SHORT TECHNICAL REPORTS

Manuscripts published in the Short Technical Reports section are shorter and less comprehensive in scope than full Research Reports.

Northern Blotting of RNA Denatured in Glyoxal Without Buffer Recirculation

BioTechniques 22:668-671(April 1997)

ABSTRACT

A rapid and easy procedure for preparing Northern blots is described. RNA is denatured with glyoxal in the presence of ethidium bromide and glycerol, then electrophoresed through agarose in a buffer that does not require recirculation. Without any additional washes, the RNA is vacuum-blotted to a nylon membrane in NaOH, which simultaneously removes the glyoxal adducts. All of these steps plus prehybridization of the filter and addition of a digoxygenin-labeled probe can be completed in one day. Using standard procedures to wash the filters and detect the probe, the entire procedure can be completed within two days.

INTRODUCTION

Two methods are commonly used to denature RNA for Northern analysis. Pretreatment of the RNA with formaldehyde and Me_2SO, followed by electrophoresis through an agarose gel containing up to 2.2 M formaldehyde (6) is the more commonly used method. Formaldehyde is now an Occupational Safety and Health Administration (OSHA)-regulated carcinogen (OSHA Standard 1910.1048), making its use undesirable. A second method using glyoxal to denature the RNA (7) offers an improvement with sharper bands, but the method is more difficult to carry out since the electrophoresis running buffer must be frequently exchanged or recirculated (7,8).

In this report, I introduce two modifications to the glyoxal procedure that significantly simplify it, making it both easier and faster than the formaldehyde procedure. First, a new running buffer is described that does not need to be recirculated or exchanged at any time during the electrophoresis of the RNA. Second, a glyoxal reaction mixture containing both ethidium bromide (EtdBr) (3,5) and glycerol can be prepared and stored as a master stock.

MATERIALS AND METHODS

RNA Isolation

Total RNA was prepared from the murine hybridoma cell line BR96 (4) and from the same cell line transfected with human immunoglobulin γ1 and κ constant region sequences to produce chimeric antibodies (2). RNA in the transfected cells was isolated from the master cell bank and from a post-production cell bank. The RNA was isolated in TRIZOL™ (Life Technologies, Gaithersburg, MD, USA) and purified by lithium chloride precipitation following the manufacturer's instructions. Polyadenylated RNA was isolated from the total RNA using an Oligotex™ mRNA Kit (Qiagen, Chatsworth, CA, USA).

Glyoxal Purification

Ten milliliters of 6 M glyoxal (Sigma Chemical, St. Louis, MO, USA) are deionized by gently mixing with 2 g of a mixed bed resin [AG-510-X8(D); Bio-Rad, Hercules, CA, USA] until the indicator dye in the resin is exhausted. The glyoxal solution is separated from the resin by filtration through a Uniflo® Plus filter (Schleicher & Schuell, Keene, NH, USA). The deionization process is repeated until the pH of the glyoxal is >5.0. The pH is monitored by mixing 200 µL of glyoxal with 2 µL of 10-mg/mL Bromcresol Green (Sigma Chemical) in deionized H_2O. When the color of the dye is blue-green to blue, the glyoxal is considered sufficiently purified. The glyoxal was deionized three times in the experiments described here.

Reagents

A 10× stock of BTPE buffer is prepared by adding 3 g of piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) free acid, 6 g of bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris) (free base) and 2 mL of 0.5 M EDTA to 90 mL of H_2O, then treating the solution with diethyl pyrocarbonate (DEPC). The 1× buffer is 10 mM with

Table 1. pH Gradient Generated During Electrophoresis

	Initial pH	Anode	Cathode
Apparatus A	6.5	5.7	7.3
Apparatus B	6.5	5.9	7.1

Minigels were run at 5 V/cm for 3 h.

respect to PIPES and 30 mM with respect to Bis-Tris. The pH of the 1× buffer is 6.5. The glyoxal reaction mixture is prepared by combining 6 mL of dimethyl sulfoxide (DMSO), 2 mL of 6 M glyoxal (deionized), 1.2 mL of 10× BTPE, 0.6 mL of 80% glycerol and 0.2 mL of 10-mg/mL EtdBr. A master stock of the mixture can be stored in 1-mL aliquots at -70°C. A working stock is prepared as 0.1-mL aliquots from a thawed tube of the master stock and stored at -70°C. Once a tube of the working stock is thawed, any unused reagent is discarded.

