Enhanced sensitivity RNA gel loading buffer that enables efficient RNA separation on native gels

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BioTechniques 36:334-336 (February 2004)

RNA gel analysis is essential for quality assessment of RNA preparations for subsequent analysis such as microarrays and real-time PCRs. The routinely used standard electrophoresis of RNA through formaldehyde-containing agarose gels is not only labor-intensive and time-consuming, but also involves sizeable quantities of hazardous materials. Above all, it is not sensitive, requiring more than 1 µg of RNA for the assay. Current gene expression profiling with microarrays and real-time PCR often involves limiting amounts of RNA. It is therefore important to have a more sensitive way to analyze RNA. Here we report an improved ethidium bromide-based RNA gel analysis system with our Superload buffer that increases sensitivity to 12.5 ng of total RNA and allows RNA analysis on a regular native Tris-acetate EDTA (TAE) agarose gel.

INTRODUCTION

RNA gel analysis is a routine procedure for biological and biomedical research. It is essential for various gene expression analyses such as microarrays and real-time PCRs. Currently used RNA gel analysis protocols generally use the formaldehyde-denaturing system adapted from Sambrook et al. (1,2). Electrophoresis of denatured RNA through formaldehyde-containing agarose gels is not only labor-intensive and time-consuming, but also involves sizeable quantities of hazardous materials. Moreover, more than 1 µg of RNA is typically needed for the assay. Current gene expression profiling with microarrays and real-time PCR often involve a limited amount of materials (3,4). It is, therefore, important to have a more sensitive RNA assay. One alternative method is to use the Model 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA), which requires only nanogram quantities of RNA. However the equipment may not be accessible to most laboratories.

Here we report a method that improves the current RNA gel analysis. We have developed an ethidium bromide (EtBr)-based RNA-denaturing and gel-loading buffer kit named Superload that could greatly simplify the RNA gel analysis procedure and increase detection sensitivity.

MATERIALS AND METHODS

RNA Preparations

Rat XC cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf

serum and antibiotics. Pig spleen was acquired from freshly slaughtered carcasses, and dog heart was harvested rapidly from dying animals. Total RNAs were isolated by the method of Guthrie et al. (5). One hundred micrograms of each total RNA were treated with 1.0 U DNase I (amplification-grade; Gibco-BRL, Bethesda, MD, USA) at 37°C for 15 min and purified using RNeasy® Mini columns (Qiagen, Valencia, CA, USA) to remove remaining DNase. The isolated RNA samples were stored at -80°C until use.

Antisense RNA (aRNA) was prepared from 100 ng of human heart total RNA (obtained from BD Biosciences Clontech, Palo Alto, CA, USA) by two rounds of amplifications with the MessageAmp™ aRNA kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions (http://www.ambion.com/techlib/prot/fm_1750.pdf). RNA ladder of 0.24–9.5 kb was purchased from Invitrogen (Carlsbad, CA, USA).

RNA Gel Analysis

Prior to gel electrophoresis, RNA samples were prepared with the traditional protocol (TRD), the modified traditional protocol (MTRD), or the Superload protocol (Superload). The TRD was a formamide-formaldehyde-based method adapted from Sambrook et al. (1) with 60 µg/mL EtBr in the gel-loading buffer. The MTRD differs from the TRD in the way the RNA samples were heat-denatured. In TRD, the EtBr-containing gel-loading buffer was added to the sample mixture that had already been heat-denatured; whereas in MTRD, the loading buffer was added to the sample prior to heat denature treatment. The Superload protocol uses the Superload kit (Viagen, Austin, TX, USA), which is composed of buffer A, containing formaldehyde, and buffer B, contain-

ing formamide and EtBr. Two volumes of RNA samples are mixed with one volume of buffer A and three volumes of buffer B, followed by a 10-min incubation at 70°C.

The denatured RNA samples were electrophoresed under either standard formaldehyde-denaturing gel condition (1) or native 1× Tris-acetate EDTA (TAE) gel conditions (2). For all experiments, 1.3% agarose gels were used. Gel images were taken with the Kodak 1D Image System (Eastman Kodak, Rochester, NY, USA) using a 300-nm UV transillumination

and an EtBr photographic filter. Image data were analyzed with Kodak 1D Image Analysis software (Eastman Kodak).

RESULTS AND DISCUSSION

In order to test the sensitivity of the Superload kit, we prepared serial dilutions of pig spleen total RNA (5, 1, 0.5, and 0.25 µg). The RNA samples were then processed with Super-

load, TRD, or MTRD, and electrophoresed under denaturing gel conditions. Figure 1A shows that 250 ng of total RNA can be easily detected with Superload treatment, whereas TRD barely detects 5 µg of total RNA with the same electrophoresis and gel image conditions (lanes 2 and 15). Interestingly, when samples were heat-denatured at 70°C after combining the traditional gel-loading buffer with the sample-denaturing buffer, as executed in MTRD, sensitivity also greatly increased (lanes 6–10). We tested a range of denaturing temperatures from 55° to 95°C, and similar sensitivity was observed (data not shown). The combination of heat and treatment with denaturing agents may facilitate the incorporation of EtBr to RNA molecules and enhance the fluorescence intensity through a mechanism that is still largely unknown. With fine-tuning of ionic strength, the Superload kit further improved the sensitivity of the EtBr-based RNA analysis (lanes 11–15). To quantify the sensitivity difference, we individually measured net intensity of 28S ribosomal RNA (rRNA) bands from nonsaturated lanes 10 and 15 (Figure 1B). Superload is eight times more sensitive than MTRD.

