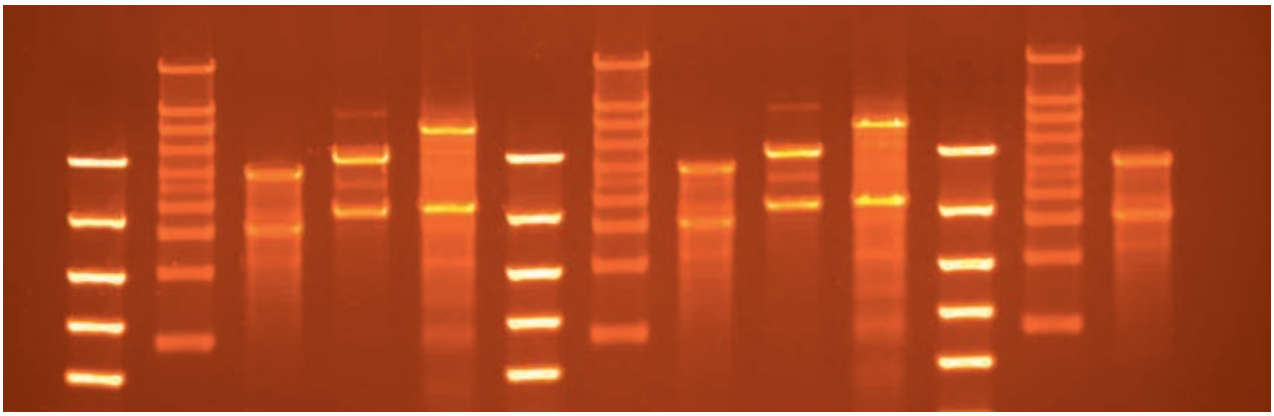


In This Section

Introduction

Separation of RNA in agarose gels is used for a number of different purposes, including Northern blots to monitor RNA expression levels, checking RNA integrity and size-selection of RNA for cloning experiments. Separation of RNA based on fragment length requires conditions that are different from DNA analysis. These include sample preparation, the use of sample and gel denaturants, electrophoresis buffers, and visualization.

Denaturing systems

The purpose of the experiment and the size of the RNA being separated are the primary drivers in determining which denaturing system to use. The most frequently used denaturants for RNA agarose gel electrophoresis are formaldehyde, formaldehyde/formamide, and glyoxal plus DMSO. In each system, the denatured RNA migrates through the agarose gel in a linear relation to the log of its molecular weight (similar to DNA). The most efficient RNA denaturant is methylmercury hydroxide. Because of the hazards associated with this denaturant, it is the least used system for RNA analysis.

The resolving powers of the glyoxal/DMSO and the formaldehyde buffer systems are nearly identical. For detection by Northern analysis, glyoxal/DMSO denaturant is preferable because these gels tend to produce sharper bands than the formaldehyde system. Glyoxal gels require more care to run than formaldehyde gels and because of the lower buffering capacity of glyoxal, these gels must be run at lower voltages than gels containing formaldehyde. Glyoxal gels require a phosphate electrophoresis buffer and the buffer must be recirculated during electrophoresis. If the pH of the buffer rises above 8.0, glyoxal dissociates from RNA, causing the RNA to renature and migrate in an unpredictable manner.

For staining purposes, either denaturant can be used. Ethidium bromide, GelStar® Nucleic Acid Gel Stain and SYBR® Green II Gel Stain bind formaldehyde-denatured RNA more efficiently than glyoxal-denatured RNA. Glyoxal denaturant can interfere with binding of the stain, but gel backgrounds are often lower in these gels than with formaldehyde-denatured gels.

It is important to minimize RNase activity when running agarose gels by following certain precautions. There are several agents on the market that effectively remove RNase's or consult Sambrook, et al.

Section VIII: **Separation of RNA in Agarose Gels**

Preparation of RNA Samples

Which sample denaturation method to choose depends on the final goal of the experiment and the secondary structure of the RNA. There are several procedures to choose from, the most useful of which are described here. Any sample denaturation method can be used with any of the gel buffering systems. If simply checking the integrity of cellular RNA, no sample denaturation is necessary and TAE or TBE Buffer can be used.

Common denaturants	Application
Formamide	Retain biological activity
Formaldehyde	Sample recovery
Glyoxal	Northern blotting; significant secondary structure

Caution: Materials and methods shown here present hazards to the user and the environment. Refer to the safety information on page 210 before beginning these procedures.

Formamide-only denaturation

Formamide denaturation is suitable for almost all RNA samples and is recommended if you need to retain biological activity. Gels can be cast and run in standard TAE or TBE Buffer Systems or MOPS Buffer. If there is a significant amount of secondary structure, another sample denaturation method should be chosen.

- Materials
  - Water bath set to 70°C
  - Ice
- Reagents
  - AccuGENE® Molecular Biology Water (RNase-free)
  - Deionized formamide
  - AccuGENE® 10X MOPS Buffer (200 mM MOPS, pH 7.0, 50 mM sodium acetate, 10 mM EDTA, 10 mM EGTA), pH 7.0

Formamide denaturation of RNA samples

1. Bring the RNA volume up to 8 µl with RNase-free water.
2. Add 2 µl of 10X MOPS Buffer.
3. Add 9 µl of deionized formamide.
4. Mix thoroughly.
5. Heat at 70°C for 10 minutes.
6. Chill on ice for at least 1 minute before loading.

Formaldehyde denaturation

Formaldehyde denaturation is suitable when samples are to be recovered. It is necessary to ensure that the formaldehyde is fully removed from the recovered RNA prior to subsequent studies. Some enzymatic reactions, such as *in vitro* transcription, may be problematic even after complete removal of the formaldehyde.

