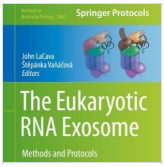

Sep 02, 2021

RNA Electrophoresis in Agarose Gels

Book Chapter

In 1 collection

Jonathan Houseley, Cristina Cruz

1 *Works for me* Share

dx.doi.org/10.17504/protocols.io.bnsxmefn

Springer Nature Books

satyavati Kharde

Citation: Jonathan Houseley, Cristina Cruz (09/02/2021). RNA Electrophoresis in Agarose Gels. https://dx.doi.org/10.17504/protocols.io.bnsxmefn

ABSTRACT

Over the past decade a plethora of noncoding RNAs (ncRNAs) have been identified, initiating an explosion in RNA research. Although RNA sequencing methods provide unsurpassed insights into ncRNA distribution and expression, detailed information on structure and processing are harder to extract from sequence data. In contrast, northern blotting methods provide uniquely detailed insights into complex RNA populations but are rarely employed outside specialist RNA research groups. Such techniques are generally considered difficult for nonspecialists, which is unfortunate as substantial technical advances in the past few decades have solved the major challenges. Here we present simple, reproducible and highly robust protocols for separating glyoxylated RNA on agarose gels and heat denatured RNA on polyacrylamide–urea gels using standard laboratory electrophoresis equipment. We also provide reliable transfer and hybridization protocols that do not require optimization for most applications. Together, these should allow any molecular biology lab to elucidate the structure and processing of ncRNAs of interest.

Introduction

Northern blotting methods allow for simultaneous quantification and molecular weight determination of RNA. Although superseded by qPCR and sequencing methods for routine mRNA quantification, northern blotting is the method of choice when complex mixtures of overlapping species are under investigation. This is particularly true when studying RNA processing by complexes such as the exosome, and generally aids in resolving the behavior of differentially expressed RNA isoforms. Historically, northern analysis has been something of a black art; running a high-quality formaldehyde gel required substantial skill and a little luck, while radioactive probing of RNA membranes often resulted in terrible cross-hybridization and invisible signals. Fortunately, technology has moved on such that modern northern analysis methods are simple and robust.

Electrophoretic separation of single-stranded RNA is more complex than double-stranded DNA as RNA forms strong secondary structures that impede separation by molecular weight in a gel matrix. For analysis of high molecular weight RNA in agarose gels, chemical modification of guanine is the preferred method to melt secondary structure, which disrupts C:G base pairing and allows single stranded RNA to migrate according to size. Although formaldehyde has been widely used for this purpose [1], it is not ideal due to issues with sample migration and batch-to-batch variation. Furthermore formaldehyde gels release toxic formaldehyde gas; this did not overly concern early investigators eager to replace the hideously toxic denaturant methyl mercury used in the first northern blotting protocols [2,3], but is clearly a problem in modern labs. To circumvent these issues glyoxal was long ago suggested as an effective RNA denaturant [4], but originally required technically awkward buffer recirculation. This problem was solved by the introduction of BPTE running buffer, which allows agarose gels of glyoxylated RNA to be run with no more difficulty than a normal DNA agarose gel [5].

High-resolution separation of small RNA fragments (~20–250 bp) is best performed on denaturing acrylamide gels, which rely on heat and urea rather than chemical modification to prevent secondary structure formation. The technique is identical to traditional sequencing gel electrophoresis [6], however, the apparatus used for sequencing is not practical for northern blotting and standard protein gel electrophoresis systems are well-suited for this purpose. Helpfully, many of the complications inherent to running a high quality sequencing gel can be safely ignored unless base-pair resolution is required.

Separated RNA is transferred to a membrane by capillary transfer for agarose gels or using an electroblotting system for acrylamide gels [7,8,9]. Various different membranes and transfer conditions have been described but we find charged nylon membrane best for all standard applications [10], and observe little difference between transfer methods. Similarly, many combinations of probes and hybridization buffers can be used to detect RNA species, each having their own strengths and weaknesses. Here we provide a protocol for using RNA probes transcribed from PCR products; in our hands these are both the most reliable and the most sensitive, and as such are the probe of choice for new users [11,12,13,14]. We also provide probing conditions for use of synthetic oligonucleotides, which are widely used in RNA processing analysis since they provide unparalleled resolution of intermediates, and for random-primed DNA probes.

