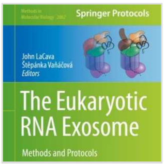


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# Transfer of RNA from Agarose Gels onto Membranes

Book Chapter

In 1 collection

Jonathan Houseley, Cristina Cruz



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 Although superseded by qPCR and sequencing methods for routine mRNA quantification, northern blotting is the method of choice when complex mixtures of overlapping species are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

**Protocols for Northern Analysis of Exosome Substrates and Other Noncoding RNAs**

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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.

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% $H_2O_2$.

## Transfer of RNA from Agarose Gels onto Membranes

1. 2 plastic boxes and 2 glass plates. Both at least 20 × 20 cm.
2. Chromatography paper (Whatman 3MM or similar).
3. Nylon membrane HYBOND N+ (GE Healthcare).
4. Guillotine paper cutter.

> If Northern and Southern blotting is going to become a regular technique, we recommend investment in a good guillotine paper cutter.

5. Paper towel.
6. Parafilm.
7. 0.5 M NaOH.
8. Neutralizing solution: 1.5 M NaCl, 0.5 M Tris pH 7.5.
9. 20× SSC: 3 M NaCl, 300 mM trisodium citrate, pH 7.
10. UV cross-linker with a 254-nm light source.

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.



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BEFORE STARTING

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

## Transfer of RNA from Agarose Gels onto Membranes

1 Cut a 15 × 15 cm piece of nylon membrane, two 15 × 15 cm pieces of Whatman paper and a 15 × 46 cm piece of Whatman paper.

> 46 cm is just the width of our paper. This piece has to be just long enough to reach the SSC on both sides.

2 Cut a corner of the nylon membrane and label the membrane with pencil.

> Cutting a corner of the membrane helps to keep track of the orientation of the gel once the blotting apparatus is disassembled. Labeling in pencil is important as ink is removed by some hybridization buffers, and should always be on the same side of the membrane as the RNA to aid orientation.

3 Soak the 15 × 15 cm Whatman paper and the membrane in 6× SSC in the same plastic box.

4 To set up the transfer apparatus (see Fig. 1), fill a box with 6× SSC up to about **2 cm** deep.



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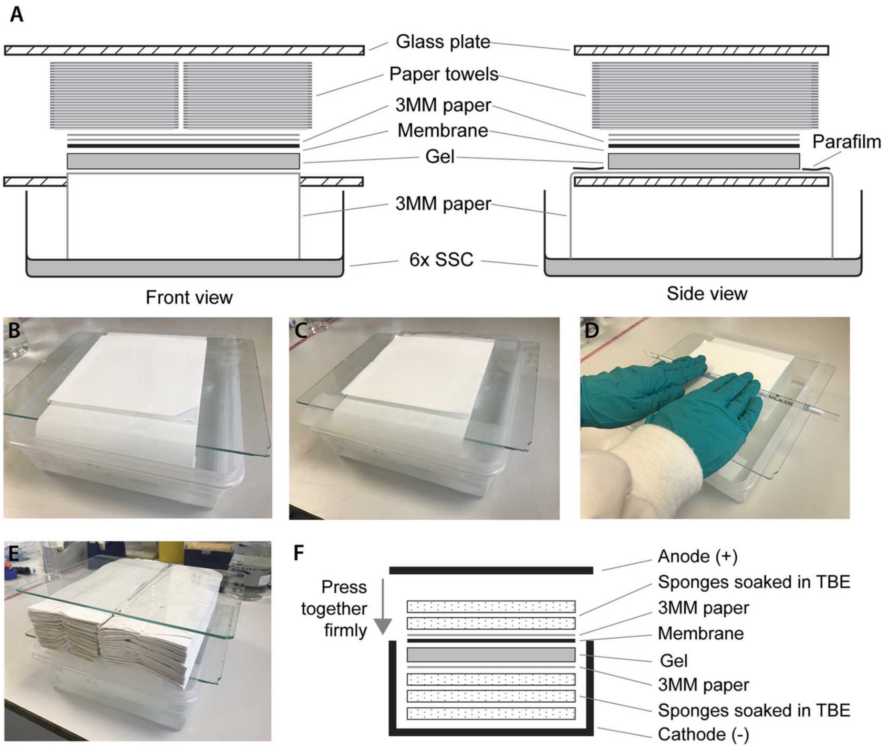



Fig. 1 Schematic representation and images of blotting apparatus for agarose and acrylamide gels.
(**a**) Schematic representation of the front and side view of a blotting apparatus for an agarose gel.
(**b**) Intermediate step with the long strip of Whatman paper soaked in 6× SSC, the gel and the membrane with the cut corner for tracking purposes.
(**c**) Intermediate step with the two squares of Whatman paper laid on the membrane and Parafilm covering the excess Whatman paper to avoid short circuiting.
(**d**) Elimination of air bubbles by rolling a Stripette over the gel "sandwich."
(**e**) Complete blotting apparatus for an agarose gel.
(**f**) Schematic representation of the blotting apparatus for an acrylamide gel.

