in RNase 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 fragments, there could be a problem with the RNase A/T1 Solution.

2. 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 usually be more intense than this full-length probe band, and both bands will be distinct, with no smearing between the two. We generally recommend using no more than 1–10 x 10^4 cpm of high specific activity probe for up to 10 µg of RNA sample. A pilot experiment using different amounts of input RNA can be done to determine the amount of probe necessary for probe to be present in molar excess over target.

3. Residual DNA transcription template is protecting the probe from RNase digestion

The experimental data in this case will look much like that described in the preceding section, a faint full-length probe band in all lanes [including the ***no target*** control lane (no target/+RNase)], and a protected fragment band in the experimental sample lanes, with little if any smearing between the bands. To avoid this, we always recommend treating in vitro transcription reactions with DNase I, and gel purifying probe for use in this procedure.

4. The RNase did not completely degrade unprotected probe

Incomplete RNase digestion is indicated if there is a full-length probe 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 Solution is used at 1:100, but if it does, use a less dilute RNase solution in subsequent experiments (e.g. 1:50).

5. Problem with probe design

The probe should be longer than the protected fragment so that an obvious difference in size is seen between the full-length undigested probe and the protected fragment. This additional nontarget-specific sequence must be cleavable by RNase A and/or T1 (RNases A and T1 do not cleave 3' of A residues). For detailed information about probe design and preparation see section IV.A on page 26.

F. Aberrant, Pointed or Smeared Bands

1. Residual salt in the RNA pellet

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. We recommend briefly respinning the final pellet after the supernatant is removed, and then thoroughly aspirating any residual supernatant using a very fine-tipped pipet. Alternatively pellets can be washed with 75% ethanol.

2. Gel quality and sample volume

See section D.1.b on page 21.

3. Radioactive material that fails to migrate into the gel

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 probes that were not gel purified tends to diminish the amount of material left in the wells. Another contributing factor seems to be residues left in microcentrifuge tubes from the manufacturing process. Autoclaving of siliconized tubes appears to exacerbate this problem.

G. Reasons for Legitimate but Unexpected Bands on Autoradiographs

Due to the extreme sensitivity of the *mir*Vana miRNA Detection assay, probe-related sequences in the sample RNA that might go undetected by other methods may be detected with this assay. Typically, these extra bands are 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 RNA(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 the assay.

1. Probe hybridizes to target RNA with differing but related sequence

If the probe is complementary to an RNA 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 RNA from the related gene(s). In some cases, the related RNA will have multiple mismatches with the probe, resulting in protected fragments that are shorter than those seen with the completely homologous RNA. Multiple mismatches in short probes can also result in a complete loss of signal (see for example mir-16 mut probe in Figure 1 on page 12). This can be advantageous for distinguishing between miRNAs with sequence similarity which run as a single band on Northern blots. Some investigators will want to use higher concentrations of RNase to maximize recognition of small differences between the probe and sample RNA, while others will want to use lower concentrations of RNase to minimize probe cleavage at mismatched positions.

2. Probe detects heterogeneity in processing of RNA transcripts

Depending on the design of the probe and the experimental RNA sample, probes may be capable of protecting fragments of different sizes that result from heterogeneity in the target RNA. Such heterogeneity could result from differential transcription initiation or termination sites or from the presence of immature RNA species in the RNA sample (e.g. precursor siRNAs or miRNAs).

3. There are mismatches between probe and target RNA

If the probe is not completely homologous to the target RNA in the sample RNA mixture, total or partial cleavage of the probe-target RNA hybrid may occur at the position of the mismatch. 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 will not be cleaved (Myers et al. 1985). If the mismatches consist of more than single-nucleotide differences, they will be cleaved much more efficiently. Because the procedure uses short probes, multiple mismatches in a probe may also result in a complete loss of target RNA protection. For example, three consecutive mismatches in the mir-16 mut probe (Figure 1 on page 12) completely abolish the detection of miR-16 miRNA in Mouse Kidney Total RNA. Cleavage at mismatch positions can often be minimized by decreasing the RNase concentration and/or the incubation time and temperature during RNase digestion.