The source of RNA used for northern blotting is rarely critical as long as it is of high quality (*see* **Note 1**), and therefore in this chapter we focus purely on the gel systems and hybridization methods.

DOI

dx.doi.org/10.17504/protocols.io.bnsxmefn

EXTERNAL LINK

https://doi.org/10.1007/978-1-4939-9822-7_5

Citation: Jonathan Houseley, Cristina Cruz (09/02/2021). RNA Electrophoresis in Agarose Gels. https://dx.doi.org/10.17504/protocols.io.bnsxmefn

PROTOCOL CITATION

Jonathan Houseley, Cristina Cruz 2021. RNA Electrophoresis in Agarose Gels. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bnsxmefn

COLLECTIONS

Protocols for Northern Analysis of Exosome Substrates and Other Noncoding RNAs

KEYWORDS

lncRNA, ncRNA, northern blot, hybridization, probes

CREATED

Oct 22, 2020

LAST MODIFIED

Sep 02, 2021

OWNERSHIP HISTORY

Oct 22, 2020	Lenny Teytelman	protocols.io
Jul 05, 2021	Emma Ganley	protocols.io
Aug 24, 2021	Satyavati Kharde	
Aug 26, 2021	satyavati Kharde	

PROTOCOL INTEGER ID

43575

PARENT PROTOCOLS

Part of collection
Protocols for Northern Analysis of Exosome Substrates and Other Noncoding RNAs

GUIDELINES

RNA Handling and RNase-Free Technique

The difficulties in handling RNA are often overemphasized. In our hands, most of the cumbersome procedures recommended for avoiding RNase contamination seem to be dispensable. We use tubes and water specifically designated for RNA work, but take few other precautions. Solutions for gels and blotting are made with milliQ water in normal lab bottles with standard chemicals weighed in disposable weighing boats. We do not DEPC treat water or solutions, or use RNase decontaminating sprays or wipes routinely. It is good to have RNase ZAP in the laboratory to clean occasional contaminations or to wipe for example tissue grinding tools that will be in direct contact with the sample. For the final resuspension of RNA samples, we use commercially available nuclease free water. On first use, electrophoresis tanks can be rendered RNase-free by treating with 3% $H_2O_2$for 10 min, then rinsed with milliQ water, then set aside for RNA work if required. Blotting and hybridization can be carried out in normal laboratory trays and glassware. We use certified RNase-free filter tips and set aside a specific set of pipettes for handling stocks of RNase and RNase-containing solutions like plasmid miniprep resuspension buffer. Our bodies are a good source of RNase contamination, so care should be taken to avoid touching the inside of the lids when handling tubes. After assembling reagents, solutions and equipment for northern blotting, it is advisable to run a test gel using RNA of known quality before handling precious samples; ribosomal RNA bands should be clearly resolved and the higher molecular weight band (28S in mammals, 25S in yeast) should be brighter than the lower band (18S). This will confirm that the reagents are sufficiently RNase free.

Conversely, nonenzymatic mechanisms of RNA degradation need to be considered: RNA hydrolysis is catalyzed by alkaline pH and/or divalent cations, particularly with increasing temperature. Therefore, store RNA in water as opposed to TE (pH 8) or similar and beware of reaction conditions involving divalent cations and heat. DNase treatment can be a problem as all DNase I buffers contain magnesium, and DNase treatment is rarely necessary for northern blots as high molecular weight genomic DNA usually resolves far from the bands of interest.

Citation: Jonathan Houseley, Cristina Cruz (09/02/2021). RNA Electrophoresis in Agarose Gels. https://dx.doi.org/10.17504/protocols.io.bnsxmefn

MATERIALS TEXT

RNA Handling and RNase-Free Technique

1. A source of RNase-free milliQ water.
2. Commercially available nuclease free water.
3. Certified RNase-free filter tips.
4. 1.5 mL microfuge tubes specifically designated for RNA work.
5. A set of pipettes that are RNase-free.
6. RNaseZAP (Sigma) or similar.
7. 3% H_2O_2.

RNA Electrophoresis in Agarose Gels

1.RNA samples (1–10 µg total RNA or 10–1000 ng poly(A)+) in 3 µL total volume or less.
2.Glyoxal mix (10 mL): 6 mL DMSO, 2 mL of 40% glyoxal solution (Sigma), 1.2 mL 10× BPTE, 0.6 mL 80% glycerol. For long-term storage store at −80 °C. For routine use store at −20 °C.
3.10 mg/mL ethidium bromide solution.

