invitrogen

NorthernMax™ Kit

USER GUIDE

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Revision	Date	Description
B.0	3 February 2023	Minor update to Optional section of Procedure.
A.0	22 December 2020	New document for NorthernMax™ Kit.

The information in this guide is subject to change without notice.

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1 Product information

Product description

The Ambion™ NorthernMax™ Kit contains a complete set of RNase-free reagents for running formaldehyde-based northern analysis. Sufficient reagents are provided to process 1000 cm^2 membrane.

Key features of the kit include:

- Increased sensitivity up to 100x over standard hybridization protocols.
- Reduced hybridization time (2 hours) for many messages.
- The kit contains ULTRAhyb™ Ultrasensitive Hybridization Buffer and gel and wash reagents for 10–20 gels.
- Compatible with isotopically or nonisotopically–labeled RNA, DNA, or oligonucleotide probes.

For additional information about northern blotting, see **https://www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/northern-blotting.html**.

Contents and Storage

The NorthernMax™ Kit (Cat. No. AM1940) 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.

Component	Amount	Storage
Positive Control RNA	11 µg	–30°C to –10°C
pTRI-GAPDH Mouse 0.5 mg/mL	10 µL	
DECAtemplate ß-actin-Mouse 10 ng/µL	10 µL	
Formaldehyde Load Dye[1]	6 mL	
ULTRAhyb™ Ultrasensitive Hybridization Buffer[1]	225 mL	4°C
10X Denaturing Gel Buffer[1]	80 mL	
10X MOPS Gel Running Buffer	600 mL	
Agarose–LE	8 g	15°C to 30°C
Transfer Buffer	1000 mL	

(continued)

Component	Amount	Storage
Low Stringency Wash Solution #1[2]	450 mL	15°C to 30°C
NorthernMax™ High Stringency Wash Buffer[2]	450 mL	
RNase*Zap*™ RNase Decontamination Solution	250 mL	
Nuclease-free Water[3]	1 mL	

[1] These reagents contain formaldehyde and/or formamide which are potentially hazardous substances. Use with appropriate caution.
[2] A precipitate may form in these solutions during shipping. If this occurs, redissolve before use by heating to 37°C and agitating as necessary.
[3] You can also store Nuclease-free Water at –30ºC to –10ºC or 4°C.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. Catalog numbers that appear as links open the web pages for those products. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item	Source
General laboratory supplies	
Nuclease–free water	AM9938
Polypropylene microcentrifuge tubes, 1.5 mL or 0.65 mL	MLS
Adjustable pipettors and tips	MLS
Disposable gloves	MLS
Blotting paper	MLS
Razor blade, scalpel, or scissors	MLS
(Optional) Ethidium bromide	MLS
Plastic wrap	MLS
Paper towels	MLS
Electrophoresis equipment	
Horizontal gel electrophoresis chamber, trays, and combs	MLS
Power source capable of delivering 5V/cm (distance measured between the electrodes of the electrophoresis chamber)	MLS
Supplies for transfer and hybridization	
BrightStar ™–Plus Positively Charged Nylon Membrane, or equivalent	AM10100
Hybridization tubes or heat sealable bags and sealer	MLS

(continued)

Item	Source
One of the following: • Hybridization oven, incubator, or water bath capable of maintaining temperatures between ambient temperature and 68°C. • Ultraviolet crosslinking apparatus.	MLS
Glass or plastic, flat bottomed container somewhat larger than the dimension of the agarose gel	MLS
Probe synthesis and detection	
Template and reagents for preparing radiolabeled or nonisotopically labeled probes	MLS
(Nonisotopically labeled probes) Detection System for nonisotopically labeled probes.	MLS
X-ray film, film cassettes, and the means to develop the film after exposure	MLS
(Radiolabeled probes) Intensifying screen	MLS

Recommended materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. Catalog numbers that appear as links open the web pages for those products. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item	Source
BrightStar ™–Plus Positively Charged Nylon Membrane	AM10100
Millennium™ Markers	AM7150
KinaseMax™ Kit	AM1520
RNase-free tips and tubes	MLS

2 Procedure

Before you begin

Use the RNase*Zap*™ RNase Decontamination Solution provided with the kit to remove any contaminating RNases from pipettors, glassware, and electrophoresis equipment used in conjunction with this kit. Spray or wipe (by applying to a paper towel) surfaces with the solution, 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.