IV. Additional Procedures

A. Preparation of Antisense RNA Probes

Because only the probe is visualized at the end of the *mir*Vana miRNA Detection procedure, the success of the experiment is largely dependent on the quality of the probe. Below are recommendations for probe design and preparation.

1. Probe size

The *mir*Vana miRNA Detection procedure is optimized for use with short antisense RNA probes. Typically these probes contain a 19–35 nt sequence that is the complement of the target RNAs of interest and 3–10 nt or more of nonspecific sequence that will not hybridize with the target RNA. The probe size must be within the effective separation range of the gel that will be used to visualize the results (12–15% polyacrylamide gels). The shorter the probe, the more tolerant the assay is to partially degraded sample RNA. On the other hand, the shorter the probe, the less sensitive the assay will be and high specific activity probes must be used.

Including sequence in the probe that does not hybridize to the target will result in probes that are longer 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 to a smaller protected fragment helps to validate that RNA from the sample is protecting the probe and is not an artifact. With short probes, a shift of 3–4 nt or more is enough for efficient resolution on 15% polyacrylamide gels.

2. Probe sequence

The nonspecific sequence in the probe must be cleavable by RNase A and/or T1 since those are the RNases used in the procedure. RNase A cleaves 3' of U and C residues; RNase T1 cleaves 3' of G residues. Thus, adding a stretch of 3–4 A residues to the probe between the target-specific sequence and the nonspecific sequence will increase the size of the protected fragment regardless of the target size. This is useful for simultaneously detecting several targets of the same size (e.g. mir-16 +4, see Figure 1 on page 12, Figure 3 on page 14, and the example below). Adding 3–4 A residues typically increases protected fragment size enough for good resolution of bands on denaturing 15% acrylamide gels (i.e. for multitarget detection of miRNA or siRNA).

In vitro transcribed probes

In general, probes prepared by in vitro transcription are designed to include a 5' non-target-specific sequence. The *mir*Vana miRNA Probe Construction Kit procedure is optimized to quickly prepare dsDNA for in vitro transcription with synthesized RNA transcripts carrying the

leader sequence 5'-GGGAGACAGG-3'. Note that this sequence is cleavable by RNases A and T1 and does not contain any U residues. It has been reported that the presence of the limiting nucleotide in the sequence within 12 bases of the RNA polymerase promoter results in increased premature termination (Ling et al. 1989). Using [α-^{32}P]UTP to prepare radiolabeled RNA probe, this design helps to ensure full-length probe synthesis. Furthermore both probe and protected fragment will have the same specific activity.

The dsDNA template included in the *mir*Vana miRNA Detection Kit will generate a 32 nt antisense RNA probe upon transcription with T7 RNA polymerase. This probe is specific for miR-16 miRNA (Lagos-Quintana et al. 2001) and also includes the 5'-GGGAGACAGG-3' nonspecific sequence.

End-labeled antisense RNA oligonucleotides

Antisense, 5' end radiolabeled probes must include the non-target-specific sequence at their 3' end because if the nonspecific sequence were at the 5' end, it would be removed during the RNase digestion. The positive control probe provided with the *mir*Vana Probe & Marker Kit is specific for miR-16 miRNA and carries the sequence 5'-CCAGAG-3' (see sequence below).

Example probe and target sequences

Sequence elements that are ***not*** derived from the target sequence are underlined.