- Materials
  - Water bath set to 70°C
  - Ice
- Reagents
  - AccuGENE® Molecular Biology Water (RNase-free)
  - AccuGENE® 10X MOPS Buffer (200 mM MOPS, pH 7.0, 50 mM sodium acetate, 10 mM EDTA, 10 mM EGTA), pH 7.0
  - 37% (v/v) formaldehyde
  - Deionized formamide

Caution: Materials and methods shown here present hazards to the user and the environment. Refer to the safety information on page 210 before beginning these procedures.

Formaldehyde denaturation of RNA samples

1. Bring the RNA volume up to 6 µl with RNase-free water.
2. Add 2 µl of 10X MOPS Buffer.
3. Add 2 µl of 37% formaldehyde.
4. Add 9 µl deionized formamide.
5. Mix thoroughly.
6. Heat at 70°C for 10 minutes. Chill on ice for at least 1 minute before loading.

Glyoxal denaturation

Glyoxal is a very efficient denaturant, but should not be used if samples are to be recovered. Glyoxal denatures RNA by introducing an additional ring into the guanosine residues, thus interfering with G-C base pairing. Glyoxal denaturation would be the recommended procedure for Northern blotting. Typically a phosphate electrophoresis buffer is recommended with recirculation to prevent formation of a pH gradient. Alternatively, a 10 mM PIPES, 30 mM bis-Tris buffer, or a 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 1 mM EGTA Buffer systems can also be used without recirculation.

- Materials
  - Water bath set to 50°C
  - Ice
- Reagents
  - AccuGENE® Molecular Biology Water (RNase-free)
  - DMSO
  - 100 mM Sodium phosphate, pH 7.0
    - Mix 5.77 ml of 1 M Na_2HPO_4 with 4.23 ml of 1 M NaH_2PO
    - Adjust volume to 100 ml with RNase-free water
  - 6 M Glyoxal, 40% (v/v) solution, deionized immediately before use
    - Pass solution through a small column of mixed-bed ion exchange resin until the pH is >5.0; large volumes can be deionized then stored frozen in aliquots at −20°C

Caution: Materials and methods shown here present hazards to the user and the environment. Refer to the safety information on page 210 before beginning these procedures.

Glyoxal denaturation of RNA samples

1. Bring the RNA volume up to 11 µl with RNase-free water
2. Add 4.5 µl of 100 mM sodium phosphate.
3. Add 22.5 µl of DMSO
4. Add 6.6 µl of deionized glyoxal.
5. Mix thoroughly
6. Heat at 50°C for 1 hour. Chill on ice for at least 1 minute before loading

Section VIII: **Separation of RNA in Agarose Gels**

Buffers for RNA Electrophoresis

The two commonly used buffer systems for RNA electrophoresis are a phosphate buffer for glyoxal/DMSO denatured RNA and a MOPS Buffer for formaldehyde or formamide denatured RNA. These buffers are very low in ionic strength. During electrophoresis, a pH gradient may be generated along the length of the gel, resulting in the hydrolysis (melting) of the agarose gel. This problem can be avoided by recirculating the buffer. For glyoxal-denatured gels, if the pH of the buffer rises above pH 8.0, the glyoxal will dissociate from the RNA, causing the RNA to renature and migrate in an unpredictable manner.

Buffer preparation

NOTE: Use RNase-free chemicals, water and containers

100 mM Sodium Phosphate Buffer, pH 7.0 (Glyoxal/DMSO denatured RNA)	g/l
1 M Na_2HPO_4	57.7 ml
1 M NaH_2PO_4	42.3 ml

— Adjust volume to 1 liter with RNase-free water

— Adjust volumes accordingly to prepare more buffer

10X MOPS Buffer (Formaldehyde or Formamide denatured RNA)	g/l
200 mM MOPS (free acid)	41.86 g
50 mM sodium acetate	6.80 g
10 mM EDTA•$2H_2O$	3.72 g
10 mM EGTA (free acid)	3.80 g

— Mix with 850 ml of distilled water

— Adjust pH to 7.0 with 10 M NaOH

— Adjust volume to 1 liter with RNase-free water

— Filter through a 0.2 µm nitrocellulose filter and store in the dark

Caution: NaOH is s corrosive material. Use safety glasses and gloves to protect from burns.

Section VIII: Separation of RNA in Agarose Gels

Electrophoresis of RNA

The choice of an agarose free of RNase contamination is of major importance. Lonza offers a variety of agarose products for RNA electrophoresis including The FlashGel® System for RNA, Reliant® and Latitude® Precast RNA Gels, Lonza agarose, AccuGENE® 10X MOPS Buffer, RNA Marker, GelStar® and SYBR® Green Nucleic Acid Gel Stains.

General guidelines

— Northern blotting requires a standard melting temperature agarose such as SeaKem® LE or NuSieve® 3:1 Agarose or Reliant® or Latitude® Precast RNA Gels

— If samples are to be recovered, a low melting temperature agarose can be used such as NuSieve® GTG® or SeaPlaque® GTG® Agarose

— A 1.5 - 2.0% gel made with SeaKem® GTG® or SeaKem® Gold Agarose or FlashGel® System, Reliant® or Latitude® Precast RNA Gels will work for RNA molecules of 500 - 10,000 nucleotides

— For RNA smaller than 500 nucleotides, use a 3 or 4% NuSieve® 3:1 or MetaPhor® Agarose Gel

— For RNA larger than 10,000 nucleotides, SeaKem® Gold Agarose and FlashGel® System, Reliant® or Latitude® Precast RNA Gels will be a better choice for tighter bands and better resolution

— If a low melting temperature agarose is required, a 1.5 or 2.0% SeaPlaque® GTG® Gel can be used for separation of RNA from 500 - 10,000 nucleotides, while a 3.0% or 4.0% NuSieve® GTG® Gel should be used for fine resolution of RNA smaller than 500 nucleotides; NuSieve® GTG® Agarose is not recommended for formaldehyde/MOPS gels

Dye mobilities for RNA gels

The following table is a migration table of single-stranded RNA in relation to bromophenol blue (BPB) and xylene cyanol (XC) in formaldehyde or glyoxal agarose gels.

