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Comparison of denaturing and non-denaturing gel electrophoresis methods for RNA analysis

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Abstract: RNA molecules form secondary structures freely by intra-molecular base pairing with complementary ribonucleotides. Secondary structures have biological and physiological significance for cells because of the involvement of RNA molecules in numerous cellular processes. However, these secondary structures make RNA analysis complicated due to the fact that hydrogen bonding, resulting from the internal base pairing, has to be denatured in order to analyze molecular structures and size of these molecules. One method affected by the secondary structure of RNA, is agarose gel electrophoresis that has been used for decades to separate different sized molecules of RNA. Commonly used methods for RNA gel electrophoresis require use of denaturing chemicals that are toxic, hazardous and potential carcinogens such as formaldehyde, glyoxal/DMSO, formamide and others. In this study we compared two denaturing and one modified non-denaturing gel electrophoresis methods for analysis of total RNA extracted from SiHa cells. We consistently and repeatedly found that RNA separation on non-denaturing gel was better than denaturing gel electrophoresis in terms of intensity and integrity of 28S and 18S rRNA bands. We routinely use this method for RNA analysis and recommend using it for research and teaching laboratories owing to the simplicity, safety and low cost of the procedure.

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INTRODUCTION

RNA is single-stranded polymer of ribonucleotides bonded together by covalent phosphodiester bonds. The ribonucleotide consists of pentose D-ribose covalently bonded at C3 or C5 to a phosphate group and one of four possible nitrogenous bases (adenine, uracil, cytosine and guanine) covalently bonded at C1. In aqueous solutions, adenine ribonucleotide forms hydrogen bonds with uracil ribonucleotide and cytosine ribonucleotide forms hydrogen bonds with guanine ribonucleotide. The ribonucleotides can form hydrogen bonds with other ribonucleotides internally or with other RNA molecules as well as with complementary deoxyribonucleotides in DNA. Internal hydrogen bonding of ribonucleotides results in secondary structures which not only provide increased stability to the single-stranded RNA molecules but also it confers functionality. For example, transfer RNA (tRNA) folds into the "cloverleaf" structure so it can bind to amino acids and transport them to the ribosomes for the synthesis of polypeptides during translation. Without the cloverleaf structure tRNA is not able to transport amino acids to the ribosome which catalyzes a peptide bond between the amino acid and the growing polypeptide chain. RNA secondary structures form spontaneously when RNA is extracted from cells. During electrophoresis RNA secondary structures can prevent RNA molecules from separating according to their size.

Gel electrophoresis procedures are used to separate RNA polymers in an agarose gel matrix according to size and net charge on the RNA molecule. The phosphodiester bonds between ribonucleotides have a negative charge and increasing the number of ribonucleotides in a RNA polymer increases the overall negative charge of the molecule. In gel electrophoresis an electric field is established and RNA migrates through the gel towards the cathode. The gel is porous and acts as a sieve allowing smaller RNA molecules to migrate faster than larger RNA molecules towards the cathode. It is commonly believed that RNA secondary structure interferes with the separation of RNA molecules; therefore, several different techniques have been developed to denature RNA prior and during gel electrophoresis. Commonly practiced RNA electrophoresis techniques use strong denaturants that are hazardous to health such as formaldehyde, glyoxal/dimethylsulfoxide (DMSO), guanidine thiocynate, urea and rarely mercuric hydroxide. Due to the toxicity and carcinogenicity associated with these chemicals, gel electrophoresis is performed inside fume hoods and the storage of these chemicals and wastes requires special handling.

Traditional method of RNA electrophoresis uses agarose gels prepared in about 7% formaldehyde and RNA is separated in electrophoresis buffer containing 3-(*N*-morpholino) propanesulfonic acid (MOPS), acetate and ethylenediamine tetraacetic acid (EDTA)[1]. Another RNA electrophoresis method utilizes agarose or acrylamide gels dissolved in glyoxal/DMSO (50:50 v/v solution) and uses NaH_2PO_4 buffer as a running buffer[2,3].

A third RNA electrophoresis method is based on dissolving agarose gels in guanidine thiocyanate, a non-volatile denaturant[4]. Finally, a

TAE/formamide-based gel electrophoresis method was introduced that offers a fairly save and non-toxic method for separation of RNA species through gel electrophoresis[5]. Still other methods include the use of urea[6], methylmercuric hydroxide[7]and formamide[8] but these are not used commonly.

In literature there are few reports of electrophoresis of non-denaturing RNA electrophoresis without the use of strong denaturants prior and during gel electrophoresis and partial comparisons have been done with different procedures. To the authors' best knowledge, there are no reports that compare the most commonly used RNA electrophoresis techniques: formaldehyde, glyoxal/DMSO and non-denaturing (TAE) agarose gel electrophoresis. We sought to develop a non-denaturing agarose gels for RNA electrophoresis and compared our simple non-denaturing TAE agarose technique with the standard denaturing agarose gel techniques. Such a non-denaturing TAE agarose technique is both simple and non-toxic. It can be reliably used in research and teaching undergraduate laboratories that lack appropriate facilities for the handling of toxic chemicals without compromising the sensitivity of the results.

MATERIALS AND METHODS

The SiHa cell line (ATCC, HTB-35) was purchased from the American Type Tissue Culture Collection to extract total RNA. The SiHa cells were cultured at 37°C, 5% CO_2 in a tissue culture incubator using Eagle's minimum essential medium (ATCC 30-2003) with 10% fetal bovine serum. When the SiHa cells were confluent they were washed with 1x phosphate buffered saline (ATCC, 30-2200) and total RNA was extracted using TRI Reagent following the manufacturer's recommendations. The total RNA was dissolved in diethylpyrocarbonate (DEPC) treated water. The RNA concentration was measured using a UV spectrophotometer at 260 nm and the RNA samples were stored at -80°C.