Prepare gel

1. Melt 1 gm agarose in 90 mL RNase-free water for every 100 mL of gel, then transfer the solution to a 50–60°C water bath until equilibrated.

   Melt in a microwave oven, hot plate or autoclave, with frequent agitation, until the agarose is completely in solution.

CAUTION! The 10X Denaturing Gel buffer contains formaldehyde. Use it in a ventilating hood.

2. Add 10 mL 10X Denaturing Gel Buffer per 100 mL of gel.

   Mix well by swirling gently; try to avoid forming bubbles in the gel solution.

3. Pour the gel to about 0.6 cm in thickness.
   - Because of the formaldehyde, the gel should be poured in a fume hood; once the agarose is solidified, it can be removed from the hood.
   - Bubbles should be eliminated by popping them with a heated glass or metal rod, or by pushing them 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.

4. 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 makes them easier to visualize. Use a pasteur pipet to gently flush out any pieces of agarose in the wells.

5. Set up the electrophoresis chamber.

   IMPORTANT! Do not let gels sit in the buffer for more than ~1 hour before loading.

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

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

   c. *(Optional)* 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.

STOPPING POINT Gels can be wrapped in plastic and stored in the refrigerator overnight.

Prepare RNA

1. Mix sample RNA with 3 volumes NorthernMax™ Formaldehyde Load Dye.

   Up to 30 µg total RNA or poly (A+) RNA can be loaded per lane. Using more than 30 µg of total RNA overloads the gel, and/or exceeds the RNA binding capacity of the membrane. If molecular weight markers are used, they should also be mixed with load dye at this step. If the total volume exceeds the capacity of the wells, the RNA must be precipitated and resuspended in a smaller volume of RNase-free water or dissolved directly in ~20 µL NorthernMax™ Formaldehyde Load Dye.

   Note: NorthernMax™ Formaldehyde Load Dye contains significantly more EDTA than most other formulations. The EDTA concentration was increased to guard against divalent cation-mediated strand scission of RNA that can occur at elevated temperatures. When ethidium bromide (EtBr) is added directly to RNA samples in the NorthernMax™ Formaldehyde Load Dye before electrophoresis, the ethidium fluorescence of the samples is reduced compared to what you may be accustomed to seeing. This effect is not seen if the gel is stained post-electrophoresis.

The sensitivity of Northern blots using the control reaction supplied with the kit is actually more sensitive using NorthernMax™ Formaldehyde Load Dye compared to typical loading dyes.

2. *(Optional)* Prepare the positive control RNA.
   a. Spin the RNA pellet to the bottom of the tube before opening it.
   b. Dissolve the pellet in 11 µL of the Nuclease-free Water supplied with the kit to yield a final concentration of 1 µg/µL.
   c. Combine 1 µL of Control RNA (1 µg) with 3 µL of NorthernMax™ Formaldehyde Load Dye.
   d. *(Optional)* If desired, ethidium bromide (EtBr) can be added to the RNA + Formaldehyde Load Dye to a final concentration of 10 µg/mL.

      If desired, ethidium bromide (EtBr) can be added to the RNA + Formaldehyde Load Dye to a final concentration of 10 µg/mL. Alternatively, the ribosomal bands may be visualized after transfer by UV shadowing of the membrane. Always treat samples and markers equally with respect to ethidium bromide staining, because it may slightly alter migration of RNA in the gel.
3. *(Optional)* Add ethidium bromide to the NorthernMax™ Formaldehyde Load Dye or to the RNA samples to a final concentration of 10 µg/mL for direct visualization of the RNA during and after electrophoresis.
4. Incubate the samples 15 min at 65°C.

   This incubation denatures RNA secondary structure. A dry heat block is recommended for this step to avoid potential contamination of the samples with water from a water bath.
5. Centrifuge briefly to collect the contents, then place on ice.

Run the gel

1. *(Optional)* Load the positive control RNA in an outside lane of the gel.

   It is important to load the Positive Control RNA in an outside lane of the blot because it is cut away from the experimental samples for hybridization with the positive control probe.
2. 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.
3. Run the gel at ~5 V/cm. This distance should be measured between the electrodes of the electrophoresis chamber.

   Free ethidium migrates in the opposite direction of the RNA and runs 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.

4. *(Optional)* For runs lasting longer than three hours, during the run, circulate the Running Buffer.