- The (22 nt) miR-16 miRNA sequence is:
  5'-UAGCAGCACGUAAAUAUUGGCG-3'
- The sequence of the (32 nt) mir-16 probe transcribed from the *mir*Vana miRNA Detection Kit Control Template is:
  3'-AUCGUCGUGCAUUUAUAACCGCGGACAGAGGG-5'
- The sequence of the *mir*Vana Probe & Marker Kit (28 nt) mir-16 control probe is:
  3'-GAGACCAUCGUCGUGCAUUUAUAACCGC-5'
- Either probe will generate the same protected, (22 nt) miR-16 specific, RNA sequence:
  3'-AUCGUCGUGCAUUUAUAACCGC-5'
- The sequence of the *mir*Vana miRNA Probe Construction Kit (36 nt) mir-16 +4 control probe is:
  3'-AUCGUCGUGCAUUUAUAACCGCAAAAGGACAGAGGG-5'
- With this probe, the protected, (26 nt) miR-16 specific, RNA sequence will be 4 nt longer:
  3'-AUCGUCGUGCAUUUAUAACCGCAAAA-5'

3. Making radiolabeled RNA probes

a. In vitro transcription

Phage RNA polymerases are widely used for the in vitro synthesis of RNA transcripts from DNA templates by run-off transcription. The template must have a double-stranded 19–23 base promoter

upstream of the sequence to be transcribed. The template is then mixed with RNA polymerase, rNTPs, and transcription buffer, and the reaction mixture is incubated for 10 min to 1 hour at 37°C. In vitro transcription kits are available from Ambion (MAXIscript Kits). Templates for in vitro transcription can be quickly prepared from a short, inexpensive, desalted DNA oligonucleotide using the *mir*Vana miRNA Probe Construction Kit; the kit also includes reagents for in vitro transcription with T7 RNA polymerase.

For reasons of practicality, the labeled NTP (generally [α-^{32}P]UTP or CTP, at about 800 Ci/mmol and 10 mCi/mL or greater) is usually present in the transcription reaction at a limiting concentration, and is therefore referred to as the "limiting nucleotide." (Note that the "limiting NTP" can be a mixture of both the labeled and unlabeled form of that NTP). The greater the concentration of limiting nucleotide, the higher the RNA yield (and proportion of full-length transcripts), but if unlabeled nucleotide is used to increase the limiting nucleotide concentration, it will lower the specific activity of the transcript.

In general, probes should be synthesized at the lowest specific activity which will give the sensitivity required to detect a particular target. When making probe to detect an unknown amount of target sequence, start with a maximum specific activity transcription reaction containing no unlabeled limiting nucleotide. If a strong hybridization signal is seen using this probe, the specific activity can be reduced in subsequent experiments by adding unlabeled limiting nucleotide to about 5–10 µM. Reducing probe specific activity will reduce assay sensitivity, but these probes will have a longer shelf life (due to less radiolytic decay), and they may also exhibit less nonspecific hybridization.

Occasionally problems may be encountered in obtaining good yields of full-length transcripts. In general this problem stems from the limiting concentration of labeled nucleotides. Because of the high cost of these nucleotides, transcription reactions are generally run at concentrations of labeled nucleotide well below the K_m of the phage polymerases. Thus, for synthesis of full-length high specific activity probes there is a trade-off between using labeled nucleotide at a low concentration to obtain a high specific activity and adding sufficient amount of the limiting nucleotide to achieve synthesis of reasonable amounts of full-length transcripts.

b. 5' end labeling

Antisense RNA probes prepared by chemical synthesis can also be used with the *mir*Vana miRNA Detection Kit. These probes are 5' end labeled by a phosphorylation reaction. In general, 1–10 pmol of gel- or HPLC-purified RNA oligonucleotide are incubated at 37°C for 1 hour with T4 polynucleotide kinase (PNK), kinase buffer

and [γ-^{32}P]ATP (6000 or 7000 Ci/mmol, 10–150 mCi/mL). Because only one radioactive phosphate is transferred per molecule of RNA probe, the reaction is performed in the absence of unlabeled ATP to ensure the highest specific activity possible. For optimal labeling of RNA probes we recommend the Ambion *mir*Vana Probe & Marker Kit. This kit is optimized to efficiently 5' end label both DNA and RNA oligonucleotides and also provides reagents for the rapid removal of unincorporated nucleotides and preparation of small RNA ladders.