For gel electrophoresis, triplicate samples of SiHa total RNA (10 µg/lane) were loaded on two denaturing agarose gels, formaldehyde and glyoxal/DMSO, and one non-denaturing TAE gel. The published protocols for the preparation and running conditions for these gels were as follows[1,2,5]. In the non-denaturing gel (1xTAE), the RNA sample was denatured by mixing with loading dye buffer and heating at 95 °C prior to loading onto the gel. Gels were run at 60 volts for 2 hours and then stained with ethidium bromide solution (0.5µg/ml) for 15 minutes. Finally the stained gels were destained in deionized water for 15 minutes with constant shaking, viewed and imaged under UV irradiation using FOTO/Analyst® FX (Fotodyne Incorporated) at the same camera settings. The experiment was repeated several times over a period of one year.

RESULTS AND DISCUSSION

The electrophoresis of SiHa total RNA in denaturing (formaldehyde, glyoxal/DMSO) and non-denaturing (TAE) conditions revealed two conspicuous bands in each of the three gels (Figure 1). These two bands represent 28S and 18S rRNA, respectively. The two bands in all three gels are distinct. Interestingly, the two bands in the non-denaturing (TAE buffer) gel (Figure 1(B)) are more distinct than the bands in the denaturing gels (Figures 1A and 1C) when exposed to UV irradiation.

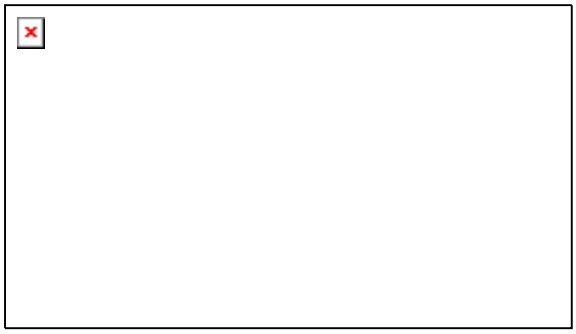

Figure 1: Comparison of SiHa total RNA separated by electrophoresis in different agarose gels: A. Formaldehyde, B. non-denaturing TAE gel, C. Glyoxal /Dimethylsulfoxide. All samples were analyzed in triplicate by using 10 µg RNA. Gel electrophoresis was carried out in specific buffers at 60 volts for 2 hours. After gel electrophoresis, gels were stained with ethidium bromide solution (0.5µg/ml) and viewed with UV light.

Gel electrophoresis of RNA is based on the principle that the RNA molecules will separate in the gel according to size only. Denaturing gel electrophoresis techniques are commonly used to eliminate RNA secondary structure which may interfere with the migration of RNA in a gel. In the denatured condition, RNA molecules separate based on their molecular weight only thus DNA can be used on the same gel for size estimation (McMaster and Carmichael, 1977). In our experiment, the non-denaturing total RNA electrophoresis has sharper bands than the two non-denaturing gels. Thus denaturing electrophoresis conditions may not be

necessary for the electrophoresis of total RNA for qualitative gels. Denaturing gels, prepared under fume hoods are quite problematic, especially when only a few gels are run occasionally. Generally, separate buffers and gel electrophoresis apparatus are dedicated for running such denaturing gels. Disposal of the used running buffer also needs special arrangements. In case of non-denaturing TAE gels, the denaturing of the secondary structures is achieved by the addition of small quantities of formamide to the samples and heating at high temperatures. Since DNA gels are also run in TAE buffers, no dedicated apparatus for RNA gels will be required. Working solutions of TAE buffer can be re-used a couple of times or disposed in the sink without any harmful effects to the environment.

In our experience we were able to obtain sharp 28S and 18S rRNA bands from TAE gels whereas these bands were quite diffuse in glyoxal/DMSO gels. Results from formaldehyde gels were in between glyoxal/DMSO and TAE gels in terms of sharpness of the 28S and 18S bands. Superior resolution from formaldehyde gels corroborates the results of previous studies[3] found that signal to noise ratio of the ethidium bromide stained gels was much better in formaldehyde gels compared with glyoxal/DMSO gels. The glyoxal/DMSO gels required 8-9 times longer destaining protocols than formaldehyde gels to diminish the background staining. Our results are not in agreement with previous studies[5] that found comparable results from RNA gel electrophoresis using formaldehyde and TAE gels. In our comparison of TAE non-denaturing gels with formaldehyde and glyoxal/DMSO denaturing gels revealed that TAE gels were superior to the denaturing gels as evidenced by the sharpness and brightness of the 28S and 18S rRNA bands from TAE gels (Figure 1).

One of the bottlenecks in research and teaching RNA analysis to undergraduates is the specific needs of equipment, fume hoods and hazardous chemicals. In the absence of such facilities, which is true for many small colleges and universities, RNA analysis is intentionally omitted from the curriculum thus keep students from learning important molecular biology techniques. Use of simple and safe techniques (such as TAE agarose gel electrophoresis) will enable students to learn about RNA molecules and their analysis techniques.

CONCLUSION

In this study it is found that non-denaturing method (TAE agarose gel electrophoresis) of RNA analysis as sensitive or more as compared with denaturing gel electrophoresis methods. Non-denaturing method does not need any separate equipment or involves the use of toxic chemicals. Separation of the molecules can be carried out in the same equipment and even along with DNA molecules[2,9]. It is inexpensive, easy to use and can be easily done in any research or teaching without dedicated equipment for RNA analysis.

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