   It is not necessary to circulate the Running Buffer during electrophoresis unless the run exceeds three hours. For long runs, the buffer should be circulated to avoid the formation of a pH gradient that can cause aberrant RNA migration. Circulation can be accomplished by manual exchange of the buffer every 15–30 minutes. Throughout the run (be sure samples have migrated into the gel first), or by continuous circulation of the buffer from one chamber to the other using a pump.

5. Stop the run when the bromophenol blue dye front (corresponding to approximately 500 nt) has migrated almost to the bottom of the gel.

6. *(Optional)* Examine the gel with UV light, and photograph it.
   - Avoid prolonged exposure of the gel to UV light during this step.
   - If ethidium bromide has been added to the samples, 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.
   - For additional information, see **https://www.thermofisher.com/us/en/home/technical-resources/technical-reference-library/nucleic-acid-purification-analysis-support-center/nucleic-acid-electrophoresis-blotting-support.html?icid=scb-nap3**.
   - If ethidium bromide was added to the positive control RNA, 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.

7. *(Optional)* If the ethidium bromide fluorescence of samples is not sufficiently bright, post-stain the gel.
   a. Soak the gel 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).

   b. Destain the gel with two 10 minute water washes.

Perform Northern transfer

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 BrightStar ™–Plus Positively Charged Nylon Membrane, and we recommend their use to minimize background and to maximize signal.

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.

Prepare blotting materials

1. Remove the unused gel above the wells.

   Note:
   - 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.

2. Cut the BrightStar ™–Plus Positively Charged Nylon Membrane (or other positively charged nylon membrane) to the same size or slightly larger than the gel.

   IMPORTANT! Nitrocellulose membranes are chemically incompatible with Transfer Buffer. Do not use.

   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.

3. Cut 8 filter paper sheets to the same size or slightly larger than the gel.

4. Cut a 3 cm high stack of paper towels to about 1–2 cm wider than the agarose gel.

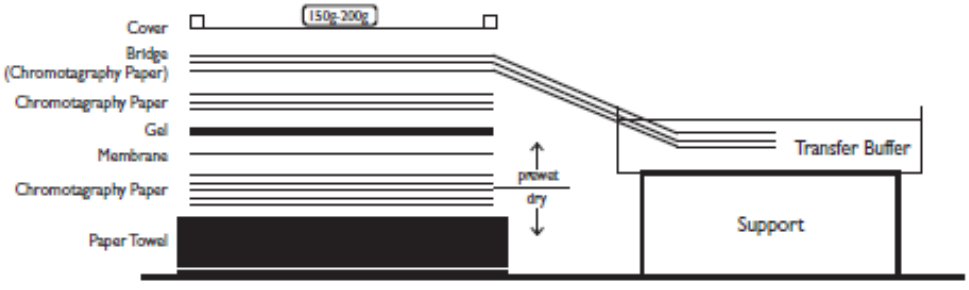
5. Put 0.5 mL Transfer Buffer per cm^2 of gel surface into a glass or plastic flat-bottomed container somewhat larger than the dimension of the agarose gel. This is the reservoir for the Transfer Buffer and is used to wet the blotting paper, membrane, and bridge.

6. Cut three filter paper bridges large enough to cover the area of the gel and to reach across into the Transfer Buffer reservoir.

Set up downward transfer apparatus

This procedure describes how to set up downward transfer. If you are using commercially available active transfer methods (electroblotter, semi-dry electroblotter, vacuum blotter, pressure blotter, etc.), follow the manufacturer's recommendations for transfer buffer and for the exact transfer setup.

The following figure shows how to set up the downward transfer apparatus.



1. Arrange a stack of paper towels and blotting paper.
   a. Stack the precut paper towels (~3 cm high) next to the Transfer Buffer reservoir.

b. Put 3 dry pieces of filter paper on top of the paper towels.

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

2. Position the membrane, then the gel on top of the blotting stack.
   a. Wet the BrightStar ™–Plus Positively Charged Nylon Membrane briefly in Transfer Buffer and place it on top of the stack of filter paper sheets. Ensure there are absolutely no bubbles trapped between the layers. A glass rod or Pasteur pipette should be used to gently roll out any bubbles.

   b. Center the trimmed gel on the membrane, aligning the notches. Ensure the gel is upright (in the same orientation as it was during electrophoresis), with the bottom of the gel in contact with the membrane. Roll out any bubbles.

