# NorthernMax®-Gly Kit

(Part Number AM1946)

*Protocol*

**P/N 1946M Revision B** **Revision Date: July 10, 2008**

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**Literature Citation:** When describing a procedure for publication using this product, please refer to it as the NorthernMax®-Gly Kit.

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# I. Introduction

## A. Background

### What is a Northern blot?

Northern blotting is a technique for size fractionating RNA in a gel, followed by transfer and immobilization on a solid support (membrane) in such a manner that the relative positions of the RNA molecules are maintained. The resulting Northern blot is then hybridized with a labeled probe complementary to the mRNA of interest. Signal generated from detection of the probe can be used to determine the size and abundance of the target RNA. A similar technique was first described by Southern in 1975 for the detection of DNA sequences. The equivalent technique for the detection of RNA sequences, quickly dubbed a "Northern," was published by Alwine, Kemp, and Stark in 1977.

### The NorthernMax®-Gly advantage

Despite the advent of powerful techniques, such as the nuclease protection assay and reverse transcription-PCR (RT-PCR), Northern analysis has remained a standard method for detection and quantitation of mRNA. This is probably because Northern analysis is the only method that provides information about mRNA size, and it is a relatively simple and straightforward procedure. However, the inefficiency of several steps (RNA transfer to the membrane, covalent binding of RNA to the membrane, blocking, hybridization, and washing steps) in the procedure and the possibility for ribonuclease contamination can lead to a loss in sensitivity of the assay. In addition, the numerous protocols available make selection and evaluation of a method laborious and time consuming. The NorthernMax-Gly kit has been optimized to maximize the efficiency of each step in the protocol, providing excellent sensitivity with both radiolabeled and nonisotopic probes.

The NorthernMax-Gly kit uses glyoxal/dimethylsulfoxide (DMSO) to denature the RNA; the gel itself does not contain a denaturant. An advantage of glyoxal/DMSO compared to formaldehyde is that a fume hood is not required to pour and run the gel, and exposure to noxious formaldehyde fumes is avoided. A disadvantage of standard protocols for running glyoxal gels is that recirculation of the electrophoresis buffer is required. The Ambion® NorthernMax-Gly kit, however, includes an improved buffer that does not require recirculation. The sensitivity of glyoxal systems is identical to that of formaldehyde-based Northerns, but glyoxal systems may show sharper bands (*Molecular Cloning*, p. 7.40).

### ULTRAhyb® Ultrasensitive Hybridization Buffer

ULTRAhyb Buffer maximizes the sensitivity of blot hybridizations by drastically increasing hybridization signal without increasing background. Research by in-house scientists and others shows that only 1 to 5% of target molecules are bound to probe using standard

hybridization buffers overnight (Vernier, et al. 1996, Brown, et al. unpublished). In contrast, 50 to 100% of target molecules are bound to probe in an overnight NorthernMax-Gly hybridization. ULTRAhyb Buffer is unique in that it can be used either in sensitive-mode: with an overnight hybridization, or in a fast-mode: with only a 2 hr hybridization. Overnight hybridization increases signal 20- to 50-fold over traditional hybridization buffers (see Figure 1). Messages that can be seen with an overnight exposure to X-ray film using traditional hybridization buffers are generally easily detected using just a 2 hr hybridization in ULTRAhyb Buffer.

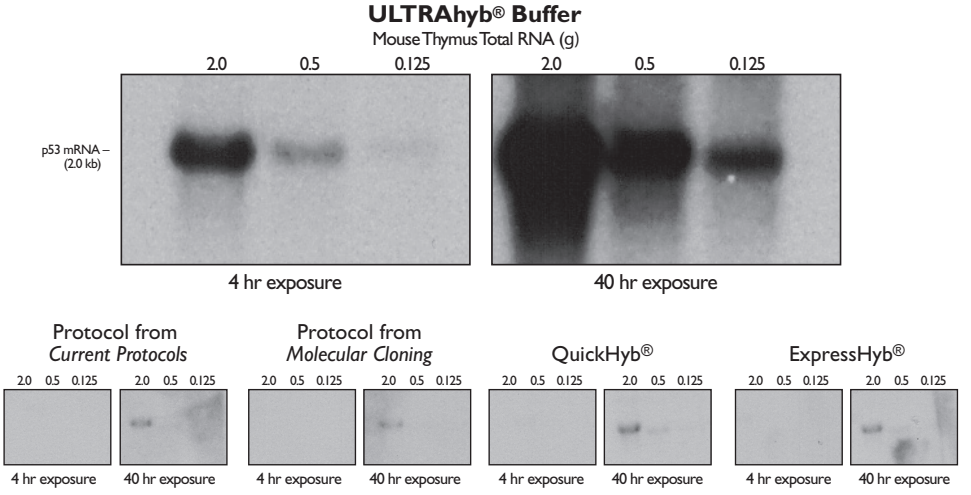



Figure 1. ULTRAhyb® vs. Other Hybridization Buffers

Six identical Northern blots were assayed for p53 using $10^6$ cpm/mL of a $^{32}P$ random-prime labeled probe. The blots were incubated in the indicated hybridization buffers following the manufacturer's recommendations for time and temperature. The ULTRAhyb blot was hybridized overnight at 42°C. Blots were washed at high stringency, and exposed to the same piece of film at –80°C with one intensifying screen for the indicated times.

## Nonisotopic Northerns

One of the problems with nonisotopic Northerns has been that since so much probe is needed, an entire labeling reaction provides probe for only a single Northern blot. ULTRAhyb Buffer requires 10- to 100-fold less DNA probe than standard hybridization buffers (see Figure 2). This means that the 10–20 ng of DNA produced in a single random-priming or PCR probe synthesis reaction will be enough probe for up to 20 Northern blots.

Nonisotopic Northerns have traditionally been plagued with low sensitivity, typically due to high background from the nonisotopic detection protocol. The transfer, hybridization, and washing steps in the NorthernMax Kit minimize one principal cause of high background, the

nonspecific hybridization of the probe molecule to the membrane. Use of the BrightStar®-Plus positively charged membrane with reagents in the BrightStar® BioDetect™ Kit dramatically reduces the other major source of background, nonspecific binding of the secondary nonisotopic detection component. Together, these improvements make nonisotopic Northern analysis a realistic option in terms of sensitivity and probe synthesis.

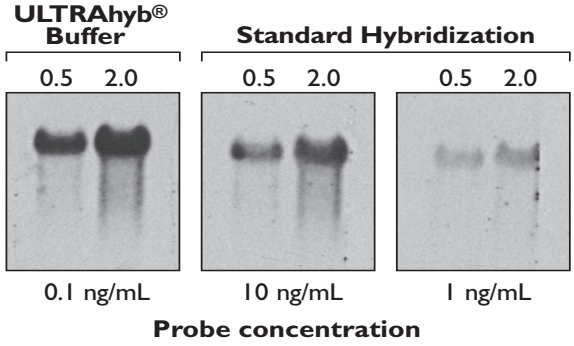



Figure 2. Effect of Hybridization Buffer on the Amount of Probe Needed for Nonisotopic Northerns

Three identical Northern blots were prepared using total mouse thymus RNA. The indicated amount of a biotinylated, random-primed probe for cyclophilin was hybridized to the blots in either ULTRAhyb® Buffer, or in a standard, formamide-based hybridization buffer. Biotinylated probe was detected using the Ambion® BrightStar® BioDetect™ Kit. Blots were exposed to a single piece of film for 15 min.

## B. Kit Contents and Storage

This kit contains reagents for preparing 1000 $cm^2$ of membrane. This is usually enough for up to 20 small blots or about 200 individual RNA samples.

| Amount | Component | Storage |
|---|---|---|
| 11 µg | Positive Control RNA | –20°C |
| 10 µL | pTRI-GAPDH Mouse 0.5 mg/mL | –20°C |
| 10 µL | DECAtemplate ß-actin-Mouse 10 ng/µL | –20°C |
| 6 mL | Glyoxal Load Dye* | –20°C |
| 225 mL | ULTRAhyb† | 4°C |
| 700 mL | 10X Gel Prep/Running Buffer | 4°C |
| 8 g | Agarose-LE | room temp |
| 1000 mL | Transfer Buffer | room temp |
| 450 mL | Low Stringency Wash Solution #1‡ | room temp |
| 450 mL | High Stringency Wash Solution #2‡ | room temp |
| 250 mL | RNase*Zap*® | room temp |
| 1 mL | Nuclease-free Water | any temp** |

* This reagent contains DMSO and ethidium bromide. Handle with care.

† This reagent contains formaldehyde and/or formamide which are potentially hazardous substances. Use with appropriate caution

‡ A precipitate may form in these solutions during shipping, if this occurs, redissolve before use by heating to 37°C and agitating as necessary.

