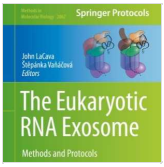

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RNA Electrophoresis in Acrylamide Gels

Book Chapter

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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 Although superseded by qPCR and sequencing methods for routine mRNA quantification, northern blotting is the method of choice when complex mixtures of overlapping species are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.

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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.

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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 Acrylamide Gels

1 .RNA samples (1–10 µg total RNA or 10–1000 ng poly(A)+) in 5 µL total volume or less.
2. 30% acrylamide and bisacrylamide solution, 29:1 (v/v) (Bio-Rad).
3. 10× and 1× TBE.
4. Urea.
5. 10% APS: Dissolve 1 g ammonium persulfate in water to 10 mL final volume. Store at 4 °C for up to 6 months.
6. TEMED.
7. Empty Gel Cassettes, mini, 1.5 mm (Thermo Fisher Scientific).
8. 50 bp DNA ladder.
9. 2× formamide loading dye: 95% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 5 mM EDTA, 0.025% SDS. Store long-term at −20 °C, up to 1 month at room temperature.
10. XCell SureLock™ Mini-Cell Electrophoresis System (Thermo Fisher Scientific) (or equivalent system).

> We have also used Bio-Rad minigel systems to good effect with the same protocol.

11. Comb with 10 or 15 wells, 1.5 mm.
12. Syringe and needle.
13. 0.5× TBE.
14. 10 mg/mL Ethidium bromide solution.

> If the concentration of the samples is very low, stain with SYBR gold (Thermo) after migration for higher sensitivity. Follow the manufacturer's instructions.

15. Imaging and quantification 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 Acrylamide Gels 1h 7m

1 In a 15 mL tube (Falcon/Corning type), make **10 mL** of an 8% acrylamide, 1× TBE, 42% urea (w/v) solution, and warm to **50 °C** until urea dissolves. Let cool to **Room temperature** .

2 ⚠

Meanwhile, prepare the empty gel cassette. It is important to have this ready, as the gel will polymerize very rapidly on addition of APS and TEMED.

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3

Add **50 µl 10% APS** to the gel, invert to mix, add **10 µl TEMED** and mix again. Pour the gel into the empty gel cassette and push in the comb carefully (at a slight angle works best), avoiding bubbles forming in the wells.

4

Mix samples with **5 µl 2× formamide loading dye** .

5

Also, mix **1 µl 50 bp DNA molecular weight marker** with **5 µl 2× loading dye** .

6 57m

Once the gel has set (**00:20:00** – **00:30:00**), denature the samples and ladder at **95 °C** for **00:05:00** then chill **On ice** for **00:02:00** .

7

Meanwhile, heat **750 mL 1× TBE buffer** to ~ **50 °C** , assemble the gel running apparatus according to manufacturer instructions and fill the inner chamber with hot buffer. Pour hot buffer in the outer chamber until it is **5 mm** from the top of the tank.

8

When the samples are on ice remove the comb from the gel, and use a needle and syringe to clean urea out of the wells (this is critical).

9 Load the samples and immediately run the gel at 300 V.

This high voltage keeps the gel hot, which is important for obtaining the best resolution. It is not disastrous if this cannot be attained with the power supplies available, most RNA species will resolve on much slower runs, but aberrant migration of the DNA ladder is often observed.

10 For the example shown in Fig. 2d the gel was migrated until the xylene cyanol was at the bottom of the gel. Tables of dye migration in different gel percentages are readily available on line, for example see http://www.elpisbio.com/brochure/gel%20electrophoresis%20buffer.pdf.