We then investigated the possibility of using Superload to analyze RNA samples on a nondenaturing gel. A series of 2-fold dilutions of pig spleen total RNA were processed with the Superload protocol and separated on a native gel. As shown in Figure 2A, Superload treatment allowed as little as 12.5 ng of total RNA to be detected. We also found that a 5-fold lower amount of RNA ladder than what was recommended by the manufacturer (600 ng versus 3 µg) was easily detectable with Superload.

One of the major applications of RNA gel analysis is for quality assessment of aRNA probe generated by in vitro transcription for gene expression microarrays. To verify the effectiveness of Superload in analyzing aRNA, we treated 1 and 0.5 µg aRNA with Superload and electrophoresed on a native gel with 250 ng of pig spleen total RNA as a control. Figure 2B shows that Superload denatured and separated aRNA, producing a smear of aRNA molecules ranging from

Figure 1. RNA analysis with Superload protocol, traditional protocol, and modified traditional protocol on a denaturing gel. (A) Six hundred nanograms of 0.24–9.5 kb RNA ladder (lanes 1, 6, 11) and four sequentially diluted pig spleen total RNA samples, 5 µg (lanes 2, 7, and 12), 1 µg (lanes 3, 8, and 13), 0.5 µg (lanes 4, 9, and 14), and 0.25 µg (lanes 5, 10, and 15) were treated with the traditional protocol (TRD), modified traditional protocol (MTRD), and Superload protocol (Superload), and electrophoresed on a standard formaldehyde-denaturing agarose gel. (B) Net fluorescence intensities of the 28S rRNA band from 0.25 µg of total RNA treated with MTRD and Superload (panel A, lanes 10 and 15, respectively) were calculated with the Kodak 1D Image Analysis software and plotted. rRNA, ribosomal RNA.

Figure 2. RNA analysis with Superload on native Tris-acetate EDTA (TAE) gels. (A) Six hundred nanograms of 0.24–9.5 kb RNA ladder (lane 1) and six sequentially diluted pig spleen total RNA samples, 400 ng (lane 2), 200 ng (lane 3), 100 ng (lane 4), 50 ng (lane 5), 25 ng (lane 6), and 12.5 ng (lane 7), were treated with Superload and electrophoresed on a native TAE agarose gel. (B) Six hundred nanograms of 0.24–9.5 kb RNA ladder (lane 1), 250 ng of pig spleen total RNA (lane 2), 1 µg (lane 3), and 500 ng (lane 4) of aRNA were treated with Superload and analyzed on a native TAE agarose gel. aRNA, antisense RNA.

Figure 3. RNA migration under both denaturing and native gel conditions after Superload treatment. Six hundred nanograms of 0.24–9.5 kb RNA ladder (lanes 1), 250 ng of total RNA from rat XC cells (lanes 2), pig spleen (lanes 3), and dog heart (lanes 4) were treated with Superload and electrophoresed on a native Tris-acetate EDTA (TAE) agarose gel (A). As a comparison, a twin set of samples treated with Superload was also electrophoresed on a standard formaldehyde denaturing gel (B). Migration distances of each band of 28S rRNA and 18S rRNA were measured, and sizes were calculated and plotted for each gel condition (C). rRNA, ribosomal RNA.

200–1500 bp. The same set of aRNA samples was also subjected to MTRD treatment and electrophoresed on the native gel; however, the sensitivity was less than Superload (data not shown).

To further assess the denaturing power of Superload and its downstream use in applications such as Northern blot analysis, the RNA ladder and two sets of total RNA from rat XC cells, pig spleen, and dog heart were treated with Superload. One set was electrophoresed under native conditions (Figure 3A), and another was electrophoresed under denaturing conditions (Figure 3B). The migration distance of each band was measured, and sizes of 28S rRNA and 18S rRNA were determined using Kodak 1D Image Analysis software using the RNA ladder as a known size standard (Figure 3C). The calculated sizes differed little between the samples run on denaturing or native gels and corresponded well to the expected sizes (4.7 kb for 28S rRNA and 1.9 kb for 18S rRNA).

In summary, the Superload protocol involves simple sample preparation, yet increases the sensitivity of RNA detection and simplifies RNA gel analysis by enabling usage of native gels.

ACKNOWLEDGMENTS

K.G. and W.Z. contributed equally to this report.

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