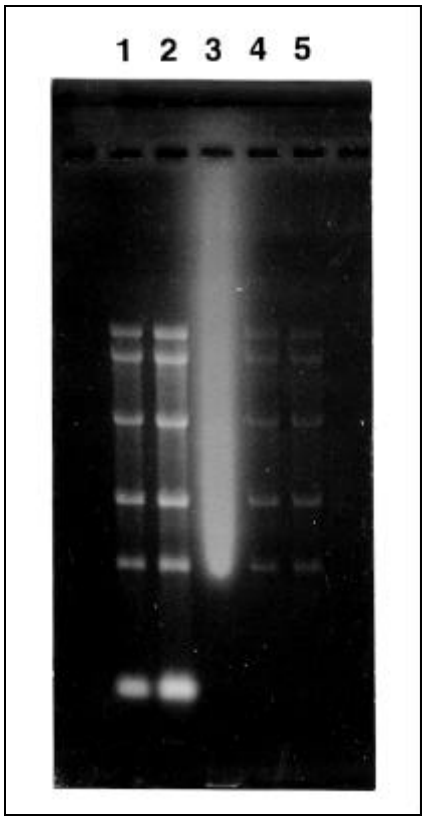

Figure 1. Comparison of procedures to denature and stain RNA for electrophoresis in agarose. 2 µg of RNA were incubated at 50°C for 1 h in the glyoxal buffers listed below. The RNA was run at 5 V/cm for 3 h through 1% agarose in 1× BTPE. After examination of the gel on a UV transilluminator, it was stained for 15 min in 1 µg/mL EtdBr and photographed. The denaturation buffers contained 1 M glyoxal, 50% Me_2SO, plus the following:

	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
Buffer	$NaPO_4$	BTPE	$NaPO_4$	$NaPO_4$	$NaPO_4$
Fluorescent dye	EtdBr	EtdBr	AcrOr	----	----
Glycerol	+	+	+	+	----

Denaturation and Electrophoresis of RNA

Two microliters of RNA and 10 µL of glyoxal reagent are mixed, incubated at 50°–55°C for 1 h, then placed on ice. No further additions are made to the sample before loading it on the gel. A 1% or 1.5% SeaKem® GTG® agarose gel (FMC BioProducts, Rockland, ME, USA) is prepared in 1× BTPE. The gel is submerged in 1× BTPE, and the samples are loaded directly. The electrophoresis is carried out at 5 V/cm for 3 h. Migration of the RNA is monitored by running dyes (bromophenol blue and xylene cyanol) in a separate lane. After completion of the run, RNA standards are visualized by placing the gel on a transparent tray on a UV transilluminator. RNA is transferred to a nylon membrane without any additional washing of the gel.

Probe Preparation

Human immunoglobulin γ1 constant-region cDNA was prepared from mRNA using a gene-specific primer. A digoxigenin (Dig)-labeled probe was prepared by polymerase chain reaction (PCR) using internal primers, DIG DNA Labeling Mix (Boehringer Mannheim, Indianapolis, IN, USA) and *Taq* Extender™ (Stratagene, La Jolla, CA, USA). A human immunoglobulin κ constant-region probe was prepared by PCR-amplifying a cloned genomic gene fragment.

Transfer and Hybridization

RNA is transferred to a positively charged nylon membrane (Boehringer Mannheim) using a vacuum blotter (Bio-Rad). The transfer solution is 7.5 mM NaOH (11), and a 5" Hg vacuum is applied for 90 min. The filter is neutralized briefly in 1 M ammonium acetate, then the RNA is UV cross-linked to the filter (125 mJ/cm^2). Hybridization is carried out according to the optimized procedure of Engler-Blum et al. (1). Detection of the Dig-labeled probe is performed with a Genius™ Kit (Boehringer Mannheim), using a slight modification of the procedure accompanying the kit. Tween® 20 (0.3%) is added to both the washing and blocking buffers (buffers 1 and 2 from Genius Kit). After incubation with the antibody, the filters are washed twice with the wash buffer plus 0.3% Tween 20 and 0.1% *N*-lauroylsarcosine. Filters are incubated overnight after application of the Lumigen PPD (Boehringer Mannheim), and the X-ray film (BioMax™ MR; Eastman Kodak, Rochester, NY, USA) is exposed for 30 min.