**Store Nuclease-free Water at –20°C, 4°C, or room temp.

## C. Materials Not Provided with the Kit

**General laboratory supplies**

- Nuclease-free water to dilute the agarose and gel running buffer to working concentrations (~1.1 liter/assay). The following procedure is supplied for your convenience:
  i. Add DEPC to double-distilled, deionized $H_2O$ to a concentration of 0.1% (i.e. add 1 mL per liter of $H_2O$).
  ii. Stir well, incubate several hours to overnight at 37–42°C.
  iii. 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.
- Polypropylene microcentrifuge tubes, 1.5 mL or 0.65 mL
- Adjustable pipettors and tips
- Disposable gloves
- Blotting paper (e.g. Whatman 3MM)
- Razor blade, scalpel, or scissors

- Plastic wrap
- Paper towels

**Electrophoresis equipment**

- Horizontal gel electrophoresis chamber, trays, and combs
- Power source capable of delivering 5V/cm (distance measured between the electrodes of the electrophoresis chamber)

**Supplies for transfer and hybridization**

- Positively-charged nylon transfer membrane
  This procedure has been developed and optimized using Ambion BrightStar-Plus membranes, and we recommend their use to minimize background and to maximize signal.
- Hybridization tubes or heat sealable bags and sealer
- Hybridization oven, incubator, or water bath capable of maintaining temperatures between ambient temperature and 68°C.
- Ultraviolet crosslinking apparatus, or oven.
- Glass or plastic, flat bottomed container somewhat larger than the dimension of the agarose gel (used as a component of the transfer set-up described in section II.E on page 10.)

**Probe synthesis and detection**

- Template and reagents for preparing radiolabeled or nonisotopically labeled probes
- Detection System for nonisotopically labeled probes, if used
- X-ray film, film cassettes, and the means to develop the film after exposure
- Intensifying screen(s), if using radiolabeled probes (optional)

## D. Related Products Available from Applied Biosystems

| | |
|---|---|
| BrightStar®-Plus Membrane<br>P/N AM10100–AM10104 | Positively-charged nylon membranes recommended for use with Ambion's BrightStar® nonisotopic labeling and detection products. These membranes are an excellent choice for Northerns and other blot hybridizations. |
| Millennium™ Markers and BrightStar® Biotinylated Millennium™ Markers<br>P/N AM7150 and AM7170 | Ambion's Millennium™ Markers are designed to provide very accurate size determination of single-stranded RNA transcripts from 0.5 to 9 kb and can be used in any Northern protocol. They are a mixture of 10 easy-to-remember sizes of in vitro transcripts: 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6 and 9 kb. |
| KinaseMax™ Kit<br>P/N AM1520 | 5' end labeling kit for DNA and RNA. This kit includes reagents for both kinase and dephosphorylation reactions. |
| RNA Isolation Kits<br>See web or print catalog for P/Ns | Family of kits for isolation of total or poly(A) RNA. Included in the product line are kits using classical GITC and acidic phenol, one-step disruption/denaturation, phenol-free glass fiber filter or magnetic bead binding, and combination kits. |
| FirstChoice® Total and Poly(A) RNA<br>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, and the purity and integrity of these RNAs are verified by Agilent bioanalyzer evaluation, denaturing agarose gel electrophoresis, or Northern analysis. FirstChoice Total RNA is prepared by methods that quantitatively recover small RNAs (miRNA, siRNA, and snRNA). FirstChoice Total and Poly(A) RNAs are ready for use in any application that requires highly purified, intact RNA. See the catalog or website (www.appliedbiosystems.com) for a complete listing of available FirstChoice RNAs. |
| BrightStar® BioDetect™ Kit<br>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. |
| High quality water<br>See web or print catalog for P/Ns | All water products are shown to be nuclease-free by stringent nuclease testing. DEPC-treated water is autoclaved both before and after packaging to assure sterility and inactivation of DEPC. Nuclease-free water that has not been treated with DEPC is also available. |
| RNase-free Tubes & Tips<br>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 the catalog or website (www.appliedbiosystems.com) for specific information. |

# II. NorthernMax-Gly Procedure

## A. Before You Start

Use the RNase*Zap*® Solution provided with the kit to remove any contaminating RNases from pipettors, glassware, and electrophoresis equipment that will be used in conjunction with this kit. Spray or wipe (by applying to a paper towel) surfaces with RNase*Zap*, then rinse twice with RNase-free water to remove any residue. The solution works on contact and can be immediately rinsed; there is no incubation time.

## B. Preparation of Gel

**1. Melt 1 gm agarose in 90 mL RNase-free water + 10 mL 10X Gel Prep/ Gel Running buffer for every 100 mL of gel**

Melt in a microwave oven, hot plate or autoclave, with frequent agitation, until the agarose is completely in solution. Transfer the solution to a 50–60°C waterbath until equilibrated.

**2. Pour the gel to about 0.6 cm in thickness**

Bubbles should be eliminated by popping them with a heated glass or metal rod, or by pushing t hem to the edges of the gel with a clean pipet tip.

The comb should be positioned ~1 cm from the top of the gel, at a height of ~2 mm. To increase well capacity, use combs with thicker teeth rather than pouring a thicker gel.

**3. Allow the gel to solidify at RT or at 4°C, remove the comb**

After the gel has solidified, carefully remove the comb; a thin layer of 1X Gel Running Buffer poured over the gel surface before removing the comb may help to prevent the wells from tearing when the comb is removed.

Examine the wells: a piece of dark paper placed under the wells will make them easier to visualize. Use a pasteur pipet to gently flush out any pieces of agarose in the wells.

**4. Set up the electrophoresis chamber**

Position the gel tray in the electrophoresis chamber with the wells next to the cathode (negative/black) lead.

Dilute the 10X Gel Prep/Running Buffer to 1X with nuclease-free water and cover the gel with about 0.5–1 cm running buffer.

NOTE

*If desired, verify that the wells of the gel are intact by loading 1–2 µL of Load Dye which can then be flushed out of the wells with buffer, or run into the gel.*

If desired, gels can be wrapped in plastic and stored for later use.

## C. Preparation of Sample RNA

**1. Mix sample RNA with an equal volume of Glyoxal Load Dye**

Up to 30 µg total RNA or poly (A+) RNA can be loaded per lane. Using more than 30 µg of sample RNA will probably overload the gel, and/or exceed the RNA binding capacity of the membrane. If molecular weight markers will be used, they should also be mixed with load dye at this step. For large sample volumes, slightly less Load Dye can be used, but do not use less than one-half volume. As the mass amount of RNA is incrementally increased from ~5 µg–30 µg, the mobility of the ribosomal RNA bands generally decreases slightly


NOTE

*Glyoxal Load Dye contains ethidium bromide.*

**2. Incubate the samples 30 min at 50°C**

This incubation denatures RNA secondary structure. If less than one volume of Load Dye was used, increase the incubation time to one hr. A dry heat block is recommended for this step to avoid potential contamination of the samples with water from a water bath. After incubation, spin briefly to collect the samples. Place samples on ice if they will not be loaded onto the gel immediately

Samples can be stored at –20°C at this stage for up to several days before electrophoresis.

## D. Electrophoresis

**1. Load the RNA samples into the wells of the gel**

Use RNase-free pipette tips. To keep the samples as dense as possible, make sure there is no air trapped in the end of the pipette tip. Place the tip just inside the top of the well, expel the sample slowly, then gently raise the pipette tip out of the well.

**2. Run the gel at ~5 V/cm**

This distance should be measured between the electrodes of the electrophoresis chamber (i.e. not just the size of the gel). In general, stop the run when the bromophenol blue dye front (corresponding to approximately 500 nt) has migrated almost to the bottom of the gel.


NOTE

*It is not necessary to recirculate the running buffer.*

Free ethidium (included in the Glyoxal Load Dye) will migrate in the opposite direction of the RNA, and will run off the top of the gel. For this reason if the gel was poured with two tiers of wells, the top tier should be used first, and removed before using the lower tier.

### 3. (optional) Examine the gel with UV light, and photograph it

Avoid prolonged exposure of the gel to UV light during this step.

The gel can be viewed and photographed under UV light before transfer. Put plastic wrap beneath the gel to eliminate possible RNase contamination of the gel by the surface of the transilluminator.

#### Expected appearance of the gel

When visualizing total RNA samples, the ribosomal RNA (rRNA) bands will be distinct and relatively sharp if the samples are undegraded (see figure 3, lane 2 on page 10). If the rRNA bands are smeared as shown in most of the lanes in figure 3, but the molecular weight marker bands are sharp and clear, the RNA is partially degraded and there is no point in carrying on with the procedure. There may be slight migrational differences between ribosomal RNA bands from species other than mouse (the control).

If poly (A+) RNA has been used, there is often enough residual rRNA to determine integrity. If no rRNA is present, there are usually a number of visible bands corresponding to abundant mRNAs. These bands should be sharp and distinct if the RNA is undegraded.

#### Photographing the gel

A transparent ruler should be placed next to the gel when it is photographed (include the wells and bottom of the gel as landmarks) so the positions of the size markers can later be transferred to the film.

#### Post-electrophoresis staining of RNA gels

If the ethidium bromide fluorescence of samples is not sufficiently bright, post-stain the gel by soaking in 1X gel running buffer containing 0.5 µg/mL ethidium bromide for ~20 min (the buffer remaining in the electrophoresis chamber can be used for this purpose). Destain the gel with two 10 min water washes.

To avoid post-staining the sample RNA, the lane containing the size markers can be cut off and stained separately.