4. After the labeling reaction

- Probes prepared by in vitro transcription should be treated with DNase I to remove the DNA template. Yield and specific activity of the radiolabeled transcript should be determined; complete instructions for this are provided in the *mir*Vana miRNA Probe Construction protocol—follow the link on our web site at: www.ambion.com/catalog/CatNum.php?1550
- For accurate quantitation of radiolabeled probes by scintillation counting, remove unincorporated nucleotides (see section IV.C on page 31).
- Probes prepared by in vitro transcription should also be gel purified to remove transcripts that are shorter than the full-length product (see section IV.B on page 29). The quality and efficient recovery of radiolabeled probes can be quickly assessed by denaturing polyacrylamide gel analysis (see section IV.F on page 34).

B. Gel Purification of Probe

Gel purification of probes prepared by in vitro transcription is recommended to separate full-length transcripts from prematurely terminated transcription products as well as from unincorporated nucleotides. This procedure can be performed directly after DNase treatment of the transcription reaction or after removal of the free ribonucleotides with spin column or ethanol precipitation (see section IV.C).

This technique can also be used to gel purify 5' end-labeled probes, unlabeled transcripts or chemically synthesized RNA oligonucleotides to be used as control sense target RNA or competitor unlabeled antisense probe.

1. Separation of the transcription products on a gel

Add an equal volume of Gel Loading Buffer II to the DNase-treated transcription reaction.

Heat for 3 min at 95–100°C and load all or part of the transcription reaction into the freshly-rinsed wells of a 0.75 mm thick denaturing polyacrylamide gel (see recipe in section IV.G.2 on page 35). A 12% polyacrylamide gel is typically used for probes 29–45 nt long (the position of bromophenol blue and xylene cyanol in 12% gels corre-

sponds approximately to 15 and 40 nt, respectively). Run the gel at 10–25 mAmp until the bromophenol blue reaches the bottom of the gel.


NOTE

If possible, use a "preparative scale" comb that will form large capacity wells. Alternatively load the material into several smaller wells, e.g. an entire DNase I treated transcription reaction (21 µL plus 21 µL Gel Loading Buffer II) can be loaded in 2 standard 10 X 5 X 0.75 mm wells.

2. Excise the gel fragment containing the full-length transcript

When the full-length transcript is visualized by autoradiography:

After electrophoresis remove one glass plate from the gel, cover the gel with plastic wrap, and expose it to x-ray film for 30 sec to several minutes; the exposure time will depend on the specific activity of the probe. The goal is to get an autoradiograph with a faint or "grey" signal so that a small discrete gel fragment can be excised. Glow-in-the-dark stickers are the easiest way to orient the film with the gel to cut out the band. Once exposed, develop the film and identify the full-length labeled transcript; it is usually the most slowly migrating, most intense band on the autoradiograph. Now, align the exposed x-ray film with the gel, and cut out the area of the gel that contains the full-length labeled probe with a razor blade or scalpel, and transfer it to an RNase-free microfuge tube. Remove the smallest possible fragment of gel that contains the full-length probe.


IMPORTANT

If the transcription reaction was not treated with DNase I the most slowly migrating band will correspond to the undigested DNA template. This band is ~20 nt longer than the expected RNA probe and is not radioactive.

When the full-length transcript is visualized by ethidium bromide staining:

After electrophoresis stain the gel for 5–10 min in a 0.5–1 µg/mL solution of ethidium bromide in 1X TBE. Wash the gel 2 times for 2 min in 1X TBE and visualize RNA using a UV transilluminator protected with a plastic wrap. The full-length transcript is usually the most slowly migrating, most intense band on the gel. Excise the smallest gel slice possible containing the full-length transcript with a clean scalpel or razor blade and transfer it to an RNase-free microfuge tube.