	Formaldehyde gels		Glyoxal gels	
Agarose (% w/v)	XC	BPB	XC	BPB
SeaKem® Gold Agarose				
1.0	6,300	660	9,500	940
1.5	2,700	310	4,300	520
2.0	1,500	200	2,300	300
SeaKem® GTG® and SeaKem® LE Agarose				
1.0	4,200	320	7,200	740
1.5	1,700	140	2,800	370
2.0	820	60	1,600	220
SeaPlaque® and SeaPlaque® GTG® Agarose				
1.0	2,400	240	4,400	400
1.5	800	80	1,900	180
2.0	490	30	1,050	120
NuSieve® 3:1 Agarose				
2.0	950	70	1,600	155
3.0	370	20	740	75
4.0	190	5	370	40
NuSieve® GTG® and MetaPhor® Agarose				
2.0	Not applicable		1,300	150
3.0	Not applicable		480	60
4.0	Not applicable		260	40

Agarose selection guide for RNA electrophoresis

	Separation	Northern Blotting	Preparative
Fragments <500 bases	NuSieve® 3:1 Agarose MetaPhor® Agarose	NuSieve® 3:1 Agarose	NuSieve® GTG® Agarose
Fragments between 500-10,000 bases	SeaKem® LE Agarose SeaKem® GTG® Agarose	SeaKem® LE Agarose SeaKem® GTG® Agarose	SeaPlaque® GTG® Agarose
Fragments >10,000 bases	SeaKem® Gold Agarose	SeaKem® Gold Agarose	SeaPlaque® GTG® Agarose

Electrophoresis of gels containing formaldehyde

A formaldehyde denaturant with a MOPS buffer is the most commonly used system for RNA electrophoresis. Care should be taken when handling gels containing formaldehyde. These gels are less rigid than other agarose gels. NuSieve® GTG® Agarose is not compatible with the MOPS buffering system.

Formaldehyde electrophoresis of RNA

NOTE: Do not exceed 20 µg of RNA per lane as larger amounts can result in loss of resolution.

1. For a 1% gel, dissolve 1.0 g of agarose in 72 ml of water. Adjust the amounts for different percent gels
2. Cool agarose to 60°C in hot water bath
3. Place in fume hood
4. Immediately add 10 ml of prewarmed 10X MOPS Buffer
5. Add 5.5 ml of prewarmed 37% formaldehyde
6. Cast gel in a fume hood
7. Denature the RNA sample following one of the methods previously described
8. Add 2 µl of formaldehyde loading buffer per 20 µl of sample
9. Mix thoroughly
10. Remove the gel comb
11. Place the gel in the electrophoresis chamber
12. Cover surface of the gel to a depth of 1 mm with 1X MOPS Buffer
13. Load the samples
14. Electrophorese at a maximum of 5 V/cm (interelectrode distance) until the bromophenol blue has traveled at least 80% of the way through the gel (see mobility table on page 135)

- Materials
  - Water bath set to 60°C
  - Fume hood
  - Accessories to cast an agarose gel
  - Electrophoresis chamber and power supply
  - Flask or beaker
- Reagents
  - AccuGENE® 10X MOPS Buffer prewarmed to 60°C
  - 1X MOPS Buffer 37% (v/v), formaldehyde prewarmed to 60°C
  - Formaldehyde Loading Buffer (1 mM EDTA, pH 8.0, 0.4% bromophenol blue and xylene cyanol, 50% glycerol)
  - Distilled water

Caution: Materials and methods shown here present hazards to the user and the environment. Refer to the safety information on page 210 before beginning these procedures.

Electrophoresis of gels containing glyoxal/DMSO

These gels should be run slower than formaldehyde gels with buffer recirculation to avoid the formation of a pH gradient. Glyoxylated RNA will give sharper bands than formaldehyde-treated RNA.

Glyoxal/DMSO electrophoresis of RNA

1. For a 1% gel, dissolve 1.0 g of agarose in 100 ml of 10 mM sodium phosphate, pH 7.0. Adjust amounts for different percent gels.
2. Cool agarose to 60°C in hot water bath.
3. Cast the gels to a thickness that will accommodate a loading volume of 60 µl.
4. Remove the comb.
5. Place the gel in the electrophoresis chamber.
6. Cover gel with 10 mM sodium phosphate buffer to a depth of 1 mm.
7. Add 12 µl of glyoxal loading buffer per 45 µl of sample.
8. Mix thoroughly.
9. Load 0.5 µg - 1.0 µg of RNA per lane.
10. Electrophorese at 4 V/cm (interelectrode distance) while the buffer is recirculated. If no recirculation apparatus is available, pause electrophoresis every 30 minutes and remix the buffer.
11. Electrophorese until the bromophenol blue has traveled at least 80% of the way through the gel (see mobility table on page 135).

- Materials
  - Water bath set to 60°C
  - Fume hood
  - Accessories to cast an agarose gel
  - Electrophoresis chamber and power supply
  - Flask or beaker
  - Recirculating unit
- Reagents
  - 100 mM sodium phosphate pH 7.0
  - Glyoxal loading buffer (10 mM sodium phosphate, pH 7.0, 0.25% bromophenol blue and xylene cyanol, 50% glycerol)

Caution: Materials and methods shown here present hazards to the user and the environment. Refer to the safety information on page 210 before beginning these procedures.

Introduction

Detection of RNA in agarose gels varies depending on the denaturant, stain and photographic conditions used. Lonza offers two highly sensitive stains, GelStar® and SYBR® Green II Gel Stains, for the detection of RNA in agarose gels. These stains exhibit higher RNA detection sensitivity than ethidium bromide, allowing you to load less RNA sample on your gel. Unlike ethidium bromide, GelStar® and SYBR® Green II Stain only fluoresce upon binding to the nucleic acid resulting in lower background fluorescence which is particularly useful when including the stain in the gel for glyoxal denatured samples or when concentrations of formaldehyde in the gel exceed 2 M. The chart below shows the detection sensitivity of various dyes for RNA using in-gel or post-staining techniques.