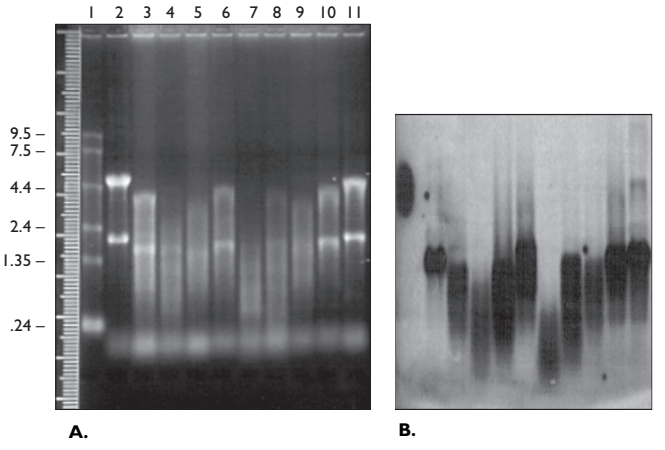



Figure 3. RNA of Variable Integrity - EtBr Stained, and Northern Blot

**A**: This gel shows total RNA samples (5 µg/lane) ranging from high quality, intact RNA (Lane 2) to almost totally degraded RNA (Lane 7). Note that as the RNA is degraded, the 28S and 18S ribosomal bands become less distinct, the intensity of the ribosomal bands relative to the background staining in the lane is reduced, and there is a significant shift in their apparent size as compared to the size standards.

**B**: This is an autorad of the same gel after hybridization with a biotinylated GAPDH RNA probe followed by nonisotopic detection. The exposure is 10 min the day after the chemiluminescent substrate was applied. Note that the signal in Lane 2, from intact RNA, is well localized with minimal smearing, whereas the signals from degraded RNA samples show progressively more smearing below the bands or no bands at all when the RNA is extremely degraded (Lane 7).

## E. Transfer of RNA to the Membrane

The RNA is transferred from the agarose gel to a positively charged nylon membrane so that the size-fractionated RNA can be hybridized to labeled nucleic acid probes. This procedure has been optimized to work with Ambion® BrightStar®-Plus membranes, and we recommend their use to minimize background and to maximize signal.

**IMPORTANT**

*Nitrocellulose membranes are chemically incompatible with the NorthernMax Transfer Buffer, and should not be used.*

We find downward transfer from gel to membrane to be superior to conventional upward capillary transfer. Alternatively, commercially available active transfer methods (electroblotter, semi-dry electroblotter, vacuum blotter, pressure blotter, etc.) can be used. Be sure to follow the manufacturer's recommendations for transfer buffer and for the exact transfer setup. Do not use transfer buffers not specifically

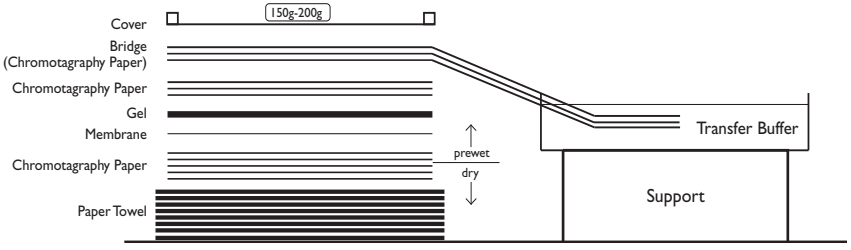
designed for Northern applications. [See Ambion Technical Bulletin #169 (www.ambion.com/techlib/tb/tb_169.html) for a semi-dry electroblot protocol.]

**1. Prepare blotting materials**

- Remove the unused gel above the wells
  Use a razor blade or scalpel to cut through the wells. This prevents Transfer Buffer from flowing through the wells instead of through the gel during transfer.
  To ensure proper orientation later on, cut a notch into the upper right hand corner of the gel and membrane.
- Cut the BrightStar-Plus Membrane (or other positively charged nylon membrane) to the same size or slightly larger than the gel. Handle the membrane only by the edges using gloved hands (preferably powder-free gloves or ones that have been rinsed in DEPC-treated water) or blunt forceps.
- Cut 8 filter paper sheets to the same size or slightly larger than the gel.
- Cut a 3 cm high stack of paper towels to about 1–2 cm wider than the agarose gel.
- Put 0.5 mL Transfer Buffer per $cm^2$ of gel surface into a glass or plastic, flat bottomed container somewhat larger than the dimension of the agarose gel. This will be the reservoir for the Transfer Buffer and will also be used to wet the blotting paper, membrane, and bridge.
- Cut three filter paper bridges large enough to cover the area of the gel and to reach across into the Transfer Buffer reservoir.

**2. Assemble the transfer materials as shown in figure 4**

Figure 4. Downward Transfer Assembly, with Modifications, from Chomczynski, 1992




**a. Arrange a stack of paper towels and blotting paper**
Stack the pre-cut paper towels (~3 cm high) next to the Transfer Buffer reservoir.
Put 3 dry pieces of filter paper on top of the paper towels.

Wet 2 more pieces of filter paper in Transfer Buffer and add them to the top of the stack.

**b. Position the membrane, then the gel on top of the blotting stack**

Wet the BrightStar-Plus membrane briefly in Transfer Buffer and place it on top of the stack of filter paper sheets. Be sure there are absolutely no bubbles trapped between the layers. A glass rod or Pasteur pipette should be used to gently roll out any bubbles.

Center the trimmed gel on the membrane, aligning the notches. Make sure the gel is upright i.e. in the same orientation as it was during electrophoresis, with the bottom of the gel in contact with the membrane. Roll out any bubbles.

**c. Put the filter paper bridge in place**

Wet 3 more pieces of filter paper in Transfer Buffer, and place them on top of the gel. Again, be sure there are absolutely no bubbles trapped between the layers.

Wet the filter paper bridges in Transfer Buffer and place them on top of the stack, with one end in the Transfer Buffer reservoir as shown in the figure. Make sure there are no bubbles trapped between any of the layers.

**d. Cover the stack with rigid light-weight plastic to prevent evaporation**

Usually the casting tray used to pour the gel works nicely.

Place a small weight (150–200 g) on top of the stack to assure even contact of all the stack components.

**IMPORTANT**

*Make sure the filter paper bridge is in contact with the Transfer Buffer in the reservoir. There should be no path for the Transfer Buffer to follow from the reservoir to the dry blotting paper and paper towels except through the gel. The most common place for "short circuiting" to occur is the area where the bridge enters the stack. If the bridge touches the blotting paper or the paper towels underneath the gel, the flow of buffer will bypass the gel - drastically reducing transfer efficiency. Laboratory film (e.g. Parafilm®) or plastic wrap can be placed around the edges of the gel to prevent this from occurring, but this is usually not necessary if the stack is assembled carefully.*

**3. Transfer for 15–20 min per mm of gel thickness**

Transfer for a typical 6 mm thick gel should be 1.5–2 hr.

Do not exceed 4 hr because this would cause hydrolysis of small RNAs, reducing their hybridization signal.

After transfer, the gel will be slightly compressed. The paper towels should be wet, but not soaked through. If the stack of paper towels are soaked through, more paper towels should be used for future transfers.

**4. Disassemble the transfer setup**

Remove the membrane with forceps and briefly (ten seconds) rinse in 1X Gel Running Buffer to remove salt and agarose. The buffer remaining in the electrophoresis chamber can be used for this purpose. Briefly blot excess liquid, but do not dry the membrane.

Verify transfer by examining the gel under UV light; there should be very little ethidium-stained material remaining in the gel at this step.

**5. Crosslink the RNA**

The damp membrane should be treated to crosslink the RNA immediately after step 4.

This can be done by one of two methods:

**a. Ultraviolet light (preferred):**
If using a commercial crosslinker, follow the manufacturer's recommendations. Ultraviolet crosslinking may also be accomplished by using a transilluminator or a handheld UV light source (Ambion Technical Bulletin #169)

**b. Baking:**
The membrane may be treated by baking at 80°C for 15 min. It is not necessary to use a vacuum oven. A conventional oven or convection oven is suitable.

Crosslinked membranes can be stored at –20°C in a vessel that will protect them from physical damage (i.e. rolled up in a 50 mL conical tube).

**6. (optional) View the blot**

If total RNA was used, the ribosomal RNA can be visualized by shining short wavelength ultraviolet light (254 nm) onto the membrane from above in the dark, using a handheld UV light source. The bands will fluoresce brightly. Apply the UV light for as short a period as possible. The position of the bands can be marked for future reference with either a sharp pencil or a black (not blue) Sharpie® marker.

At this point, any lane(s) which are to be hybridized separately can be cut away from the rest of the blot.

## F. Prehybridization and Hybridization

**Temperatures for prehybridization and hybridization**

| Probe type | Prehyb/hyb temperature |
|---|---|
| DNA probes larger than ~50 bp* | 42°C |
| RNA probes larger than ~50 bases | 68°C |
| oligonucleotide probes up to ~50 bases† | 37°C to 42°C |

* DNA probes prepared by random-primed labeling will be on average about half the size of the template used in the labeling reaction

† Use a 37°C hybridization temperature initially, and raise the temperature if cross-hybridization is seen. More information on hybridization temperatures for oligonucleotide probes is provided in section V.D. on page 32

**1. Preheat ULTRAhyb to 68°C**

Swirl the bottle to help dissolve any precipitated material. ULTRAhyb will remain stable even after repeated heating to 68°C, thus the entire bottle can be preheated for convenience.

**2. Prehybridize ≥30 min at the appropriate temperature**

Use ~10 mL preheated ULTRAhyb Buffer per 100 $cm^2$ of membrane. It is not necessary to add any additional blocking agents to ULTRAhyb Buffer for either the prehybridization or the hybridization.

Either heat-sealable bags in a water bath, or hybridization tubes in a roller oven, can be used for prehybridization and hybridization.