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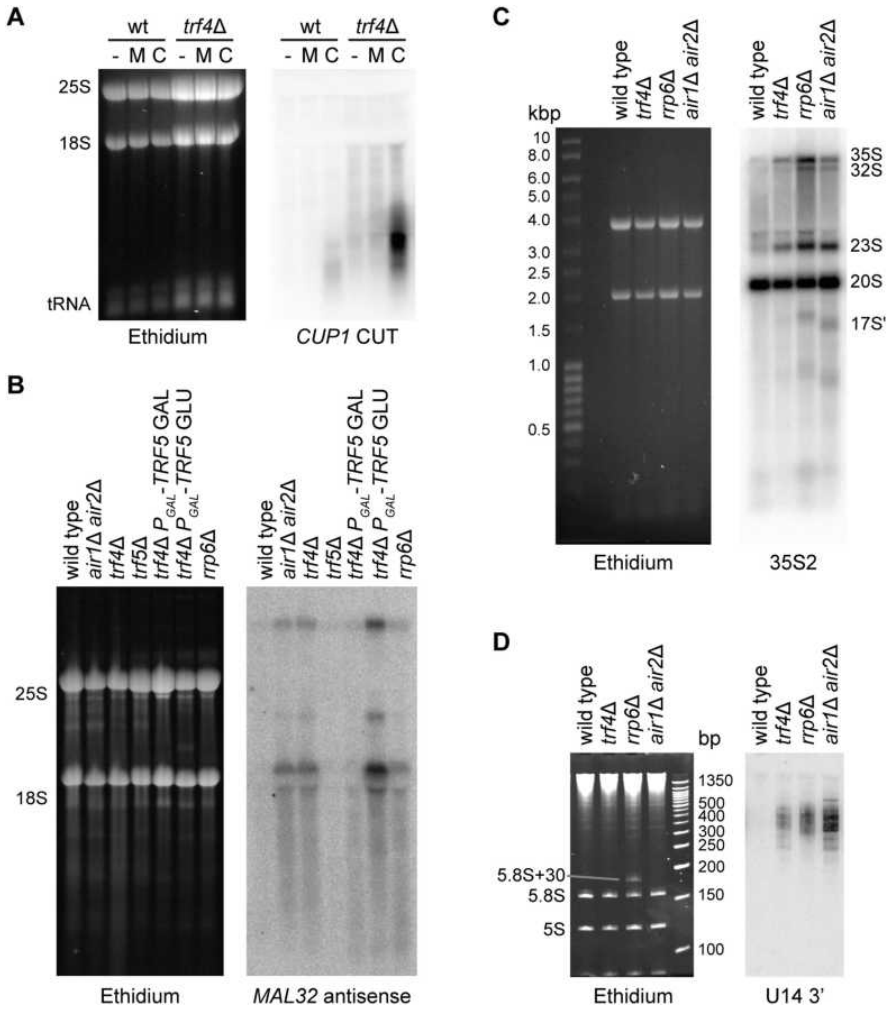

Fig. 2 Example images of northern blots hybridized with RNA probes against exosome substrates.
(**a**) 10 µg total RNA isolated using the hot phenol method from wild-type yeast and TRAMP mutant *trf4*Δ, grown to mid-log in YPD at 25 °C and treated with methotrexate (M) or copper sulfate (C), separated on a 1.2% agarose gel, transferred and probed for *CUP1* upstream cryptic unstable transcript (CUT) in ULTRAHyb at 65 °C using an RNA probe [15]. CUTs have no defined termination site and therefore are of very heterogeneous length [16], note that the signal for the CUT is only visible on *CUP1* induction using copper, and some degradation products are also visible in the wild type + copper lane.
(**b**) 10 µg total RNA isolated using the GTC phenol method from a variety of TRAMP mutants along with exosome mutant *rrp6*Δ, grown to mid log at 25 °C in YPD except *trf4*ΔP_{GAL}-*TRF5* which was grown either in YPGal or shifted for 24 h from YPGal to YPD), separated on a 1.2% agarose gel, transferred and probed for *MAL32* antisense in ULTRAHyb at 65 °C using an RNA probe. Multiple low abundance isoforms are readily detected in TRAMP mutants.
(**c**) 1 µg total RNA isolated using a mirVANA kit from wild-type yeast, exosome mutant *rrp6*Δ, and TRAMP mutants *trf4*Δ and *air1*Δ *air2*Δ grown to mid-log in YPD at 25 °C separated on a 1.2% agarose gel, transferred and probed using 35S2 which binds to a region of the pre-ribosomal RNA. Various aberrant rRNA intermediates are visible in the absence of exosome activity [17]. Due to the high signal from pre-rRNA species, hybridization was performed at 65 °C in Church Hyb.
(**d**) RNA samples from C separated on an 8% acrylamide/urea gel, transferred and probed using U14 3' which binds immediately downstream of the U14 snoRNA sequence. This is an example of a difficult sequence, being 70% AT in the probe binding region. A weak signal was obtained initially from probing at 65 °C in ULTRAHyb, and the membrane was immediately hybridized with the same probe at 60 °C in ULTRAHyb. Discrete bands are visible in TRAMP mutant samples, but not in *rrp6*Δ cells where these species are polyadenylated [17]

11 Dismantle the apparatus according to the manufacturer's instructions, put the gel in 0.5× TBE containing ethidium bromide (1 µL per 100 mL) and shake gently for **00:15:00** . 15m

12 Replace the staining solution with fresh 0.5× TBE and destain for **00:15:00** on a shaker. 15m

13

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Acquire the gel image using a transilluminator or fluorescence imager.

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