Stain	Method	Native RNA	Glyoxal RNA
GelStar® Stain	in-gel	10 ng	10 ng
Ethidium bromide, no destain	in-gel	30 ng	150 ng
Ethidium bromide, with destain	in-gel	30 ng	50 ng
SYBR® Green II Stain	post stain	15 ng	50 ng

Limits are based on optimal detection methods for each stain. Samples detected with SYBR® Green Stain were post-stained, samples detected with ethidium bromide or GelStar® Stain were detected by in-gel staining (post-stained gels showed similar results).

FlashGel® System (see page 25), uses a similar stain system and is capable of RNA detection <10ng.

Tips for staining gels with GelStar® or SYBR® Green II Gel Stains

Follow the guidelines below to increase the detection sensitivity of GelStar® or SYBR® Green II Gel Stains.

- New clear polypropylene containers (e.g., Rubbermaid® Recycling #5 Plastics) should be obtained for use with GelStar® and SYBR® Green Stains. When stored in the dark in polypropylene containers, the diluted stain can be used for up to 24 hours and will stain 2 to 3 gels with little decrease in sensitivity. The containers should be rinsed with distilled water (do not use detergents) after each use and dedicated to GelStar® or SYBR® Green Stain use only
- These stains bind to glass and some non-polypropylene (polystyrene) plastics, resulting in reduced or no signal from the nucleic acid
- A 2X working solution of GelStar® or SYBR® Green Stain should be prepared just prior to use from the 10,000X stock solution by diluting in 1X MOPS Buffer or a pH 7.5 to 8.5 buffer (e.g., TAE, TBE or TE).
- For nondenaturing or denaturing polyacrylamide gels, dilute GelStar® or SYBR® Gel Stain 1:10,000X in 1X electrophoresis buffer.
- Agarose gels should be cast no thicker than 4 mm. As gel thickness increases, diffusion of the stain into the gel is decreased, lowering the efficiency of RNA detection.
- Optimal sensitivity for GelStar® and SYBR® Green Stains is obtained by using the appropriate photographic filters for each stain.
  - GelStar® Stain: Wratten® or Tiffen® #9 Filter
  - SYBR® Green Stains: Wratten or Tiffen #15 Filter
- We do not recommend photographing gels with a 254 nm transilluminator. An outline of the UV light source may appear in photographs. A filter that will allow a 525 nm transmission and exclude infrared light is required.

Procedure for staining RNA with GelStar® or SYBR® Green II Gel Stains

For optimal resolution, sharpest bands and lowest background, stain the gel with GelStar® or SYBR® Green II Gel Stain following electrophoresis.

The photographs below demonstrate the detection sensitivity of various stains with different sample denaturants.

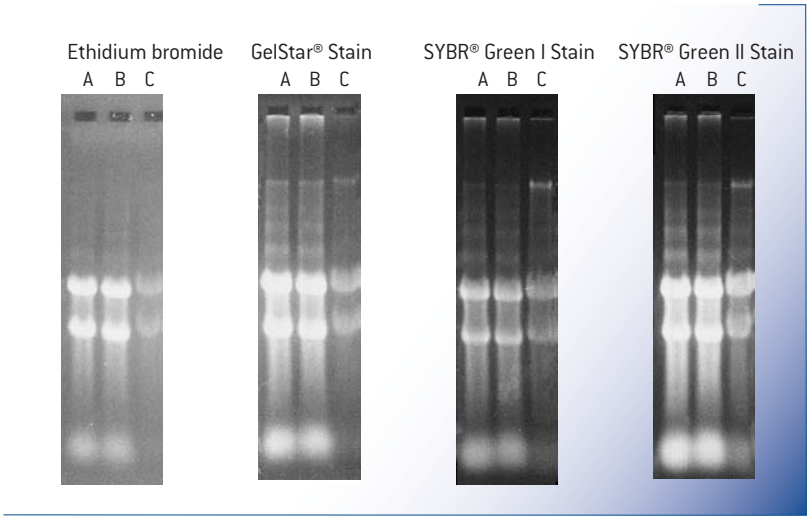
Follow the steps below to stain RNA after electrophoresis

1. Remove the concentrated stock solution of the stain from the freezer and allow the solution to thaw at room temperature.
2. Spin the solution in a microcentrifuge to collect the dye at the bottom of the tube.
3. Dilute the 10,000X concentrate to a 2X working solution (2 µl/10 ml) in 1X MOPS Buffer, in a clear plastic polypropylene container. Prepare enough staining solution to just cover the top of the gel.
4. Remove the gel from the electrophoresis chamber.
5. Place the gel in staining solution.
6. Gently agitate the gel at room temperature.
7. Stain the gel for 60 minutes.
8. Remove the gel from the staining solution and view with a transilluminator, CCD camera or Dark Reader™ Transilluminator.

NOTE: Gels stained with GelStar® or SYBR® Green II Gel Stains do not require destaining. The dyes' fluorescence yield is much greater when bound to RNA than when in solution.

- Materials
  - Clear polypropylene container (e.g., Rubbermaid® Recycling #5 Plastics)
  - GelStar® Photographic Filter (Wratten® #9 equivalent) or SYBR® Green Photographic Filter (Wratten® #15 equivalent)
  - Microcentrifuge
  - UV transilluminator Dark Reader® Transilluminator (Clare Chemical Research, Inc.) or CCD imaging system
- Reagents
  - 1X MOPS Buffer
  - GelStar® or SYBR® Green II Gel Stain stock solution

Caution: Materials and methods shown here present hazards to the user and the environment. Refer to the safety information on page 210 before beginning these procedures.