3. Put the filter paper bridge in place.
   a. Wet 3 more pieces of filter paper in Transfer Buffer, and place them on top of the gel. Ensure there are absolutely no bubbles trapped between the layers.

   b. 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. Ensure there are no bubbles trapped between any of the layers.

4. To prevent evaporation, cover the stack with rigid light-weight plastic, such as the casting tray used to pour the gel, then 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 bypasses the gel - drastically reducing transfer efficiency. Laboratory film (Parafilm™, or equivalent) 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.

Transfer RNA to the membrane

1. 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 is 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.

2. Disassemble the transfer setup.
   a. 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.

b. For samples stained with EtBr, verify transfer by examining the gel under UV light; there should be very little ethidium-stained material remaining in the gel at this step.

3. Crosslink the RNA using one of the following methods.

   - Ultraviolet light—If using a commercial crosslinker, follow the instructions for that crosslinker. Ultraviolet crosslinking may also be accomplished by using a transilluminator or a handheld UV light source.
   - 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.

STOPPING POINT Crosslinked membranes can be stored at –20°C in a vessel that protects them from physical damage (for example, rolled up in a 50–mL conical tube).

4. *(Optional)* View the blot.

   If total RNA was used, the ribosomal RNA can be visualized directly by UV shadowing. This is done by shining short wavelength ultraviolet light (254 nm) onto the membrane from above in the dark, using a handheld UV light source. The bands appear as purple shadows against the lighter background of the membrane. If ethidium bromide was used to stain the RNA, the bands 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) marker.

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

5. *(Optional)* If you are using the positive control, cut away the strip of membrane corresponding to the positive control RNA from the rest of the membrane.

(Optional) Prepare probe for the positive control RNA

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

1. Prepare the GAPDH probe by in vitro transcription. For more information see the *MEGAscript™ Kit User Guide* (Pub. No. 1330M).

   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.

2. Prepare the β-actin probe by random-primed labeling or nonisotopic labeling. For more information, see **https://www.thermofisher.com/us/en/home/technical-resources/technical-reference-library/nucleic-acid-purification-analysis-support-center/nucleic-acid-labeling-quantification-support/nucleic-acid-labeling-quantification-support-troubleshooting.html**.

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

Prehybridize the blot

1. Preheat ULTRAhyb™ Ultrasensitive Hybridization Buffer to 68°C. Swirl the bottle to help dissolve any precipitated material.

   ULTRAhyb™ Ultrasensitive Hybridization Buffer remains stable even after repeated heating to 68°C. Consequently, the entire bottle can be preheated for convenience.

2. Prepare prehybridization apparatus using one of the following methods.

   - Heat-sealable bags—There should be no air bubbles in the bag and the membrane should be entirely covered with ULTRAhyb™ Ultrasensitive Hybridization Buffer. Shaking is not critical as long as the membrane is level in the water bath and is evenly immersed in a film ofULTRAhyb™ Ultrasensitive Hybridization 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.
   - Roller bottle-type hybridization oven—Follow the manufacturer's recommendations.

3. Prehybridize ≥30 minutes at the following temperature. Use ~10 mL preheated ULTRAhyb™ Ultrasensitive Hybridization Buffer per 100 cm^2 of membrane.

   Note: If you used the positive control, perform the prehybridization reaction separately from the membrane with the samples.

   It is not necessary to add any additional blocking agents to ULTRAhyb™ Ultrasensitive Hybridization Buffer for either the prehybridization or the hybridization.

Probe type	Temperature
DNA probes larger than ~50 bp[1]	42°C
RNA probes larger than ~50 bases	68°C
oligonucleotide probes up to ~50 bases[2]	37°C to 42°C

[1] DNA probes prepared by random-primed labeling are on average about half the size of the template used in the labeling reaction.

[2] Use a 37°C hybridization temperature initially, and raise the temperature if cross-hybridization is seen.

Hybridize the blot

1. Add probe to the prehybridized blot. Add the amounts in the following table. Follow the instructions for your probe.

   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.

Probe type	Final concentration
Radiolabeled RNA and DNA[1] probes	10^6 cpm per mL
Nonisotopically labeled RNA probes	0.1 nM
Nonisotopically labeled DNA[1] probes	1.0–10 pM[2]

[1] Double-stranded DNA probes must be denatured before they are added to the hybridization probes.

[2] 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.