3. Elute the RNA from acrylamide gel slice

To elute the full-length transcript, add 100–150 µL Probe Elution Buffer to the gel slice and incubate at 37°C for 30 min. Transfer the Probe Elution Buffer, which contains the eluted RNA, to a clean microfuge tube, and repeat with 50–100 µL of Probe Elution Buffer. Pool the two elution fractions and determine the cpm/µL of the recovered RNA by scintillation counting using 1–2 µL of the eluted material.

With this elution procedure about 50% of the RNA will usually be recovered—enough to set up many hybridization reactions. Since longer RNA molecules elute more slowly from the gel we recommend

increasing the elution time for transcripts longer than 40 nt to at least 1 hr. For convenience, or to maximize recovery of RNA from the gel, incubation can be carried out overnight with ~200 µL or more Probe Elution Buffer. The Probe Elution Buffer contains EDTA and SDS which will inactivate low levels of nuclease contamination.

4. Storage of RNA transcript

Eluted RNA can be stored at –20°C in the Probe Elution Buffer up to 10 days. Alternatively probes can be precipitated and resuspended in nuclease-free water or TE. Since the Probe Elution Buffer already contains 0.5 M ammonium acetate simply add a carrier, such as yeast RNA or glycogen, and 3 volumes of 100% ethanol and follow steps 4–8 of the procedure described in section IV.C below.

C. Removal of Free Ribonucleotides from Probe Preparations

There are a number of ways to remove free nucleotides from transcription reactions, including spin columns (such as with the *mir*Vana Probe & Marker Kit), gel purification on a denaturing polyacrylamide gel or two successive ammonium acetate/ethanol precipitations. Here we provide a protocol for ammonium acetate/ethanol precipitation.

IMPORTANT

Always add 5 µg of yeast RNA or other carrier (such as Ambion GlycoBlue™ Coprecipitant) prior to precipitating RNA. Due to the low mass and small size of the transcription products, adding a carrier will greatly improve recovery.

1. Add RNase-free water to the transcription or labeling reaction to bring the volume to 50 µL.
2. Add 5 µL 5 M ammonium acetate and a carrier reagent.
3. Add 3 volumes of 100% ethanol and vortex to mix.
4. Chill the solution at –20°C for 30 min or longer.
5. Spin for >15 min at maximum speed in a 4°C microcentrifuge.
6. Carefully remove the supernatant and discard (dispose of radioactive supernatants appropriately).
7. Wash the pellet by adding 200 µL cold 75% ethanol and repeating steps 5 and 6. Allow to air dry for 5 min.
8. Resuspend the pellet in 20–50 µL of nuclease-free water or TE (10 mM Tris-HCl pH 7, 0.1 mM EDTA).
9. For complete removal of unincorporated ribonucleotides repeat steps 1–7.

Storage

Unlabeled RNA should be stored in aliquots at –70°C. The currently used aliquot can be stored at –20°C. The RNA should be stable for at least 6 months at –70°C in the absence of RNase contamination.

Isotopically labeled RNA should be stored at –20°C until use. Due to high specific activity of the probe, radiolytic degradation will occur. It is therefore advisable to use the probe within 1 week after synthesis, or to gel purify the remaining full-length probe a second time just before use.

D. Quantitation of Target RNA

Absolute quantitation using a standard curve

Several different procedures exist for determining the abundance of a particular target RNA in a heterogeneous RNA mixture. The preferred approach is based on construction of a standard curve using known amounts of in vitro transcribed or chemically synthesized sense strand RNA in a *mir*Vana miRNA Detection reaction. Hybridization reactions containing various amounts of the experimental 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 to estimate the absolute amount of the protecting RNA species in the sample RNA. The assay can be quantitated using a phosphorimager, by densitometric scanning of an autoradiograph, or by excising the regions of the gel that contain the protected fragments and scintillation counting (Hershey and Krause 1989, Roller et al. 1989). Alternatively, a standard curve can be constructed by TCA precipitating and counting the protected fragments (Lee and Costlow 1987). For accurate quantitation, it is critical that hybridization reactions are performed in the presence of an excess of labeled antisense probe, and that they proceed to completion.