> After purchasing a bottle of 10 mg/mL ethidium bromide, split into aliquots and freeze. An aliquot can be freeze-thawed but discard if the DNA molecular weight marker stops resolving. In our experience, old ethidium solutions compromise glyoxylation reactions.

4. Molecular weight marker—MassRuler.
5. 6× bromophenol blue dye: 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue.

> Xylene cyanol comigrates with rRNA and can interfere with quantification, so it is best to omit from this tracking dye.

6. 10× BPTE (1 L): 30 g PIPES free acid (Melford), 60 g Bis-Tris free base (Melford), 20 mL 0.5 M EDTA pH 8.0. Water to 1 L, stir to dissolve, check pH is around neutral. The components will not dissolve until the EDTA is added.
7. Molecular biology grade agarose.
8. Gel imaging and quantification system.
9. Sub-Cell®GT Horizontal Electrophoresis System, 15 × 15 cm tray, with casting gates (Bio-Rad), or equivalent electrophoresis system.

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

IMPORTANT: Make sure you have appropriate training to work with radioactivity under the local rules and legislation for your institution, and perform all radioactive work in the designated area.

BEFORE STARTING

It is recommended to read through the guidelines before starting any work with RNA.

RNA Electrophoresis in Agarose Gels 3h 30m

1

> Note that the method described below is for a 15 × 15 cm gel.

2 Add ethidium bromide to an aliquot of glyoxal mix to a final concentration of [M]**20 µg/mL** .

Citation: Jonathan Houseley, Cristina Cruz (09/02/2021). RNA Electrophoresis in Agarose Gels. https://dx.doi.org/10.17504/protocols.io.bnsxmefn

3 1h

Mix **15 µl glyoxal/EtBr** with **3 µl RNA sample** (can use less of RNA sample), then incubate at **55 °C** for **01:00:00** .

The ratio of glyoxal mix to RNA should be at least 5:1, but varying the ratio does not alter the result so samples do not all need to be at the same concentration. Glyoxylation at 55 °C can be left for longer than an hour if the gel is not ready to be loaded. Do not take the samples out and let them sit at room temperature. In our experience, if samples are left for too long at room temperature migration is compromised.

4

Mix **3 µl DNA ladder** with **15 µl glyoxal mix** , add **0.3 µl 10 mg/mL ethidium bromide** and incubate at **55 °C** along with the RNA samples.

5

Make a 15 × 15 cm 1.2% agarose gel by mixing **1.8 g agarose** with **150 mL 1× BPTE** .

Making a thicker gel to be able to fit more sample in is not a good idea as RNA transfer can be compromised. Also, make sure the agarose is fully melted as agarose lumps impair the transfer.

6 Fill the tank with 1× BPTE.

7

Centrifuge the samples briefly and load on the gel. If a spare lane is available, add **5 µl bromophenol blue loading dye** to track migration.

8 1h 30m

Run the gel at 150 V until bromophenol blue is ~ **2 cm** from the bottom (about **01:30:00**). This keeps all RNA on the gel, or migrate further if required. The voltage for this electrophoresis should be no higher than 6 V/cm, based on the distance between the electrodes.

9

Acquire a digital image of the ethidium staining pattern using a trans-illuminator or fluorescence imager to allow quantification and assessment of molecular weight based on the marker.

10 20m

Citation: Jonathan Houseley, Cristina Cruz (09/02/2021). RNA Electrophoresis in Agarose Gels. https://dx.doi.org/10.17504/protocols.io.bnsxmefn

Wash the gel **00:20:00** with **[M]75 Milimolar (mM) NaOH** .

It is critical that the NaOH wash is done at the specified concentration and length of time for the RNA to break into the right size fragments. This is to ensure good transfer and good hybridization of the probe.

11

Rinse the gel with milliQ water.

12 20m

Wash twice with neutralization solution for **00:20:00** , all on a shaker.

(Wash 1/2)

The neutralization solution washes can be longer. Up to 1.5 h works fine in our hands.

12.1 20m

Wash twice with neutralization solution for **00:20:00** , all on a shaker.

(Wash 2/2)

The neutralization solution washes can be longer. Up to 1.5 h works fine in our hands.

Citation: Jonathan Houseley, Cristina Cruz (09/02/2021). RNA Electrophoresis in Agarose Gels. https://dx.doi.org/10.17504/protocols.io.bnsxmefn