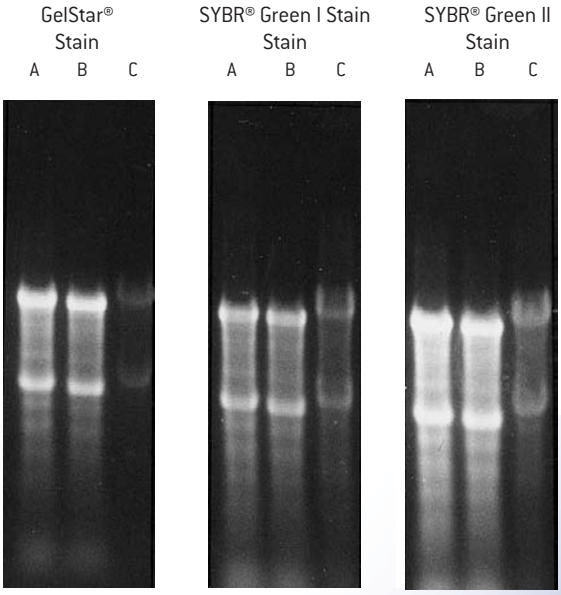
Detection of RNA with GelStar® or SYBR® Green II Gel Stains — continued

Follow this procedure when including GelStar® Nucleic Acid Gel Stain in the agarose gel

NOTE: Unlike ethidium bromide, GelStar® Nucleic Acid Gel Stain does not interact with glyoxal.

1. Remove the concentrated stock solution of GelStar® Stain from the freezer and allow the solution to thaw at room temperature.
2. Spin the solution in a microcentrifuge tube.
3. Prepare the agarose solution.
4. Once the agarose solution has cooled to 70°C, add the stain by diluting the stock 1:5,000 into the gel solution prior to pouring the gel (2 µl per 10 ml).
5. Slowly swirl the solution.
6. Pour the gel into the casting tray.
7. Load samples onto the gel .
8. Run the gel.
9. Remove the gel from the electrophoresis chamber.
10. View with a 300 nm UV Transilluminator, CCD camera or Clare Chemical's Dark Reader® Transilluminator.

NOTE: GelStar® Stained Gels do not require destaining. The dye's fluorescence yield is much greater when bound to RNA than when in solution.



Samples of *E. coli* total RNA were denatured using the following denaturants: Lane A: Formaldehyde/Formamide; Lane B: Formamide; Lane C: Glyoxal. Samples were loaded at 2 mg/lane for the formaldehyde/formamide and formamide only denatured samples, and 4 mg/lane for the glyoxal denatured samples. Reliant® RNA Precast Agarose Gels were run at 7 V/cm for 40 minutes in 1X MOPS Buffer (prepared from AccuGENE® 10X MOPS Buffer) and post-stained using GelStar® or SYBR® Green II Stain or ethidium bromide. The left series of photographs were taken using a Polaroid® Camera, SYBR® Green Photographic Filter on a UV light box. The right series of photographs were taken using the SYBR® Green Photographic Filter on the Clare Chemical's Dark Reader® Transilluminator.

Visualization by photography

Gels stained with GelStar® and SYBR® Green II Gel Stains exhibit negligible background fluorescence, allowing long film exposures when detecting small amounts of RNA. Use the appropriate photographic filter for the stain you are using (see page 138).

The table below provides suggested film types and photographic conditions

Polaroid® Film	f-stop	Exposure time
Type 57 or 667	4.5	0.5-2 seconds
Type 55	4.5	15-45 seconds

Visualization by image capture system

GelStar® and SYBR® Green II Nucleic Acid Gel Stains are compatible with most CCD and video imaging systems. Due to variations in the filters for these systems, you may need to purchase a new filter. Lonza does not sell filters for this type of camera. Contact your systems manufacturer and using the excitation and emission information listed, they can guide you to an appropriate filter.

Stain	Emission (nm)	Excitation (nm)
GelStar® Stain	527	493
SYBR® Green I Stain	521	494
SYBR® Green II Stain	513	497

Application notes

— The fluorescent characteristics of GelStar® and SYBR® Green II Gel Stains make them compatible with argon ion lasers

— Gels previously stained with ethidium bromide can subsequently be stained with GelStar® or SYBR® Green II Gel Stain following the standard protocol for post-staining. There will be some decrease in sensitivity when compared to a gel stained with only GelStar® or SYBR® Green II Gel Stain

— We recommend the addition of 0.1% to 0.3% SDS in the prehybridization and hybridization solutions when performing Northern blots on gels stained with these dyes

- Materials
  - Staining vessel larger than the gel
  - UV transilluminator, Dark Reader® Transilluminator (Clare Chemical Research, Inc.) or CCD imaging system
  - Magnetic stir plate
  - Magnetic stir bar
- Reagents
  - Ethidium bromide stock solution (10 mg/ml) (1.0 g Ethidium bromide,100 ml distilled water, stir on magnetic stirrer for several hours, transfer the solution to a dark bottle, store at room temperature)
  - Distilled water
  - 0.1 M ammonium acetate

Caution: Materials and methods shown here present hazards to the user and the environment. Refer to the safety information on page 210 before beginning these procedures.

Section VIII: **Separation of RNA in Agarose Gels**

Detection of RNA with Ethidium Bromide

Introduction

Ethidium bromide does not stain RNA as efficiently as it does DNA, so be certain that sufficient RNA is loaded to see the band of interest. Although as little as 10 ng - 20 ng of DNA can usually be visualized by ethidium bromide fluorescence, as much as 10-fold more RNA may be needed for good visualization. Visualization of poorly staining RNA is made even more difficult by the higher background fluorescence of RNA gels. In formaldehyde gels, background fluorescence can be minimized by dropping the formaldehyde concentration in the gel from 2.2 M to 0.66 M. Staining with ethidium bromide is not recommended when performing Northern blot analysis onto nylon membranes.