RESULTS AND DISCUSSION

Gel electrophoresis of glyoxal-treated RNA must be carried out in a low-ionic-strength buffer to prevent renaturation of the RNA (3). Furthermore, it is important to maintain the pH < 8.0 to prevent the removal of the glyoxal adduct from the RNA. As a result, all electrophoresis running buffers in use until now have needed to be recirculated to prevent the formation of a large pH gradient across the gel. Without recirculation, the pH of the standard 10 mM phosphate buffer (7) can reach a pH of 8.0 at the cathode in as few as 25 min (3). Aside from the low ionic strength, the primary reason for the pH gradient is the presence in running buffers of small, highly charged ions, such as sodium phosphate (7) or sodium *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (3). A more stable buffer can be prepared by combining a weak acid and base with similar pK's. For this reason, PIPES (pK = 6.8) and Bis-Tris (pK = 6.5) were chosen for evaluation. These were combined in a proportion that produced a buffer with a final pH of 6.5. Since none of the buffer components contain primary amines, the solution can be treated with DEPC to inactivate any contaminating

RNase activity. The ability of the 1× buffer to resist the formation of a large pH gradient was tested in two different minigel systems with duplicate runs in each apparatus. After electrophoresis at 5 V/cm for 3 h, 5 mL of buffer were removed from each buffer well, and the pH was measured. The duplicates matched to within 0.1 pH unit. Table 1 shows that a relatively small pH gradient formed in either apparatus, and both systems were sufficiently stable to allow electrophoresis of the RNA without recirculation of the buffer.

Several methods for preparing glyoxal adducts of the RNA were compared. The standard procedure (7) calls for causing a reaction between the RNA and 1 M glyoxal in the presence of 50% Me_2SO and 10 mM sodium phosphate, pH 7.0 at 50°C for 1 h. Glycerol and dyes were added before loading the samples, and the RNA was visualized by staining the gel with either EtdBr or Acridine Orange (AcrOr). Adding EtdBr to the denaturing reaction in either formaldehyde (5) or glyoxal (3) simplifies the procedure and increases the intensity of the UV fluorescence of the RNA. Figure 1 shows a comparison of several methods for denaturing the RNA (0.24–9.47-kb RNA Ladder; Life Technologies). All of the procedures are variations of the standard procedure (7). For the first three lanes, the denaturing solution contained glycerol and a fluorescent dye in addition to the glyoxal and buffer. For lane 4, only glycerol was added to the denaturing reaction, and in lane 5, no addition was made. All of the procedures but one (adding AcrOr to the reaction—lane 3) produced similar migration and sharp bands. The reactions that contained EtdBr in the denaturation solution produced strong bands, even before staining the gel for visualization of the last two lanes. It is interesting to note that the high concentration of EtdBr in the loading buffer did not significantly affect the mobility of the RNA as it would in a formaldehyde gel (9). In a side-by-side comparison (not shown), the RNA migrated only slightly (about 2%) slower when the denaturation solution contained 50 µg/mL EtdBr (S. Tonzi, personal communication). This lower concentration also produces acceptable results, although the reaction mixture is less visible when loaded onto the gel. Addition of glycerol during the denaturation of the RNA also did not affect its subsequent migration. The denaturation reaction for lane 2, which used 1× BTPE instead of sodium phosphate, worked just as well and produced a slightly stronger UV signal. Glyoxal is an unstable reagent that can be oxidized by exposure to air (7). Most procedures suggest deionizing the reagent immediately before using it. To simplify the procedure, a frozen reagent stock was prepared with all of the components already added. Prepared in this manner, the denaturing solution is effective for at least three months and is easy to use.