If ***heat-sealable bags*** are used, there should be no air bubbles in the bag and the membrane should be entirely covered with ULTRAhyb Buffer. Shaking is not critical as long as the membrane is level in the water bath and is evenly immersed in a film of ULTRAhyb Buffer at all times. A gel casting tray on top of the hybridization bag with a light weight placed on top can be used to accomplish this. More than one membrane can be hybridized simultaneously as long as the membranes can freely move about in the bag.

If using a ***roller bottle-type hybridization oven***, follow the manufacturer's recommendations.

**3. Add probe to the prehybridized blot**

Use the following amounts of probe:

| Type of probe | Final concentration |
|---|---|
| radiolabeled RNA and DNA* probes | $10^6$ cpm per mL |
| nonisotopically labeled RNA probes | 0.1 nM |
| nonisotopically labeled DNA* probes | 1.0–10 pM† |

* Double-stranded DNA probes must be denatured before they are added to the hybridization - see below.

† This is approximately 0.1–1 ng/mL of a 300 nt probe.
Up to 10 pM probe can be used for probes made by enzymatic incorporation of nonisotopically-modified nucleotide, whereas 1 pM should be used for probes made by chemical labeling methods such as Ambion Psoralen-Biotin.
Note that this is significantly less nonisotopic probe than the amount often suggested in blot hybridization protocols.

Probes are added directly to the ULTRAhyb Buffer used for prehybridization. It is not necessary to change the buffer, nor is it necessary to add any additional blocking agents.

**a. RNA probes and single-stranded DNA probes**

Mix the probe with ~1 mL of ULTRAhyb. The probe amounts shown in the preceding table are concentrations in the total volume of ULTRAhyb Buffer used for the hybridization.

Immediately transfer the diluted probe solution to the container with the prehybridized blot and mix well.

**IMPORTANT**

*If heat sealable bags are used, there should be no air bubbles in the bag, and the membrane should be entirely covered with hybridization solution.*

**b. Double-stranded DNA probes**

dsDNA probes must be denatured by heat treatment or by incubation in alkaline solution. After denaturation by either method described below, ***immediately*** add the denatured probe directly to the ULTRAhyb Buffer used to prehybridize the blot, and mix well.

i. Heat Denaturation

Dilute the probe ~10-fold with 10 mM EDTA (use a minimum of 50 µL).

Incubate the diluted probe at 90°C for 10 min.

Centrifuge briefly to collect the solution at the bottom of the tube.

Add ~0.5 mL ULTRAhyb Buffer to the denatured probe, mix and transfer the diluted probe to the prehybridized blot in ULTRAhyb Buffer. (It's fine to use ULTRAhyb Buffer from the prehybridization to dilute the probe.)

ii. Alkaline Denaturation

Dilute the probe with 10 volumes nuclease-free water.

Add 10% volume 3M NaOH. Vortex and centrifuge briefly.

Incubate 5 min at room temp.

Neutralize by adding an equal volume of 1M Tris-HCl (pH 7) and mixing briefly.

**4. Hybridize 2 hr to overnight**

For maximum sensitivity, do an overnight hybridization (14–24 hr). Messages that can be detected in an overnight exposure after an overnight hybridization in traditional hybridization solutions, usually yield equivalent signal from a 2 hr ULTRAhyb Buffer hybridization.

After the incubation, remove the ULTRAhyb Buffer to an appropriate container for disposal.

## G. Washing and Exposure to Film

Use 20 mL per 100 $cm^2$ membrane of wash solution for all washes.

Be sure that the Wash Solutions are completely in solution before using them. If a precipitate is visible, redissolve it before use by heating to 37°C and agitating as necessary.

**1. Low stringency washing**

Do one 10 min, room temperature wash with Low Stringency Wash Solution #1; use agitation.

Low Stringency Wash Solution #1 is equivalent to 2X SSC, 0.1% SDS or 2X SSPE, 0.1% SDS. Its purpose is to remove ULTRAhyb Buffer and unhybridized probe molecules. When using radiolabeled probes, this wash will be radioactive and should be discarded appropriately.

**2. High stringency washing**

**DNA probes longer than 50 bp***

Do two, 15 min, 42°C washes with High Stringency Wash Solution #2; use agitation.

**RNA probes longer than 50 bases**

Do two, 15 min, 68°C washes with High Stringency Wash Solution #2; use agitation.

**Oligonucleotide probes up to 50 bases**

Do one, 2 min, wash with Low Stringency Wash Solution #1 at the hybridization temperature; use agitation.

High Stringency Wash Solution #2 is equivalent to 0.1X SSC, 0.1% SDS or 0.1X SSPE, 0.1% SDS.

**3. Seal radiolabeled blots in plastic and expose X-ray film**
**Proceed with detection of nonisotopically labeled blots**

Remove the blot from the final wash.

***If a radiolabeled probe was used,*** wrap the blot in plastic wrap or in a sheet protector (seal edges) to prevent drying out. If blots are allowed to dry out at any time, it will become difficult or impossible to strip the blot for analysis with other probes.

The blot may now be exposed to film for autoradiography. Usually intensifying screens are used to decrease exposure times.

***If a nonisotopic probe was used,*** follow the manufacturer's recommendations for detection. Do not allow the blot to dry out or it will become difficult or impossible to strip.

* DNA probes prepared by random-primed labeling will be on average about half the size of the template used in the labeling reaction

# III. Analysis of Northern Blots

## A. Expected Results

The bands revealed by Northern analysis should be relatively sharp with a minimum of downward smearing (which would indicate degradation of the RNA). There should be little or no cross hybridization to either the 28S or 18S ribosomal subunits.

## B. Estimating Target Size

Determine the size of an RNA by comparison to RNA size standards. The best size standards will have a relatively large number of evenly spaced bands that are both larger and smaller than the size of the band in question. Comparing the migration of the known standards to the signal generated by the probe is accomplished by plotting, on semi-log paper, the $\log_{10}$ of the size of each standard band against the distance it traveled in the gel. Use the photograph of the gel and ruler as a reference. These data points are connected with a smooth curve. This "standard curve" can then be used to determine the size of any unknown bands on that particular gel by measuring the distance traveled on the autoradiograph or film. The 28S and 18S ribosomal bands can be used to prepare a crude size standard in the absence of other RNA markers.

## C. Quantitation of Target Abundance

### Relative quantitation

#### Hardware and software

Relative quantitation of data from both radioisotopic and nonisotopic Northern analysis can be acquired by densitometric scanning of the film after development. This can be done directly, using instruments designed for this purpose, or indirectly by computer scanning of the film followed by computer analysis of the scanned image. A number of analysis programs are available commercially, or via the Internet (i.e. NIH Image).

It is also possible to obtain images directly, with a phosphoimager. It should be noted that detection of chemiluminescent signals with a phosphorimager requires the purchase of a specific screen designed for that purpose. Typically, phosphorimagers are equipped with data acquisition and analysis software.

#### Normalization

To assure equal loading and transfer, the data is often normalized by comparison to signals obtained from internal controls within each lane. Filters are usually stripped and rehybridized with an internal control standard. These are probes for cellular RNAs assumed to be expressed at a constant level between samples, e.g., ß-actin, GAPDH, cyclophilin, or

ribosomal RNA. Alternatively, a photograph of the ethidium stained gel taken prior to transfer can be used to normalize for equal loading based on the staining of 28S and/or 18S ribosomal subunits.

**Linear range of X-ray film**

Accurate quantitation requires that the relationship between the signal seen and the amount of RNA loaded be in the linear range of the detection. Loss of linearity will occur, for example, if the X-ray film is overexposed. Linearity of exposure, and x-ray film sensitivity, can be increased by pre-exposure to a hypersensitizing flash of light, "preflashing". The best way to assure that signals obtained are in the linear range of the film is to prepare a blot using a titration, covering a wide range of known amounts of the target RNA in question. This blot is hybridized, using the same conditions as those used in the experiment, to generate a standard curve. Densitometric scanning of this blot will reveal the range over which a particular target:probe hybridization is linear (i.e. a given increase in target input generates a proportional increase in signal). As long as signals from the experimental blot correspond in intensity to signals from the standard curve blot that have been demonstrated to fall within the linear range, it can be assumed that the experimental results are linear.

## Absolute quantitation

Absolute quantitation requires the generation of a standard curve using carefully quantitated synthetic sense strand RNA. These transcripts are usually quantitated by incorporation of a trace amount of radiolabel during in vitro transcription. The synthetic RNA is electrophoresed, transferred, and hybridized with a complementary probe of similar composition to that being used in the experimental assay (i.e., single- or double-stranded DNA or RNA, radiolabeled or nonisotopically labeled, similar $T_m$, etc.) Ideally, the artificial sense strand construct will be complementary to the probe being used in the experimental assay and can be treated in an identical fashion. Comparisons can then be made between signals of equal intensity and identical exposure times. Corrections must be made for differences in probe specific activity and length. For accurate absolute quantitation, as for accurate relative quantitation, it is still necessary to demonstrate that signals in the standard curve demonstrate a linear response.

# IV. Troubleshooting

## A. Using the Positive Control

**1. Resuspend the Positive Control RNA**

Spin the RNA pellet to the bottom of the tube before opening it. Dissolve the pellet in 11 µL of the Nuclease-free Water supplied with the kit to yield a final concentration of 1 µg/µL.