   - RNA probes and single-stranded DNA probes—Mix the probe with ~1 mL of ULTRAhyb™ Ultrasensitive Hybridization Buffer. The probe amounts shown in the preceding table are concentrations in the total volume of ULTRAhyb™ Ultrasensitive Hybridization Buffer used for the hybridization. Immediately transfer the diluted probe solution to the container with the prehybridized blot and mix well.
   - Heat denaturation of double-stranded DNA probes—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.)
   - Alkaline denaturation of double-stranded DNA probes—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.

2. 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™ Ultrasensitive Hybridization Buffer hybridization.

3. After the incubation, remove the ULTRAhyb™ Ultrasensitive Hybridization Buffer to an appropriate container for disposal.

Wash and expose film

1. Add Low Stringency Wash Solution #1 (2X SSC, 0.1% SDS or 2X SSPE, 0.1% SDS), 20 mL per 100 cm^2 of membrane.
2. Incubate at room temperature for 5 minutes with agitation.
3. Discard the wash solution and dispose of the wash solution properly.
4. Repeat step 1–step 3.
5. Perform high-stringency wash with agitation (20 mL per 100 cm^2 of membrane).

Probe type	Time	Temperature	Wash solution	Number of washes
DNA probes longer than 50 bp[1]	15 minutes	42 °C	NorthernMax™ High Stringency Wash Buffer[2]	2
RNA probes longer than 50 bases	15 minutes	68 °C	NorthernMax™ High Stringency Wash Buffer[2]	2
Oligonucleotide probes up to 50 bases	2 minutes	Hybridization temperature	Low Stringency Wash Solution #1	1

[1] DNA probes prepared by random-primed labeling are on average about half the size of the template used in the labeling reaction.

[2] 0.1X SSC, 0.1%SDS or 0.1X SSPE, 0.1% SDS.

6. Remove the blot from the final wash the perform one of the following actions.
   - Radiolabeled probes—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 becomes difficult or impossible to strip the blot for analysis with other probes.
   - Nonisotopic probe—Follow the manufacturer's recommendations. Do not allow the blot to dry out or it becomes difficult or impossible to strip.
7. Expose the blots to film.
   - Radiolabeled probes—The blot may now be exposed to film for autoradiography. Usually intensifying screens are used to decrease exposure times.
   - Nonisotopic probe—Follow the manufacturer's recommendations for detection.

3 Data Analysis

Expected results—Samples

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.

Expected Results—Control

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

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 β-actin mRNA present in 1 µg total mouse liver RNA is approximately 2–6 pg.

Estimating target size

The size of an RNA is determined 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 log10 of the size of each standard band against the distance it traveled in the gel. The photograph of the gel and ruler is used 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.

Quantification of target abundance—Relative quantification

Hardware and software:

Relative quantification 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, such as NIH Image, are available commercially or via the Internet.

It is also possible to obtain images directly, with a phosphorimager. 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 ensure equal loading and transfer, the data are 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, such as β-actin, GAPDH, cyclophilin, or ribosomal RNA. Alternatively, a photograph of the ethidium stained gel taken before 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 quantification 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 preexposure 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. For example, 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.

Quantification of target abundance—Absolute quantification

Absolute quantification requires the generation of a standard curve using carefully quantified synthetic sense strand RNA. These transcripts are usually quantified 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; for example, single- or double-stranded DNA or RNA, radiolabeled or nonisotopically labeled, similar Tm, 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 quantification, as for accurate relative quantification, it is still necessary to demonstrate that signals in the standard curve demonstrate a linear response.

Troubleshooting and FAQs

Visit our online Support Centers and FAQ database for tips and tricks for conducting your experiment, troubleshooting information, and FAQs. The online FAQ database is frequently updated to ensure accurate and thorough content.

- For the Nucleic acid purification and analysis Support Center: **http://thermofisher.com/napsupport**
- For FAQs for this product: **http://thermofisher.com/AM1940faqs**
- To browse the FAQ database and search using keywords: **thermofisher.com/faqs**

B

Supplemental information

Optimization

For information about probe selection and optimization of conditions, see **http://thermofisher.com/napsupport**.

Additional procedures

Visit **http://thermofisher.com/napsupport** for information about the following procedures.

- RNA purification
- Precipitation and storage of RNA
- Stripping, reprobing, and storage of blots

Quality control

Relevant kit components are tested using the following 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.

C

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

D Documentation and support

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.