To determine whether RNA that was prepared and run in this manner was suitable for subsequent Northern analysis, polyadenylated RNA was denatured and run on a 1.5% agarose gel. Approximately 1 ng of RNA (the amount that had been isolated from 0.1 µg of total RNA) was loaded into each lane. This amount of RNA could not be visualized under UV light. The RNA ladder was run in one lane both as a molecular weight marker and to monitor the transfer efficiency of the vacuum blotter. The transfer buffer contained 7.5 mM NaOH, which can partially fragment the RNA to facilitate its transfer to the nylon membrane. When the RNA has been denatured with glyoxal, the added advantage of removal of the glyoxal adducts occurs (11), leaving the RNA sequences available for hybridization to the probe.

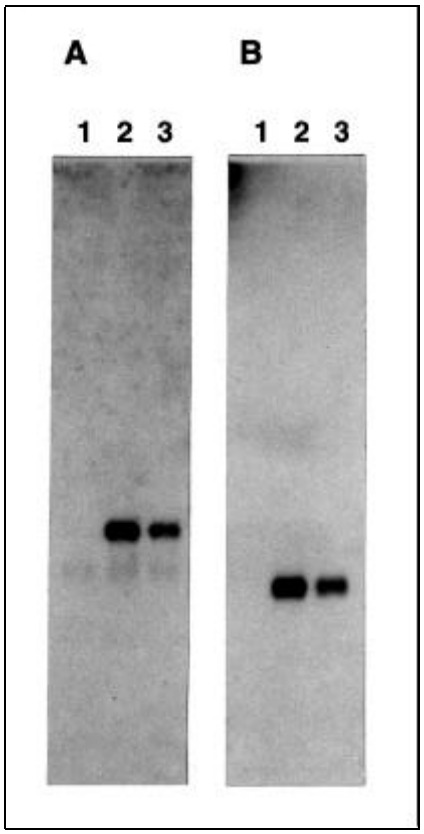

Figure 2. Northern blot of chimeric BR96 mRNA. The RNA was denatured as described in Materials and Methods and electrophoresed for 3 h at 50 V through 1.5% agarose. Polyadenylated RNA isolated from 0.1 µg of total RNA was loaded into each lane: lane 1, murine BR96 RNA; lane 2, chimeric BR96 RNA (master cell bank); and lane 3, chimeric BR96 RNA (post-production cell bank). The filters were probed with Dig-labeled (A) human immunoglobulin γ1 constant region or (B) human immunoglobulin κ constant region probes.

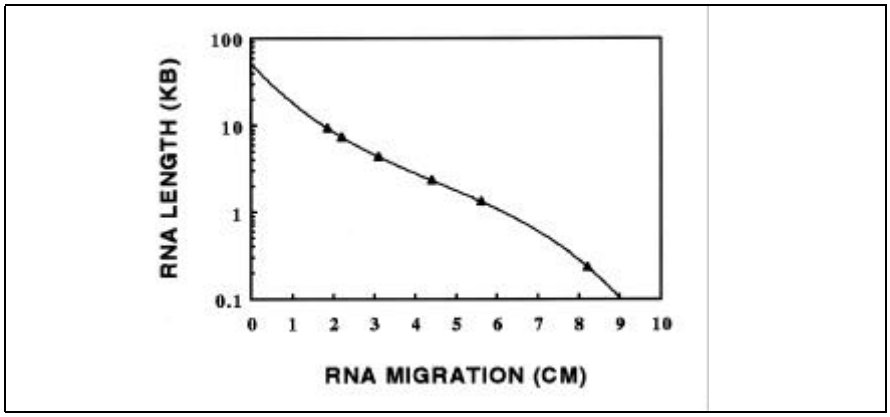

Figure 3. Migration of RNA through 1.5% agarose. 2 µg of the RNA ladder were run for 3 h at 5 V/cm through 1.5% agarose in 1× BTPE. A curve-fitting program was used to plot the migration distance vs. log(RNA length) as a third-order polynomial. The derived equation was: $\log y = 1.725 - 0.516x + 0.0701x^2 - 0.00512x^3$.