**2. Run 1 µL (1 µg) of the Positive Control RNA in an outside lane of a Northern blot**

It is important to load the Positive Control RNA in an outside lane of the blot because it will be cut away from the experimental samples for hybridization with the positive control probe.

Follow the instructions for gel and sample preparation, and electrophoresis found in sections II.B, C, and D, starting on page 7.

**3. View the gel**

The lane containing the Positive Control RNA should have two distinct, strongly visible bands at 4718 and 1847 nucleotides, representing the 28S and 18S ribosomal subunits. Ribosomal RNA makes up approximately 80% of total RNA. Depending on the amount loaded in experimental lanes, there may be a visible haze or background concentrated around and between the ribosomal subunits. This represents the mRNA in the sample. In mammalian cells, mRNA comprises between 0.5 and 3% of the total RNA (Mehra, 1996).

**4. Transfer the RNA to the membrane**

(See section II.E on page 10.)

After transfer and crosslinking, cut away the strip of membrane corresponding to the positive control RNA from the rest of the membrane.

Start prehybridization at either 65°C (if an RNA probe will be used) or at 42°C (if a DNA probe will be used).

**5. Prepare GAPDH probe**

Two probe templates are provided with the NorthernMax-Gly kit to accommodate users who are using either RNA or DNA probe synthesis reagents.

- The ***pTRI-GAPDH Mouse*** is a template designed for the production of labeled antisense RNA probes via in vitro transcription. This template consists of a plasmid containing 316 bp of GAPDH coding sequence downstream of tandem SP6, T7, and T3 polymerase promoters. This plasmid has been linearized 3' of the GAPDH sequence. Any one of the three bacteriophage RNA polymerases can be used to produce antisense GAPDH probes; SP6, T7, and T3 will produce 417 nt, 387 nt, and 359 nt transcripts respectively.

- The ***ß-actin Mouse DECAtemplate*** contains a 1076 bp piece of ß-actin coding sequence. It has been gel purified away from the vector in which it was grown, and is designed for use in random-primed labeling, or Psoralen Biotin† reactions.

**6. Hybridize control probe with the Positive Control RNA blot strip for 2 hr**

Hybridize overnight at 68°C (RNA probe) or 42°C (DNA probe) with probe made from one of the positive control templates.

For radiolabeled RNA or DNA probes, use $10^6$ cpm/mL.

For nonisotopically labeled ß-actin DNA probes made from the ß-actin Mouse DECAtemplate, use 1 pM (~650 pg/mL).

For nonisotopically labeled GAPDH RNA probes made from the pTRI-GAPDH template, use 0.1 nM (~14 ng/mL).

**7. Wash blot, and detect hybridization signal**

Wash blot, and visualize results following the instructions in section II.G on page 15.

**8. Expected Results**

- The GAPDH mRNA is 1.4 kb in size.
- The ß-actin mRNA is 2.1 kb.

Biotinylated probes should generate a band at these positions after a 10–30 min exposure using Ambion® BioDetect reagents. Radiolabeled probes should be exposed to film at room temperature for ~30 min. The band should be relatively sharp, with a minimum of downward smearing. The amount of GAPDH and ß-actin mRNA present in 1 µg total mouse liver RNA is approximately 2–6 pg.

## B. Problems During Electrophoresis

**1. Smearing of ribosomal bands, not attributable to degradation**

Before assuming that samples are degraded, be sure that smearing is not simply due to overloading, incomplete denaturation, or improper electrophoresis. Do not attempt to load more than 30 µg of RNA.

Make sure the sample is diluted with the proper volume of Load Dye (section II.C on page 8). The temperature and incubation time of RNA denaturation in Glyoxal Load Dye are also important; it should be 30 min at 50°C. Cabinet-type incubators work well, but somewhat longer incubation times may be required due to the lower heat transfer capacity of air. After incubation, transfer the samples immediately to an ice bath.

Run the gel with a constant voltage set at a maximum of 5 volts/cm as measured between the electrodes.

† Psoralen Biotin is manufactured by Schleicher and Schuell for Ambion, and its use for labeling nucleic acids is covered by U.S. patent #4,599,303.

**2. Sample RNA and molecular weight marker stain poorly with ethidium bromide**

There is some ethidium bromide in the Glyoxal Load Dye, however, it may not be enough to give optimal ethidium bromide staining. Samples can be post-stained by incubation in running buffer containing 0.5 µg/mL ethidium bromide to increase the fluorescence of the RNA.

**3. Sample RNA appears degraded, Control RNA is undegraded**

Most likely, the RNA was degraded before electrophoresis, this can occur before or during isolation from the biological source. See section *VI.A. RNA Purification* on page 34 for tips on RNA isolation.

It is also possible that the RNA was stored inappropriately, see section *VI.B. Precipitation and Storage of RNA* on page 35 for relevant information.

**4. Sample RNA appears degraded, Control RNA is also degraded**

Usually the result of gross contamination of solutions or equipment with ribonuclease. Consider everything that comes into contact with the samples, directly or indirectly, to be potentially contaminated. This includes pipette tips and tubes, pipettors, electrophoresis equipment, flasks and graduated cylinders used to make or dilute solutions, etc. These can all be decontaminated before use with the RNase*Zap* Solution provided with the kit. All stock solutions should be handled so as not to introduce ribonucleases. Use good lab practices; always wear gloves ("fingerases" are a primary source of ribonuclease contamination); keep reagents closed tightly when not in use; remove only the reagents needed for a single experiment from stock containers into disposable, single-use containers to avoid cross contamination; and follow proper storage and use recommendations.

**5. Intermittent RNA degradation, no clear pattern among samples**

This is consistent with the use of contaminated microcentrifuge tubes and/or pipette tips. We have found frequency of ribonuclease contamination in microcentrifuge tubes ranging from none to up to 50% of the tubes! Tips are less likely to be contaminated, although occasionally this is a problem, especially with tips that have been purchased in bulk and repackaged. Purchase RNase-free tips and tubes for use in preparing Northerns from a reputable supplier (e.g., Applied Biosystems).

Intermittent RNA sample degradation may also be seen on blots with RNA from different tissue sources. Some tissues contain more endogenous RNase than most, making it more difficult to isolate undegraded RNA. See section *VI.A. RNA Purification* on page 34 for tips on RNA isolation.

## C. Problems During Transfer

### 1. Poor transfer/spotty or irregular transfer/ missing bands

Make sure that the directions for assembly of the downward transfer setup have been followed explicitly. Be sure to use an adequate volume of Transfer Buffer (0.5 mL per $cm^2$ of membrane). If the Transfer Buffer can follow any path from the reservoir to the dry paper towels other than through the gel (a "short circuit"), transfer efficiencies will suffer. Parafilm or plastic wrap should be placed around the edges of the gel to prevent this. Make sure that there are absolutely no bubbles trapped between any of the wetted layers of the transfer setup. This will result in void areas on the blot and missing bands. A clean glass rod or glass pipette can be used to roll out any bubbles. Be sure to use positively charged nylon membranes (Ambion® BrightStar®-Plus Membranes are strongly recommended) with the NorthernMax system, especially in nonisotopic applications.

**IMPORTANT**

*Nitrocellulose and supported nitrocellulose membranes are chemically incompatible with the Transfer Buffer, and should not be used. Neutrally charged nylon membranes may give suboptimal results.*

### 2. Residual RNA remaining in the gel after transfer

Virtually all of the RNA should be transported out of the gel and deposited on the membrane, although residual high molecular weight material may remain in the gel near the wells after transfer. A significant amount of residual RNA remaining in the gel after transfer can be caused by a number of factors.

#### Inadequate volume of Transfer Buffer for the membrane transfer

Use at least 0.5 mL/$cm^2$ gel surface of Transfer Buffer, and make sure that there are no short circuits in the setup. There should be good contact between all layers of the transfer setup, including the dry paper towels. This allows an unrestricted flow of Transfer Buffer from the reservoir through the gel.

#### Too large a weight was used on top of the transfer assembly

Do not use a large weight on top of the assembly as would be used in a conventional (upward) capillary transfer. This will compress the gel, decreasing the effective pore size and restricting transport out of the gel. The purpose of the cover on top of the downward transfer setup is to prevent evaporation of the Transfer Buffer.

#### Gel was too thick or contained too much agarose

Do not pour gels more than 6 mm thick, or with an agarose percentage over 1.5%. After transfer, the xylene cyanol and bromophenol blue dyes should have migrated to the membrane. The paper towels should be wet with a substantial amount of Transfer Buffer (about 40–50% of the

initial volume used). Do not attempt to increase transfer efficiency by allowing the transfer to proceed for a longer time; 15 min per mm of gel thickness is adequate. Hybridization signals may drop if the transfer is too long, due to over-hydrolysis of the RNA.

## D. Poor Signal

**1. Hybridization temperature not optimal**

Temperatures of 42°C for DNA probes and 68°C for RNA probes usually give excellent results in ULTRAhyb Buffer; however, hybridization conditions that are substantially above or below the optimum for a given probe can lead to reduced signals. This is most often encountered with probes having unusually high GC or AT content, probes that have a high degree of mismatch with the target, or oligonucleotide probes. Hybridization conditions for these probes may be best determined empirically. See section *V.D. Determining Optimum Hybridization Temperature* on page 32, for more information.

**2. Probe degraded**

Use radiolabeled probes quickly. The high specific activity probes used in Northerns degrade rapidly due to autoradiolysis, resulting in low signal and/or high background. Make sure nonisotopically labeled probes have not been degraded by nuclease contamination.