Relative quantitation with multiprobe assays

The relative abundance of protecting RNA species in an experimental total RNA sample can also be estimated by a multiprobe assay with probes of known specific activity. For example in the experiments shown in section II.F, the mir-16, mir-16 +4 and mir-22 antisense probes all possess 7 U residues, none of which are present in the leader sequence they share 5'-GGGAGACAGG-3' (the mir-16 +4 also contains 4 additional A residues). Therefore all of the probes and protected fragments have the same specific activity and their relative intensity can be directly compared, i.e. mir-22 has roughly the same level of expression as mir-16 in mouse lung, but miR-22 is about 2 fold more abundant than miR-16 in mouse kidney.

Relative quantitation with housekeeping gene probes

The same approach can be extended to well characterized, constitutively expressed, housekeeping RNA species (e.g. GAPDH mRNA). To do this, design a reference probe to generate short protected RNA fragments slightly longer than the experimental protected fragment with which it will be compared. This can be accomplished by extending the sequence of the probe complementary to the target, or by adding several A residues to the probe (see section IV.A for probe design suggestions).

The sequence of the reference target should be carefully chosen to have roughly the same base content as the experimental target RNA—most importantly, the number of A residues in the sequence will dictate the specific activity of probes prepared by in vitro transcription with [α-^{32}P]UTP. For linear detection of both protected fragments by autoradiography, the level of expression of the reference and target RNA should be in the same range. Alternatively the specific activity of one or both probes can be modified or another probe sequence can be chosen.

Conversion of molar or mass amounts of protecting RNA in the total RNA sample into number of target RNA 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 Rymaszewski et al. (1990).

E. Calculating the Concentration of Unlabeled RNA from a Spectrophotometer Reading

IMPORTANT

If calculating the concentration of in vitro transcribed RNA by A_{260}, DNA and free nucleotides must first be removed.

Dilute the sample 1:50 to 1:500 in water to bring the concentration into the linear range of the spectrophotometer. Quantitate by measuring A_{260} units. Multiply the reading by the dilution factor to give A_{260}.

The RNA concentration can be calculated with either of the following equations:

$$C\ (\text{mol/L}) = A_{260} / (\varepsilon \times l)$$

$$C\ (\mu\text{g/mL}) = \frac{A_{260}}{\varepsilon \times l} \times M \times 1000 = \frac{1000 \times M}{\varepsilon \times l} \times A_{260}$$

Where:

- ε = extinction coefficient (L x mol^{-1} x cm^{-1})
- l = path length (cm): All modern spectrophotometers have a path length of 1 cm.
- M = molecular weight (g/mol)
- C = concentration

Precise quantitation

For accurate quantitation of small RNA transcript, we recommend using the exact molecular weight and extinction coefficient for each RNA sequence. A web-based calculator for calculating molecular weights and extinction coefficients is available at the following address:

www.ambion.com/techlib/misc/RNA_calculator.html

Approximate quantitation

Because for short oligonucleotides (<80 nt), 1000 X M/ε X l ≈ 33, the approximate concentration can be determined with the following formula:

C (µg/mL) ≈ 33 x A_{260}

[Use C (µg/mL) ≈ 40 x A_{260} for long ssRNA, and
C (µg/mL) ≈ 50 x A_{260} for long dsDNA.]

F. Gel Analysis of Small RNA Transcripts or RNA Oligonucleotides

The best way to quickly analyze small RNA transcripts or RNA oligonucleotides is to run an aliquot of the prep on a denaturing 12 or 15% polyacrylamide gel (see section IV.G on page 35 for recipes). The procedure below can be used to analyze radiolabeled or unlabeled RNA transcripts before or after gel purification, to check the quality of a chemically synthesized RNA oligonucleotide or to validate its integrity after a labeling reaction and removal of free nucleotides.