> If the SSC runs out, the transfer will be compromised, though this is not terminal. Transfer over the weekend is not a problem though more 6× SSC is required.

5 Put a glass plate over the top leaving a gap to fit the Whatman paper through into the 6× SSC.

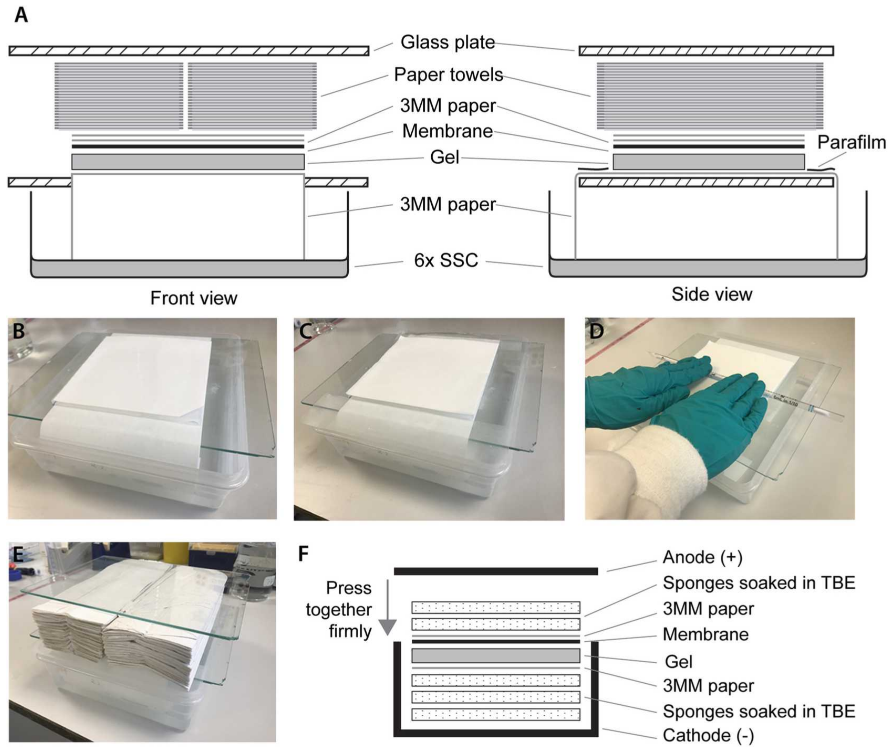
6 Wet the long strip of Whatman paper with 6× SSC and place over the plate such that both ends are in the 6× SSC reservoir.

7 Very carefully flip the gel and place it onto the Whatman paper with the wells facing down.

> Flipping the gel, although not absolutely necessary, aids transfer.

8 Place the wetted membrane over the gel using the cut corner to track the orientation of the wells (see Fig. 1b).

Before laying the membrane over the gel make sure there are no dry patches on the membrane as these impair transfer.

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(**d**) Elimination of air bubbles by rolling a Stripette over the gel "sandwich."
(**e**) Complete blotting apparatus for an agarose gel.
(**f**) Schematic representation of the blotting apparatus for an acrylamide gel.

9 Place the two square pieces of Whatman paper on top of the membrane. Roll a Stripette over the gel "sandwich" to get rid of bubbles.

This is important as bubbles completely inhibit the transfer and leave a blank patch in the signal.

10 Place two strips of Parafilm covering the ends of the long strip of paper that are not covered by the gel (see Fig. 1c, d).

The Parafilm is placed to avoid contact between paper towel and Whatman paper to prevent a short circuit that would compromise the transfer.