**3. Probe specific activity too low**

The specific activity of the probe should be at least $10^8$ cpm/µg and preferably greater than $10^9$ cpm/µg. If using end-labeled oligonucleotide probes, switch to a more sensitive probe type such as internally labeled, longer probes.

**4. Probe concentration too low**

Calculate cpm (radiolabeled probes) or concentration by $A_{260}$ (nonisotopic probes) after removal of unincorporated nucleotides. Use $10^6$ cpm/mL for radiolabeled probes, 1 pM for nonisotopically labeled DNA probes, and 0.1 nM for nonisotopically labeled RNA probes.

**5. Procedural Problems**

**a. Incomplete denaturation of double-stranded probes**
Double-stranded probes that are not denatured usually yield little to no hybridization signal. If the probe is only partially denatured, the effective probe concentration will be reduced. Instructions for denaturation of probes is found in section II.F.3 on page 14.

**b. Sub-optimal transfer of RNA to membrane**
See section *IV.C. Problems During Transfer* on page 22.

**c. Inadequate RNA crosslinking/Over-exposure to UV light**
Be sure to follow recommendations for crosslinking of RNA to the membrane. If an uncalibrated handheld UV source or transilluminator is being used for UV crosslinking, do the pilot experiment for calibrating UV sources (Ambion Technical Bulletin #169). Minimize exposure to UV light during UV shadowing.

**d. Transfer too long/too short**
Transfer for 15 min per mm of gel thickness.

**e. Incompatible membrane**
A positively charged nylon membrane, such as Ambion BrightStar-Plus Membranes, is strongly recommended, particularly for nonisotopic Northerns. Nitrocellulose membranes are ***not*** compatible with the NorthernMax Transfer Buffer and should not be used with these kits.

**f. Failure to follow nonisotopic detection protocols**
Follow the manufacturers recommendations closely. Do not attempt to expose nonisotopically labeled blots to film in a freezer as with radiolabeled blots. Low temperatures will stop the enzymatic reactions and no light will be emitted.

**6. Insufficient exposure**

Low copy number RNAs can take >3 days to show up with $^{32}$P-labeled probes at –80°C and intensifying screens. With chemiluminescent detection systems there is often a delay before peak light emission is reached. Low copy number RNAs often take 30 min to 1 hr to show up with the BrightStar system after an initial 2 to 4 hr delay.

**7. Too little target RNA**

Load up to 30 µg total RNA. If this is not enough to give a strong signal, poly (A+) RNA should be used (mRNA represents only about 0.5–3% of total RNA). If this is still not enough, switching to a more sensitive technique such as RT-PCR should be considered.

**8. Message comigrates with ribosomal RNA**

Electrophoresis and/or transfer of target RNA can be hindered by the large amount of ribosomal RNA. Messages that co-migrate with ribosomal RNA may give better signals when poly (A+) selected RNA is used rather than total RNA.

**9. Inappropriate use of intensifying screens, and exposure temp.**

Intensifying screens are only effective if they are incubated with the membrane and film at –70°C. Conversely, if screens are not used, the X-ray film will be much less sensitive at –70°C than at room temperature.

When using two intensifying screens, the exposure setup should be as follows: blot - screen - film - screen. The radioactive energy from the blot will go through the adjacent intensifying screen, expose the film and then be reflected back and forth between the two intensifying screens.

## E. High Background in Lanes

**1. Suboptimal hybridization temperature**

Hybridization temperatures of 42°C for DNA probes and 65°C for RNA probes usually give excellent results; however, hybridization conditions that are substantially below the optimum for a given probe can lead to high background and/or substantial cross-hybridization. Hybridization conditions for these probes may need to be determined empirically. See sections V.D on page 32, and V.E on page 33, for more information.

**2. Probe concentration is too high**

Use $10^6$ cpm/mL for radiolabeled probes, 1 pM for nonisotopically labeled DNA probes made by chemical labeling (such as Psoralen-Biotin), 10 pM for nonisotopically labeled DNA probes made by enzymatic incorporation of nonisotoppic nucleotides, and 0.1 nM for nonisotopically labeled RNA probes. Using more probe may increase hybridization signals, but there may be a proportional increase in background.

**3. Probe contains extra sequence**

Remove plasmid sequence from the probe template before labeling.

## F. High Background Both in Lanes and on the Entire Blot

**1. Prehybridization too short**

Prehybridize at the hybridization temperature for at least 30 min.

**2. Overexposure of film**

Make a shorter exposure. The optimal exposure times using chemiluminescent detection are often very short, 1–30 min. Make a number of exposures, of various times, to obtain the one with the highest signal-to-noise ratio.

**3. Failure to remove free nucleotides from the probe preparation**

Although it is a common practice to leave unincorporated nucleotides in the probe preparation, usually with good results, there are reports that this may lead to high background.

**4. ULTRAhyb Buffer was not completely solubilized**

ULTRAhyb Buffer should be heated to 68°C for 15–30 min before it is added to the blot for prehybridization to completely solubilize all components.

## G. Background not Associated with the Lanes

**1. Incompatible/low quality membrane**

Positively charged nylon membrane such as Ambion BrightStar-Plus Membrane is strongly recommended, particularly for nonisotopic Northerns. Nitrocellulose membranes are ***not*** compatible with the NorthernMax Transfer Buffer and should not be used with this kit.

**2. Membrane dried out during the procedure**

Do not allow the membrane to dry out at any time between prehybridization and exposure. If the membrane becomes dry in the hybridization or washing steps, or during the nonisotopic detection procedure, severe background will often result.

**3. Reagents not evenly distributed**

Do not add probe directly to the membrane and prehybridization solution; dilute it in ~1 mL of ULTRAhyb Buffer immediately before adding it to the prehybridization buffer. Be sure that all solutions are free to move over the entire surface of the membrane during each step in the procedure, and use gentle agitation of the membranes for each incubation. If necessary, increase volumes or switch to another container. If treating more than one membrane at a time, be sure they do not stick together. Make sure there are no folds, creases, or bubbles present if hybridizing in plastic bags.

**4. ULTRAhyb Buffer was not completely solubilized**

ULTRAhyb Buffer should be heated to 68°C for 15–30 min before it is added to the blot for prehybridization to completely solubilize all components.

**5. Microbial contamination of reagents**

This is especially problematic if blocking buffers used in nonisotopic detection systems become contaminated with fungi or bacteria. Follow the manufacturers recommendations for use and storage of reagents. Replace any reagents that appear contaminated (cloudy, filmy, overly viscous, etc.:not due to precipitation)

**6. Particulate matter deposited on membrane**

Be sure to handle membranes only by the edges using powder-free or rinsed gloves and forceps. Protect wet membranes from coming in contact with dust (i.e. the floor). Store membranes in a clean environment at all times.

**7. Precipitates present in nonisotopic detection reagents**

Follow manufacturers recommendations for filtration or centrifugation of reagents, particularly blocking buffer and secondary detection reagents.

**8. Agarose or Transfer Buffer dried on membrane**

Rinse membrane briefly in 1X Gel Running buffer (it is okay to use buffer from the electrophoresis chamber) after transfer and before crosslinking.

**9. Static charges exposing film during development**

Wipe the plastic film covering the membrane with a damp tissue, and allow to air dry before applying film.

**10. Blot too wet when exposed to film**

Blot membrane briefly on filter paper until it is damp but not dripping. Wrap immediately in plastic and expose to film. There should not be any moisture on the outside of the plastic covering the membrane when the film is applied. Carefully blot dry any liquid that seeps out of the edges of the plastic wrap.

## H. Cross-hybridization (Extra bands)

**1. Probe concentration too high**

Reduce probe concentration to suggested levels:

- $10^6$ cpm/mL for radiolabeled probes
- 1 pM for nonisotopically labeled DNA probes made by chemical labeling such as the Ambion Psoralen-Biotin.
- 10 pM for nonisotopically labeled DNA probes made by enzymatic incorporation of nonisotopically labeled nucleotide
- 0.1 nM for nonisotopically labeled RNA probes

**2. Hybridization/washing conditions not stringent enough**

Hybridization temperatures of 42°C for DNA probes and 68°C for RNA probes usually give excellent results; however, hybridization and/or washing conditions that are substantially below the optimum for a given probe can lead to substantial cross-hybridization. Hybridization conditions for some probes must be determined empirically. See sections V.D on page 32, and V.E on page 33, for more information.

**3. There are multiple targets in the RNA sample**

The message could be from a multigene family, be differentially processed, or the probe may have significant homology to related sequences. Redesign probe to avoid areas of high homology. Further optimize hybridization and/or washing conditions to enable discrimination between related sequences. Decreasing probe concentration may help. Using double stranded probes may also help to differentiate between related sequences (Dyson, 1991)

**4. Probe contains too much non-homologous sequence**

Purify templates for random priming and nick translation by restriction digest and gel purification of the cDNA insert. Templates for primer extension and in vitro transcription must be linearized downstream of the insert to generate "run-off transcripts" containing as little vector sequence as possible. PCR generated probes should contain minimal intron or other non-homologous sequences.