1. Mix an aliquot of the RNA with Gel Loading Buffer II in a final volume of 5–10 µL.
   - use 1–5% of a transcription or labeling reaction for radiolabeled RNA
   - use ≥10% of the transcription reaction for unlabeled RNA
   - use 50–200 pmol for RNA oligonucleotides (e.g. 0.5–2 µL of a 100 µM solution)
2. Heat sample for 3 min at 95–100°C
3. Load the sample on a denaturing 12 or 15% polyacrylamide gel and electrophorese at 20–25 mAmp.
4. Stop electrophoresis when the bromophenol blue dye front has migrated to the bottom of the gel.
5. Expose to X-ray film for one to several minutes for radiolabeled transcripts or stain the gel as described below for unlabeled RNA.
6. Soak the gel for 5 min in a 0.5–1 µg/mL solution of ethidium bromide in 1X TBE.
7. Wash the gel for 2–5 min in 1X TBE.
8. Visualize the RNA using a UV transilluminator.

G. Additional 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 mixes

The following instructions are to prepare 15 mL of gel mix with the indicated percentage of acrylamide and 8 M urea. 15 mL is enough gel solution for one 13 x 15 cm x 0.75 mm gel. Ambion offers reagents for acrylamide gel preparation; see our latest catalog or our web site (www.ambion.com) for specific information.

Amount		
12% gel	15% gel	Component
7.2 g	7.2 g	Urea
1.5 mL	1.5 mL	10X TBE
4.5 mL	5.6 mL	40% acrylamide (acryl:bis acryl = 19:1)
Adjust to 15 mL with nuclease-free water		
Stir to mix, then add:		
75 µL	75 µL	10% ammonium persulfate
15 µL	15 µL	TEMED
Mix briefly after adding the last 2 ingredients, and pour gel immediately.		

V. Appendix

A. References

Hershey AD and Krause JE (1989) Molecular Characterization of a Functional cDNA Encoding the Rat Substance P Receptor. *Science* **247**: 960–962.

Lagos-Quintana M, Rauhut R, Lendeckel W, and Tuschl T. (2001) Identification of novel genes coding for small expressed RNAs. *Science.* **294**(5543):853–8.

Lee JJ and Costlow NA (1987) A Molecular Titration Assay to Measure Transcript Prevalence Levels. *Meth. Enzyme* **152**: 633–648.

Ling M, Risman SS, Klement JF, McGraw N, and McAllister WT (1989) Abortive Initiation by Bacteriophage T3 and T7 RNA Polymerases under Conditions of Limiting Substrate. *Nucl. Acids Res.* **17**: 1605–1618.

Myers RM, Larin Z, and Maniatis T (1985) Detection of Single Base Substitutions by Ribonuclease Cleavage at Mismatches in RNA:DNA Duplexes. *Science* **230**: 1242–1246

Roller RJ, Kinloch RA, Hiraoka BY, Li SS-L, and Wassarman PM (1989) Gene Expression During Mammalian Oogenesis and Early Embryogenesis: Quantification of three messenger RNAs Abundant in Fully Grown Mouse Oocytes. *Development* **106**: 251–261.

Rymaszewski Z, Abplanalp WA, Cohen RM, and Chomezynski P (1990) Estimation of Cellular DNA Content in Cell Lysates Suitable for RNA Isolation. *Anal Bioch* **188**: 91–96.

Zinn K, DiMaio D, and Maniatis T (1983) Identification of Two Distinct Regulatory Regions Adjacent to the Human-Interferon Gene. *Cell* **34**: 865–879.

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 Analysis

Each component is tested in a functional RPA following the protocol. The Mouse Kidney Total RNA is used as sample and the probe is transcribed from the Control Template.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with 25 ng labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with 300 ng supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with 40 ng labeled *Sau*3A fragments of pUC19, and analyzed by PAGE.

Protease testing

Meets or exceeds specification when a sample is incubated with 1 µg protease substrate and analyzed by fluorescence.