Follow the steps below to stain RNA after electrophoresis

1. Prepare enough working solution of ethidium bromide (0.5 mg/ml of ethidium bromide in 0.1 M ammonium acetate) to cover the surface of the agarose gel.
2. Remove the gel from the electrophoresis chamber.
3. Submerge the gel for 30 - 45 minutes in the ethidium bromide solution.
4. Remove the gel from the solution.
5. Submerge the gel for 60 minutes in a new container filled with distilled water.

NOTE: For gel concentrations of 4% or greater, these times may need to be doubled. If after destaining the background is still too high, continue to destain.

6. Repeat in fresh distilled water.
7. Gels can be viewed with a UV light transilluminator, Dark Reader® Transilluminator (Clare Chenical Research, Inc.) or CCD imaging system.

Follow the steps below when including ethidium bromide in the agarose gel

1. Prepare agarose solution.
2. While the agarose solution is cooling, add ethidium bromide to a final concentration of 0.5 µg/ml to the solution.
3. Slowly swirl the solution.
4. Pour the gel into the casting tray.
5. Add ethidium bromide to the running buffer to a final concentration of 0.5 µg/ml.
6. Load and run the gel.
7. Destain the gel by submerging the gel in distilled water for 60 minutes.
8. Repeat in fresh distilled water.
9. Gels can be viewed with a UV light transilluminator, Dark Reader® Transilluminator (Clare Chemical Research, Inc.) or CCD imaging system.

Decontamination of GelStar® and SYBR® Green Gel Stains and ethidium bromide solutions

Staining solutions should be disposed of by passing through activated charcoal followed by incineration of the charcoal. For absorption on activated charcoal, consult Sambrook, *et al.*, pp. 6.16 - 6.19, (1989). Follow state and local guidelines for decontamination and disposal of Nucleic Acid Staining Solutions.

Recommended agaroses for Northern blotting

When transferring RNA from an agarose gel to a membrane, a standard melting temperature agarose should be used. Standard melting temperature agaroses have higher gel strength than low melting temperature agaroses and do not fracture during transfer. The table below is a list of Lonza agaroses and precast gels that are recommended for blotting.

Agarose	Size Range (bases)
NuSieve® 3:1 Agarose	<500
SeaKem® LE Agarose	500 - 10,000
SeaKem® GTG® Agarose	500 - 10,000
SeaKem® Gold Agarose	500 - 10,000
Reliant® & Latitude® RNA Gels	25 - 10,000

Tips for agarose gel preparation

— Use the lowest agarose concentration required to resolve your fragments

— If staining with ethidium bromide prior to transfer, thoroughly destain the gel with distilled water

— Avoid casting thick gels (>4 mm) unless absolutely necessary. Thick gels not only require longer electrophoretic times, but may interfere with the free transfer of nucleic acids to the hybridization membrane

— Do not use more than a 500.0 gm weight on top of the stack. Excess weight can compress the gel. This will increase the agarose concentration and decrease the pore size, which can inhibit movement of the buffer and RNA

Choosing the appropriate membrane

When transferring RNA, nylon membranes have several advantages over nitrocellulose:

Nylon	Nitrocellulose
Uncharged/charged	Supported/unsupported
High strength	Good strength*
Good for reprobing	Poor for reprobing
UV crosslinking	Baking in vacuum oven at 80°C
50 nucleotides**	500 nucleotides**

*Good for supported nitrocellulose, poor for unsupported nitrocellulose.
**Lower size limit for efficient nucleic acid retention.

Choosing a blotting method

Consider the following when choosing a blotting method:

- Gel concentration
- Fragment size
- Speed and transfer efficiency

Capillary transfer

Traditional passive capillary transfer uses paper towels to draw the transfer buffer from a reservoir through the gel. Passive capillary transfer takes 16 - 24 hours to complete. The most common complaints are poor transfer efficiency for larger RNA molecules and long transfer time.

Vacuum transfer

Vacuum transfer systems use negative pressure to pull the transfer buffer through the gel along with the nucleic acid. These systems take approximately 30 - 60 minutes to complete. Vacuum blotting can be useful for high concentration gels where compression of the gel is a concern.

Electroblotting

Electroblotting is most often used for polyacrylamide gels. A voltage gradient pulls the sample out of the gel and onto a membrane. Semi-dry electroblotting requires minimal buffer with a low voltage and current. Care should be taken so smaller fragments are not pulled completely through the membrane.

General guidelines for Northern blotting

— It is important to minimize RNase activity by following certain precautions. There are several agents on the market that effectively remove RNase's or consult Sambrook, *et al*

— **Wear gloves** throughout the procedure. RNAs are not safe from nuclease degradation until they have been immobilized on the membrane

— Cut or mark membrane for orientation. Do this before wetting the membrane

— The blotting membrane should be in contact with the underside of the gel. Since nucleic acids will concentrate near the bottom of the gel, there is less distance for them to travel during the transfer

— Cut all papers and membranes to the correct size, such that, the only thing pulling the transfer buffer is the buffer solution

— Avoid bubbles, ensure that there is even contact between all the layers of the blotting system

— Incorrect denaturation of probe can cause poor transfer results

— Glyoxal gels can be transferred immediately after electrophoresis. The glyoxal will be removed in the post transfer, prehybridization wash

— Destain gels that contain formaldehyde or ethidium bromide to avoid sample loss and inefficient transfer efficiency

NOTE: Soak gel 3 times for 5 - 10 minutes each in either 1X MOPS, transfer buffer, or sterile water. This will remove most of the formaldehyde and excess background fluorescence seen with ethidium bromide. Complete destaining usually takes 2 hours or longer.

— Lonza's Reliant® and Latitude® Precast RNA Gels do not contain any denaturants or stains and do not require destain prior to transfer.