# V. Optimizing Reaction Conditions

## A. Selection of Probe Type

Three types of probes are commonly used in Northern analyses: double-stranded DNA, single-stranded DNA (including oligonucleotides), and single-stranded RNA. Probes may be either radiolabeled or nonisotopically labeled. Using traditional hybridization buffer, single-stranded probes are much more sensitive in blot hybridizations than double-stranded probes. This is because with double-stranded probes, unlabeled template can hybridize to the target, effectively lowering the target concentration. Sense-strand probe molecules may also re-anneal to the antisense probe, effectively reducing probe concentration. Ambion ULTRAhyb Ultrasensitive Hybridization Buffer (included in the NorthernMax-Gly kit) drastically reduces the disparity in sensitivity between single-stranded and double-stranded probes by pushing the hybridization equilibrium from 1–5% complete, with traditional hybridization buffers, to 50–100% complete with ULTRAhyb. As few as 100,000 molecules can be detected on a blot. This advance in blot analysis makes the decision to use RNA or DNA probes primarily a matter of preference for a particular labeling technique (see Figure 5 on page 29).

### 1. RNA probes

Typically, RNA probes are made by in vitro transcription from cloned or PCR-generated DNA templates. During the transcription reaction, a modified nucleotide (radiolabeled, or nonisotopically labeled) is incorporated into the probe molecule which can later be detected. Alternatively, unlabeled RNA can be synthesized for labeling with Psoralen Biotin to produce biotinylated RNA probe.

### 2. Double-stranded DNA probes

Double-stranded DNA (dsDNA) probes are commonly used as probes in Northerns, because very high specific activity (2–3 x $10^9$ cpm/µg) probes can be made in random primed labeling reactions that are quick and reliable. Random priming can be used to incorporate either isotopically-labeled or nonisotopically-modified nucleotides. dsDNA probes have the drawback of requiring denaturation before they can be used in hybridization.

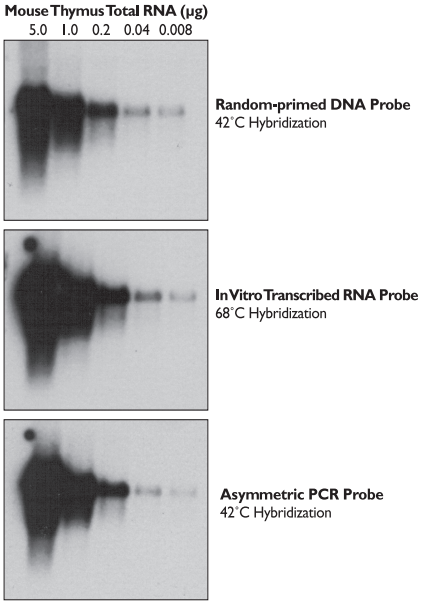



Figure 5. Comparison of Different Probe Types

Identical Northern blots were hybridized overnight with $10^6$ cpm/mL of the indicated probe types. The blots were washed to high stringency, and exposed to the same piece of film for 2.5 hr at –80°C with an intensifying screen.

### 3. Single-Stranded DNA probes

Single-stranded DNA (ssDNA) probes are typically prepared by the following methods:

Primer extension from cloned or PCR generated templates

Linear amplification (Asymmetric PCR)

End-labeling of oligonucleotides

Single-stranded probes do not need to be denatured prior to use. In addition, ssDNA probes made by primer extension can be gel-purified after synthesis to remove template DNA, providing higher specific activity. Both primer extension and linear amplification give probes with very high specific activities (up to $4 \times 10^9$ cpm/µg).

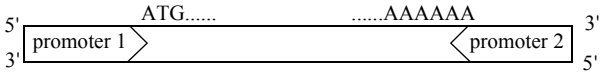
***Oligonucleotides*** are convenient and relatively inexpensive to synthesize. They are a good choice when very little target sequence information is available, or to differentiate messages that share sequence homology. Oligonucleotides are routinely labeled at the 5' end with polynucleotide kinase (for example with the Ambion KinaseMax™ Kit). End-labeled probes have only one detectable residue per molecule, thus they are generally less sensitive than internally-labeled probes. Because of their short length, optimal hybridization conditions for oligonucleotide probes can be more difficult to predict. Changes in base

composition, sequence, and degree of mismatch all affect the $T_m$ of an oligonucleotide more drastically than in longer probes. This means that hybridization conditions for oligonucleotide probes must often be determined empirically.

## B. Probe Design Considerations

### 1. Probes must contain the antisense sequence

To detect mRNA, probes must be "antisense", i.e. complementary to the target mRNA. Double-stranded probes will contain both the sense and the antisense sequence. When designing templates for single-stranded probe, be sure that the antisense strand will be synthesized. Promoters or primers used to generate single-stranded antisense probes must be located at the 3' end of the coding strand. The following schematic illustrates how to choose a promoter site for in vitro transcription, and a parallel strategy should be used to pick the primer for linear or asymmetric PCR probe synthesis.




Transcription using the RNA polymerase corresponding to promoter 1 will make sense RNA (the same sequence as the mRNA). If the RNA polymerase for promoter 2 is used, antisense RNA will be transcribed.

### 2. Probe length

In general, probes that are 100–1000 nucleotides in length will work well. As the length of a probe increases, the signal generated will increase proportionately. Probes over 1000 nucleotides may cause background in the lanes because their length will make it difficult to dissociate probe hybridization to heterologous sequences (*Current Protocols in Molecular Biology*). If probe will be prepared by random-primed labeling, be aware that the average size of probe molecule produced will be about half the length of the template. Because of this, templates longer than 1.5 kb can be random-prime labeled, but templates smaller than ~100 bp should be treated like oligonucleotides in terms of hybridization temperature and washing.

### 3. Probe sequence

Probes should contain a minimal degree of nonhomologous sequence (vector sequence, intron sequence, etc.). Although nonhomologous sequences may increase the signal by making the probe molecule longer (thus containing more labeled residues) this advantage may be outweighed by higher background signals.

Unless it is an aim of the experiment to detect similar mRNAs, probes should contain minimal levels of complementarity to closely related genes or highly repeated sequences.

Probe sequences should contain as few mismatches with their target as possible.

**4. Labeling**

For maximum sensitivity, probes should be labeled to the highest practical specific activity. This will vary depending on the labeling method used. In general, the specific activity should be at least 1 x $10^8$ cpm/µg and preferably >1 x $10^9$ cpm/µg. Nonisotopically labeled probes should have the maximum degree of substitution that will not interfere with hybridization.

**5. Oligonucleotide probes**

Oligonucleotide probes should be at least 15 nt in length. Do not expect a high degree of sensitivity when using end-labeled oligonucleotide probes since only a single labeled residue will hybridize per target.

## C. Preparation and Purification of Probes

**1. Random primed labeling (e.g. Ambion DECAprime™ II Kits)**

Prior to labeling by nick translation or random priming, DNA or cDNA probe templates in plasmid vectors are often separated from as much vector sequence as possible by restriction digestion and gel purification. Otherwise, the vector sequences will be labeled with the probe sequence, leading to higher background.

To ensure maximum specific activity when preparing probes by random priming, be sure that template is limiting the reaction, not the labeled nucleotide. Specific activities can be reduced drastically by excess DNA template in the random priming reaction. Unlabeled template molecules will compete with labeled RNA probe molecules for the RNA target sequences, reducing the signal obtained in blot hybridizations.

**2. In vitro transcription (e.g. Ambion and MAXIscript® Kits)**

Prior to labeling by in vitro transcription, plasmid templates should be linearized by restriction digestion of the vector near the 5' end of the DNA insert (to avoid labeling of excess vector sequence).

Single-stranded RNA probes may be DNase treated or gel purified to remove template DNA. This will prevent hybridization of the unlabeled DNA template to the mRNA target. To make highest specific activity radiolabeled RNA probes, reduce or eliminate the "cold" nucleotide corresponding to the labeled nucleotide (for example, if labeling with $^{32}$P-UTP, don't add any cold UTP).

**3. PCR**

**Linear amplification**

This is PCR using only one primer. The template must be linearized downstream of the sequence to be amplified so that vector sequence is not included in the probe. Typically free nucleotides (left over from PCR for example) must also be removed so that good incorporation of the labeled nucleotide can occur.

**Asymmetric amplification**

Asymmetric PCR uses both upstream and downstream primers, but a much higher concentration of the downstream primer is used to favor the synthesis of antisense probe molecules. Typically PCR or reverse transcription products are used as template. Any free nucleotides remaining from the template production reaction must be removed to get good incorporation of the labeled nucleotide.

**4. Primer extension**

Prior to labeling by primer extension, plasmid templates should be linearized by restriction digestion of the vector near the 5' end of the DNA insert to avoid labeling of excess vector sequence.

Single-stranded DNA probes should be gel purified after synthesis to remove template. This removes the complementary strand which will compete with the target for probe during hybridization.

## D. Determining Optimum Hybridization Temperature

For most probes, the general hybridization guidelines listed in section II.F.2 on page 14 (and repeated below) work very well:

| Probe Type | Prehyb/Hyb Temperature |
|---|---|
| DNA probes larger than ~50 bp* | 42°C |
| RNA probes larger than ~50 bases | 68°C |
| oligonucleotide probes up to ~50 bases | 37–42°C† |

* DNA probes prepared by random-primed labeling will be on average about half the size of the template used in the labeling reaction

† Use 37°C for hybridization initially, and raise the temperature if cross hybridization is seen.

There are exceptions to the above suggestions. The following paragraphs describe possible problems, and how we often solve them.

**RNA probes**

RNA probes with high AT content or multiple mismatches sometimes fail to hybridize at 68°C. If your experiments generate less signal than you expect, try decreasing the hybridization and wash temperatures to 60°C.