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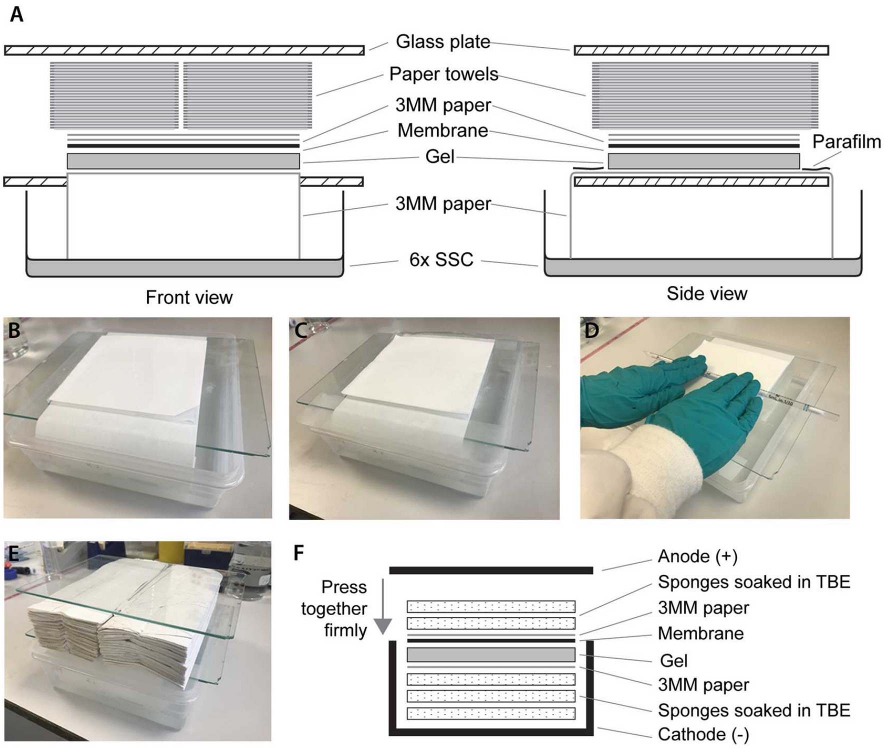


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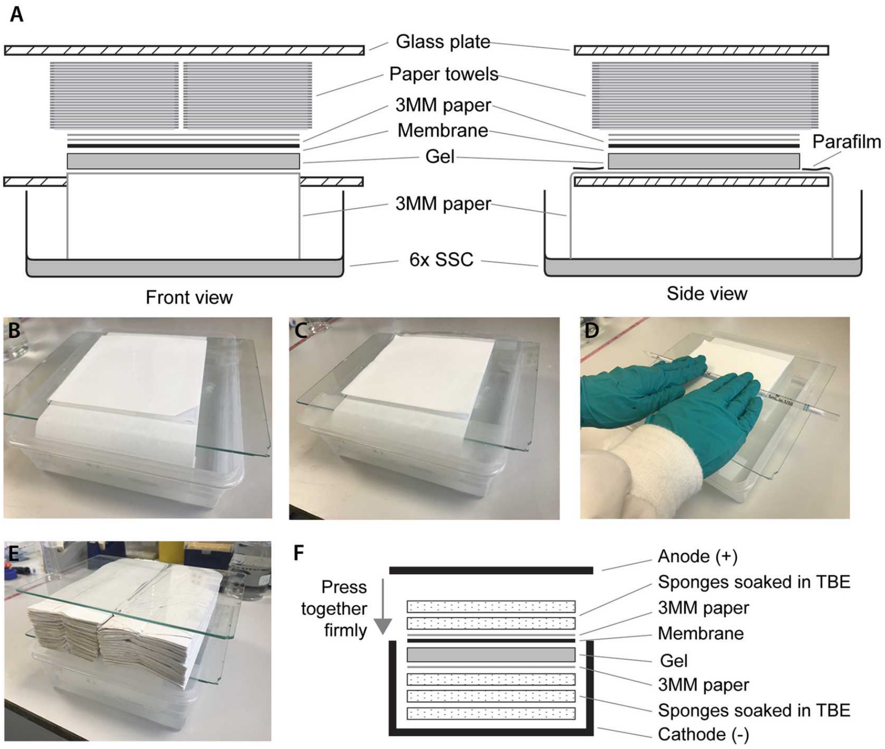
11 Place two stacks of paper towel of about 5 cm high next to each other over the "sandwich".

> It is important to have sufficient paper towel. When the blotting apparatus is disassembled there should still be some dry paper towels at the top of the stack. If all the paper towels are soaked in SSC your transfer might not be complete. However, over-weekend transfers always completely soak the paper towels.

12 Place another glass plate on top and leave the transfer apparatus overnight (see Fig. 1a, b, e).

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13 Pick up the two pieces of Whatman paper and the membrane together and place on the floor of the cross-linker with the RNA side facing upward, enabling direct irradiation of the RNA by the ultraviolet bulbs. Irradiate using Auto-crosslink mode for a Stratalinker or 120,000 µJ/cm$^2$ for other cross-linkers.

> Stratagene suggest that cross-linking works best if the paper and membrane are lightly damp, not dripping or dry after the transfer.

14

Wrap the membrane only in cling film and keep at **Room temperature** for probing on the same day **or** at **-20 °C** for long-term storage.



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