- Materials
  - Nitrocellulose or nylon membrane
  - 2-3 glass dishes larger than gel
  - Paper towels
  - Whatman® 3MM Chromatography Paper
  - Glass or plastic pipette
  - Flat ended forceps
  - Glass plate to serve as a platform for the gel
  - Plastic wrap
  - <500.0 g weight
  - Orbital or rocking platform shaker
- Reagents
  - AccuGENE® Molecular Biology Water
  - AccuGENE® 20X SSPE or AccuGENE® MOPS Buffer

Procedure for RNA transfer by passive capillary electrophoresis transfer buffer

The concentration of the transfer buffer will vary depending on the method and type of membrane used. The capillary method outlined here uses a nylon membrane and 5X SSPE Buffer.

20X SSPE	
(3 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$, 0.2 M EDTA • $2H_2O$)	
175.3 g	NaCl
27.6 g	$NaH_2PO_4 \cdot H_2O$
7.4 g	EDTA • $2H_2O$
Adjust to pH 7.4 with 10 N NaOH	
To 1 liter with distilled water	

Follow the steps below for gel preparation and setting up the transfer

1. Cast a standard melting temperature agarose gel no thicker than 4 mm.
2. Electrophorese RNA following standard protocols.
3. For formaldehyde gels, follow the steps below prior to transfer. For glyoxal gels, proceed to step 4.
4. Soak the gel in an excess of 1X MOPS Buffer, distilled water or 5X SSPE for 10 minutes.
5. Repeat three times with new wash solution each time.
6. Float membrane in RNase-free water for 5 minutes.
7. Equilibrate membrane in 5X SSPE for 5 minutes. The membrane may remain in the transfer buffer until it is used.
8. Set up transfer using either an upward capillary transfer set up or downward capillary transfer set up.
9. Allow the transfer to proceed as follows:

Transfer set-up	Time
Upward	16 - 24 hours
Downward	1 - 4 hours depending on gel thickness and concentration
Turboblotter™ System	1 - 3 hours

10. Remove the paper toweling or chromatography paper.
11. Remove the gel and membrane together.
12. Mark the positions of the wells on the membrane with a pencil for orientation then remove the gel from the transfer setup.
13. Rinse the membrane for 30 - 60 seconds in transfer buffer.
14. Place the membrane on a sheet of Whatman® 3MM Chromatography Paper.
15. Treat membrane as described in Immobilizing RNA on a membrane, next section.

References

Ausubel, F.E., *et al., Current Protocols in Molecular Biology*, J. Wiley and Sons, Inc.,1998.
Farrell, R.E. Jr., *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*, 2nd Edition, Academic Press, 1998.
Sambrook, J., *et al., Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989.
White, H.W., *et al., BioTechniques* **26**: 984 - 988, 1999.
Grundemann, D. & Koepsell, H., *Anal. Biochem.* **216**: 495 - 461, 1994.

Materials

- Membrane
- Plastic wrap
- UV light source
- Vacuum dryer set to 80°C or hot oven at 65°C.

Reagents

- AccuGENE® 1 M Tris pH 8.0
- RNase-free water
- AccuGENE® Molecular Biology Water or AccuGENE® 10X Mops Buffer
- AccuGENE® 20X SSPE

Immobilizing RNA on a membrane

The methods and procedures for immobilizing RNA on the membrane are essentially the same as they are for DNA.

If samples have been denatured under the formaldehyde system, immobilization can take place immediately after transfer without any pretreatment steps. The formaldehyde has been removed from the system prior to transfer during the gel destaining steps. If the glyoxal denaturing system was used, the glyoxal must now be removed from the filter. Follow one of the procedures below to remove glyoxal from the membrane.

Option 1

1. Air dry the membrane.
2. Bake the membrane as follows:

Membrane Type	Temperature	Time
Nitrocellulose	80°C	2 hours under vacuum
Nylon	65°C	1 - 1.5 hours

3. Immerse the filter in 200 ml of preheated 20 mM Tris, pH 8.0.
4. Cool to room temperature.

Option 2

1. Immobilize RNA on membrane either by UV irradiation or baking.
2. Wash membrane 65°C for 15 minutes in 20 mM Tris, pH 8.0.

The FlashGel® System completes RNA analysis in less than 30 minutes and requires one-fifth the amount of total RNA for detection. The FlashGel® System is recommended for verification and analysis of total RNA (Figure 1), quick checks of native RNA (Figure 3) and checks for RNA degradation (Figure 2).

Rapid RNA analysis

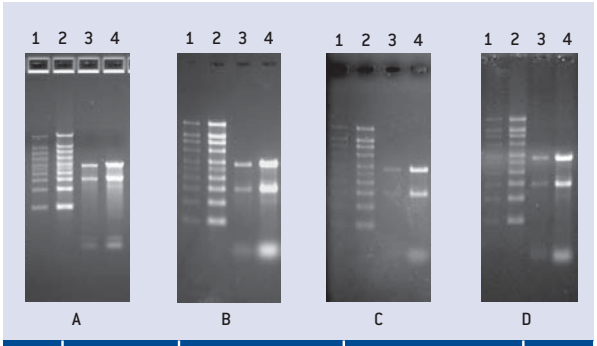
The FlashGel® System for RNA separates up to 34 samples of RNA in 8 minutes or less. RNA samples are visible on the FlashGel® Dock for up to 4 minutes, after which they fade and then reappear with increasing intensity following a 10-20 minute post-run hold. Full analysis is complete in less than 30 minutes, compared to the 1-3 hours required for typical agarose gels. Figure 1 demonstrates analysis time of the FlashGel® System compared to other agarose gel methods for RNA.

Sensitive detection

RNA quantities <10 ng per band are clearly detected on the FlashGel® System for RNA. Some RNA preparations deliver such low yields that there may not be the surplus RNA needed to assess integrity. The FlashGel® System for RNA uses a stain that reduces the amount of required RNA by a factor of five or greater compared to ethidium bromide. Figure 1 illustrates detection sensitivity of the FlashGel® System for RNA compared to other agarose gel and staining methods.