**DNA probes**

Cross-hybridization occasionally occurs at 42°C. To eliminate cross-hybridization in subsequent experiments, try increasing the hybridization and wash temperatures to 45–48°C to reduce non-specific probe interactions.

**Oligonucleotide probes**

Oligonucleotide probes with relatively low melting temperatures can fail to hybridize at the recommended hybridization temperatures. If you are getting low or no signal using 37°C for hybridization and washing,

dilute the ULTRAhyb Buffer with an equal volume of 2.5X SSC/7% SDS, and repeat the hybridization at 37–42°C with the diluted ULTRAhyb.

**2.5X SSC/7% SDS**

| for 10 mL | Component |
|---|---|
| 3.5 mL | 20% SDS |
| 1.25 mL | 20X SSC |
| 5.25 mL | nuclease-free water |

## E. Determining Optimum Washing Conditions

The stringency of the second set of washes can be modified by changing the incubation temperature. Typically, high stringency washes are done at a standard temperature of 68°C for RNA probes and 42°C for DNA probes‡. These wash temperatures are about 10–20°C below the $T_m$ of most probe:target duplexes. This is generally a good range, since the purpose of the stringent wash is to remove probe that is inappropriately bound to non-target sequences and the membrane while leaving correctly paired duplexes intact. As temperatures approach the $T_m$ of a duplex, unacceptable loss of signal from correctly paired duplexes will begin to occur.

It is generally better to employ stringent hybridization conditions to reduce cross-hybridization, and to use standard washing conditions rather than depending on very stringent washes to remove nonspecifically bound probe. The hybridization incubation provides ample opportunity for probes to correctly hybridize, even at temperatures relatively near the $T_m$ since high concentrations of probe are driving the reaction towards hybridization. During the washes, probe concentration is very low, so a substantial amount of signal can be lost as temperatures approach the $T_m$.

‡ These conditions are for probes of average GC content that are 100% homologous to the target.

# VI. Additional Procedures

## A. RNA Purification

### Overview

Obtaining high quality, intact RNA from tissue or cell cultures is the first step in Northern analysis. The extraction of RNA is potentially more problematic than DNA extraction since RNA is more labile than DNA, especially at elevated temperature and/or pH. RNA is also prone to attack from a variety of ribonucleases, which are ubiquitous both in tissues and in the environment, and are difficult to inactivate. Despite these problems, it is possible to isolate RNA consistently and reliably, even from tissues with high levels of endogenous ribonuclease activity, if the right procedures and precautions are followed.

Although there are a great number of protocols, techniques, and commercially available kits that can be used to isolate RNA, they all share these common attributes (Farrell, 1993):

1. Cellular lysis and membrane disruption
2. Inhibition of endogenous ribonuclease activity
3. Deproteinization
4. Recovery of intact RNA

The first two steps have historically proven to be the most problematic. Most tissues contain ribonucleases, normally sequestered within the cell but released upon cell lysis, that must be immediately inactivated to prevent degradation of cellular RNA. This is done using a strong chaotropic (biologically disruptive) denaturant such as guanidine thiocyanate, which disrupts cells or tissue samples very rapidly while inactivating even high concentrations ribonucleases. The resulting lysate contains both RNA (cytoplasmic and nuclear) and DNA (nuclear and mitochondrial) as well as proteins and other cellular components. Contaminants are removed with acidic phenol or binding the RNA to a silica matrix and washing. Finally, the RNA is recovered aqueous phase and is typically precipitated with alcohol. This procedure results in very good yields of high quality, biologically active RNA.

The Ambion ToTALLY RNA™ Kit (P/N AM1910) for RNA isolation is based on the classic Chomczynski and Sacchi (1987) method. The RNAqueous® series of RNA isolation kits uses a lysis and denaturation buffer that is derived from this method also. Ambion TRI Reagent® (P/N AM9738) is a monophase solution of phenol and guanidine thiocyanate that facilitates cell lysis and immediate inhibition of ribonucleases. It can be used for isolation of RNA, or sequential isolation of RNA, DNA, and protein. The following series of tips and suggestions pertain to Ambion RNA isolation kits, and to most other RNA isolation procedures.

**Tissue collection**

Tissues must be handled from the moment they are collected, in a manner that will prevent ribonucleolytic degradation. Ambion's revolutionary reagent for tissue storage and RNA stabilization, RNA*later*® Solution, rapidly permeates tissue to stabilize and protect the RNA in fresh tissue samples. It provides a convenient and practical way to collect tissue without flash freezing in liquid nitrogen. Alternatively, tissue must be immersed in liquid nitrogen immediately after dissection, and stored at –70 to –80°C to forestall any degradation. Once a sample has been frozen, it should not be thawed at any time as cells disrupted by freezing have already released ribonucleases that will become active when the temperature is raised.

**RNase precautions**

Using "RNase-free technique" during the initial tissue disruption is not essential since the guanidine solution will inactivate not only the endogenous ribonucleases, but any exogenous ribonucleases that have been inadvertently introduced. However, we do recommend Ambion RNase*Zap*® Solution for decontaminating all equipment and work surfaces prior to RNA isolation. Adequate cleaning of mortar and pestles, homogenizers, Dewar flasks, spatulas, centrifuge tubes, etc. can also be accomplished by scrubbing with soap and hot water, brief rinsing with 1% SDS, followed by rinsing with distilled water. Baking or autoclaving this type of equipment is generally not necessary.

**Tissue/cell disruption**

A major concern is that the tissue disruption be rapid and complete enough to expose all intracellular contents to the denaturant before endogenous ribonucleases have a chance to degrade the RNA. The key to achieving this is to rapidly disrupt tissue once it is placed in the lysis solution. Typically, a rotor-stator device (e.g. a polytron), or a manual homogenizer is used. Frozen (–80°C) samples must be ground to a powder in liquid nitrogen before processing in a homogenizer.

Some tissues, especially tumor samples, are very difficult to break, even when made brittle by freezing in liquid nitrogen. These samples are best prepared by crushing the frozen sample in a transparent bag as described in Gramza et al., 1995 before homogenization.

## B. Precipitation and Storage of RNA

**Precipitation**

It may be necessary to concentrate RNA samples that are too dilute to be loaded in a given volume. This can be done by alcohol precipitation and resuspension at a higher concentration. To ethanol precipitate RNA:

1. Add 5 M $NH_4OAc$ to a final concentration of 0.5 M
2. Mix well, and add 2.5 volumes of ice-cold EtOH
3. Mix again, and chill for at least 20 min at –20°C

To increase the efficiency of precipitation of very low concentrations of RNA, an inert carrier such as glycogen (10–20 µg/mL) or linear acrylamide (10–20 µg/mL) can be added to the precipitation mixture just before the ethanol is added.

To recover the RNA, centrifuge the samples for 15 min at maximum speed (at least 10,000 rpm) in a microcentrifuge at 4°C if possible. All tubes should be positioned the same way during the spin, with the hinges on the attached lids facing away from the center of rotation. The pellets will then form at the back of the tubes, directly below the hinges.

RNA pellets do not adhere tightly to the walls of standard polypropylene microcentrifuge tubes, so remove the supernatant carefully (for example by gentle aspiration rather than by pouring). When most of the supernatant has been removed, re-spin the tube for a few seconds at room temperature, and remove any residual fluid. RNA pellets recovered in this manner do not require further drying. Removing the last traces of supernatant fluid by vacuum-drying is not recommended, because it will concentrate any salts present in the supernatant, which can lead to aberrant migration of the sample during electrophoresis.

### RNA Storage

For long term storage, the RNA may be kept as an alcohol precipitate and stored at –80°C. Otherwise, RNA may be suspended in formamide, which protects against degradation (Chomczynski, 1992) or in an aqueous solution such as THE RNA Storage solution, TE Buffer or 0.1 mM EDTA. These aqueous solutions all contain chelators of divalent cations, which can catalyze RNA strand breakage. We have found that properly prepared RNA, stored at –80°C in water/0.1 mM EDTA, is still completely intact after more than two years.

## C. Stripping, Reprobing, and Storage of Blots

After analysis, the membrane may be stripped of probe so the same blot may be probed again. Never let blots that will be stripped become dry, if they do dry out, it will be nearly impossible to strip the probe from the membrane.

For DNA probes, stripping is best accomplished with 0.1% SDS in DEPC-treated water. Several hundred milliliters of this solution is brought to boiling and poured over the membrane. The solution is allowed to cool to room temperature.

RNA probes are extremely difficult to remove and may have to be stripped by placing the membrane in the above solution in an autoclavable container and autoclaving for 15 min. The membrane is blotted with filter paper to remove excess liquid and may be stored dry, between two sheets of clean filter paper for up to several months.

After stripping, removal of radioactive probes may be verified by exposing the stripped blots to film for a period of time at least equal to that of the initial exposure.

Removal of nonisotopically labeled probes may be verified by reconjugation, detection and exposure of nonradioactively probed blots for a period of time at least equal to that of the initial exposure. These blots should be briefly rinsed with DEPC-treated water before prehybridization.

# VII. 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 Testing**

Tested using a high specific activity rat GAPDH RNA transcript made with the Ambion® MAXIscript® kit. Detection limit is reached at 0.125 µg of mouse liver total RNA. There is no detectable cross hybridization to ribosomal RNA (GAPDH is known to cross hybridize if wash conditions are not sufficiently stringent).

**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.