After a 90-min transfer, a small amount of the largest bands (4.4–9.5 kb) was retained in the gel, indicating that a longer transfer time or higher molarity NaOH transfer buffer may be necessary for very large RNAs. A Northern blot was prepared with the filter using Dig-labeled human immunoglobulin κ light-chain or γ1 heavy-chain, constant-region probes (Figure 2). Both probes produced a strong signal with low background, indicating that the glyoxal adducts had been removed and that the RNA sequences were available for hybridization. The bands were as intense as those in Northern blots prepared from formaldehyde gels (not shown). The position of the hybridizing bands is consistent with the expected sizes of the chimeric immunoglobulin light- and heavy-chain mRNAs.

The absence of buffer recirculation does not appear to affect the migration of the RNA standards. A plot of log(RNA length) vs. mobility is close to linear between 1.35 and 7.4 kb on the 1.5% gel. In Figure 3, the migration distance for all of the RNA bands in the standard was evaluated with a curve-fitting program (SlideWrite Plus™; Advanced Graphics Software, Carlsbad, CA, USA). The best fit (r = 0.999998) was obtained when the log(RNA length) vs. mobility was solved as a third-order polynomial. This produces a sigmoidal curve, which compensates for the anomalous migration rates of the largest and smallest standards. Other methods for fitting the data points have been described (3).

The procedure described above is significantly faster and easier than previously described methods. Gels can be run on a laboratory benchtop, and no buffer-recirculation apparatus is necessary. Northern blots can be prepared in two days. The procedure was used to detect two abundant mRNAs from a hybridoma cell line. Other procedures to increase the sensitivity of the probe detection in Northern blots (1,10) may be useful for rare mRNA species. These should not increase the time needed to perform a Northern analysis.

REFERENCES

1. **Engler-Blum, R., M. Meier, J. Frank and G.A. Muller.** 1993. Reduction of background problems in nonradioactive northern and Southern blot analyses enables higher sensitivity than ^{32}P-based hybridizations. Anal. Biochem. *210*:235-244.
2. **Fell, H.P., S. Yarnold, I. Hellstrom, K.E. Hellstrom and K.R. Folger.** 1989. Homologous recombination in hybridoma cells: heavy chain chimeric antibody produced by gene targeting. Proc. Natl. Acad. Sci. USA *86*:8507-8511.
3. **Grundemann, D. and H. Koepsell.** 1994. Ethidium bromide staining during denaturation with glyoxal for sensitive detection of RNA in agarose gel electrophoresis. Anal. Biochem. *216*:459-461.
4. **Hellstrom, I., H.J. Garrigues, U. Garrigues and K.E. Hellstrom.** 1990. Highly tumor-reactive, internalizing, mouse monoclonal antibodies to Le^y-related cell surface antigens. Cancer Res. *50*:2183-2190.
5. **Kroczek, R.A. and E. Siebert.** 1990. Optimization of northern analysis by vacuum-blotting, RNA-transfer visualization, and ultraviolet fixation. Anal. Biochem. *184*:90-95.
6. **Lehrach, H., D. Diamond, J.M. Wozney and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions. Biochemistry *16*:4743-4751.
7. **McMaster, G.K. and G.G. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA *74*:4835-4838.
8. **O'Connor, J.L., M.F. Wade and Y. Zhou.** 1991. Control of buffer pH during agarose gel electrophoresis of glyoxylated RNA. BioTechniques *10*:300-302.
9. **Ogretmen, B., H. Ratajczak, A. Kats, B.C. Stark and S.M. Gendel.** 1993. Effects of staining RNA with ethidium bromide before electrophoresis on performance of Northern blots. BioTechniques *14*:932-935.
10. **Reed, K.C. and D.A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. *13*:7207-7221.
11. **Vrati, S., D.A. Mann and K.C. Reed.** 1987. Alkaline northern blots: transfer of RNA from agarose gels to Zeta-Probe membrane in dilute NaOH. Mol. Biol. Rep. *1*:1-4.

I am grateful to Drs. P. Thammana and J. Tabor for critically reviewing the manuscript.

Address correspondence to William V. Burnett, Bristol-Myers Squibb, 6000 Thompson Rd., East Syracuse, NY 13057-5050, USA. Internet: wburnett@usccmail.uscc.bms.com

Received 20 June 1996; accepted 6 November 1996.

William V. Burnett
Bristol-Myers Squibb Company
Syracuse, NY, USA