Clean, enclosed system

The FlashGel® System for RNA eliminates the hazards and tedium associated with RNA gel preparation. The cassettes fully enclose the gel and running buffer, eliminating operator exposure to hazardous reagents, and protecting samples from contaminating RNases. The cassettes contain a 1.2% agarose and buffer blend which are selected for purity, manufactured in a dedicated clean room, and guaranteed RNase-free. The FlashGel® Dock does not come in contact with samples or gels, so it is not necessary to dedicate a unit for RNA work.



Panel	Gel	Sample preparation	Run conditions	Total time
A	FlashGel® System for RNA	Formaldehyde Sample Buffer (Cat.# 50571) Samples denatured 5 Min. at 65° C.	8 min run at 225 V. Cassette held at ambient temp for 20 min prior to imaging.	28 min
B	Reliant® 1.25% MOPS Gel (Cat. #54922)		60 min run at 5 V/cm. 60 min post-stain with GelStar® Stain (Cat. #50535) 1:5000 dilution	120 min
C	Reliant® 1.25% MOPS Gel		60 min run at 5 V/cm. 60 min EtBr post-stain and 60 min de-stain.	108 min
D	Hand-cast 1% SeaKem® LE Agarose Gel (Cat. #50002)	10µg/ml EtBr added to Formaldehyde Sample Buffer. Samples denatured 5 Min. at 65° C.	Denaturing gel prepared at 2.2 M Formaldehyde in 1X MOPS buffer (Cat. #50876). 60 min run at 6 V/cm.	120 min

Figure 1. Sample of RNA Marker (Cat. # 50575) lanes 1 & 2, and E. coli Total RNA (Ambion) Lanes 3 & 4 contain 50 ng (lanes 1 & 3 or 250 ng (lanes 2 & 4) of RNA per 5µl load.

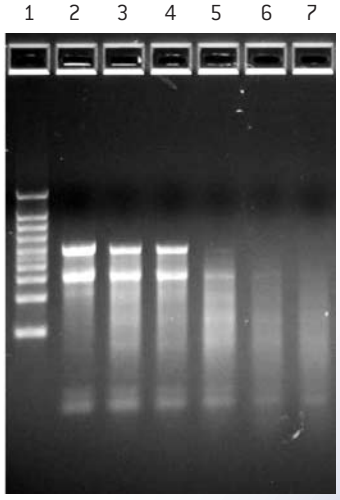


Figure 2. Checking Sample Quality with the FlashGel® System for RNA Sample degradation is clearly visible on the FlashGel® System for RNA. 8 min run at 225 V, followed by 20 min hold prior to imaging. Lane 1: RNA Marker (Lonza); Lane 2: 250 ng *E.coli* Total RNA (Ambion); Lanes 3-7: 250 ng E.coli Total RNA incubated with increasing levels of RNase A. Intact, denatured RNA shows sharp, clear bands on the FlashGel® System. Partially degraded RNA has a smeared appearance, and completely degraded RNA appears as a low molecular weight smear.

Simple Protocol

Procedure

1. Insert cassette into FlashGel® Dock.
2. Pre-load wells with RNase-free Water (Cat. #51200).
3. Load samples[1].
4. Plug in and turn on light and power (225 V).
5. Run for 8 minutes[2].
6. Turn off power and hold for 10-20 minutes or until desired RNA bands are visible on the gel.
7. Photograph as usual[3].

1. Formaldehyde Loading Buffer and RNA Markers are recommended for best performance. Denatured RNA samples will migrate according to their true size on FlashGel® RNA Cassettes.
2. The FlashGel® DNA Marker may be used to monitor separation since it will immediately be visible on the dock in real time and be visible for the full length of the run - stop the run when the 100 bp band reaches the end of the gel. The visible blue xylene cyanol band may also be used to track migration - stop the run when the blue band has migrated approximately 1/3 the distance of the gel.
3. RNA bands are visible for up to 4 minutes in real-time runs (see Figure 3). If desired bands are seen within that time, then a 10-20 minute hold time prior to imaging is not required. The longer the post-run incubation period (up to 20 minutes), the brighter the RNA bands become. Unlike typical agarose gels where samples diffuse immediately, RNA samples on FlashGel® Cassettes remain intact for >2 hours.

Caution: Materials and methods shown here present hazards to the user and the environment. Refer to the safety information on page 210 before beginning these procedures.

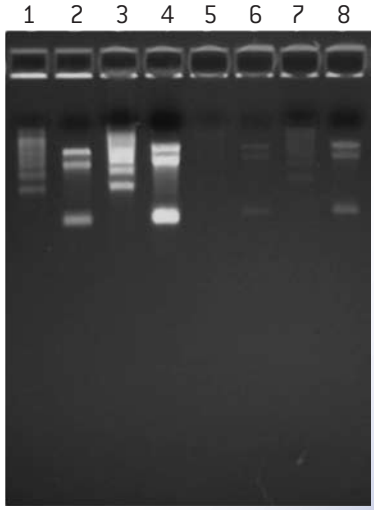


Figure 3. Quick checks of Native RNA on the FlashGel® System for RNA Native and formaldehyde denatured RNA samples were run at 225 V for 4 min. Native, nondenatured RNA samples do not require additional incubation time compared to formaldehyde denatured samples, and may be suitable for very fast checks of RNA integrity. After 20 additional minutes, the denatured samples would absorb stain and increase in visibility.

Lane	Sample	Load volume	Preparation
1	RNA Marker (Lonza)	1.25 µl	Native samples prepared with FlashGel® Loading Dye
2	*E. coli* Total RNA (Ambion)	1.25 µl	
3	RNA Marker	5 µl	
4	*E. coli* Total RNA	5 µl	
5	RNA Marker	1.25 µl	Formaldehyde denaturation at 65°C for 5 minutes
6	*E. coli* Total RNA	1.25 µl	
7	RNA Marker	5 µl	
8	*E. coli* Total RNA	5 µl	