Chapter 7

Northern Blotting Analysis

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Abstract

Northern blotting analysis is a classical method for analysis of the size and steady-state level of a specific RNA in a complex sample. In short, the RNA is size-fractionated by gel electrophoresis and transferred by blotting onto a membrane to which the RNA is covalently bound. Then, the membrane is analysed by hybridization to one or more specific probes that are labelled for subsequent detection. Northern blotting is relatively simple to perform, inexpensive, and not plagued by artefacts. Recent developments of hybridization membranes and buffers have resulted in increased sensitivity closing the gap to the more laborious nuclease protection experiments.

Key words: Gel electrophoresis, northern blotting, probe, hybridization analysis, mRNA.

1. Introduction

Northern blotting analysis is a method for obtaining information on the size and abundance of a specific RNA in a complex mixture. In short, the RNA sample (e.g. whole cell RNA or a fraction hereof) is size-fractionated by gel electrophoresis. Then, the RNA is transferred onto a membrane ("blotted") and analysed by binding ("hybridization") of one or more labelled probes specific for the RNA in question. Northern blotting (1, 2) is a further development of a similar technique for DNA analysis, Southern blotting, developed by Ed Southern (3). Thus, its name, like western blotting, was introduced as a joke. Because northern blotting, unlike Southern blotting, does not refer to a person's name, it is spelled in lower case.

Northern blotting analysis gives information on the length of the RNA molecule and the possible existence of length variants

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because the RNA is electrophoresed under denaturing conditions in parallel with a molecular weight marker (an RNA ladder). RNA molecules that are branched or circular have aberrant mobility's and their analysis requires specialized procedures (4). Northern blotting is frequently used simply to demonstrate the presence of a specific RNA in a sample, but the method also allows for quantitative measurements. It is the steady-state level of the RNA, that is the sum of its production and removal, that is being measured. If the transcriptional activity is of interest, this is measured by e.g. nuclear run-on experiments. Decay of the RNA is measured independently by measurement of the transcript level at various timepoints following addition of a transcription initiation inhibitor. When used as a quantitative method, northern blotting is usually used to compare the RNA level in different experimental situations rather than for determination of absolute amounts. This is because hybridization to filter-bound RNA is suboptimal and strongly dependent on experimental parameters, such as degree of covalent attachment to the filter. Addition of known amounts of an internal standard of different length but with similar sequence made by in vitro transcription could be used to estimate absolute transcript levels, but this is rarely seen.

An outline of a northern blotting analysis is shown in Fig. **7.1**. Only the most common variations of the technique are covered by this chapter. For a more comprehensive description, *see* (5–7). The input RNA can be of any kind, but mostly it is whole cell RNA ("total RNA") prepared by the acid phenol/guanidinium thiocyanate extraction method (8) or by purification on an affinity matrix as in many commercial kits for preparation of RNA. If an mRNA is being analysed and the sensitivity of the analysis is an issue, polyA+ RNA (mostly mRNA) is isolated by oligo(dT) chromatography (9) prior to gel electrophoretic fractionation. Cytoplasmic, polyadenylated RNA constitute approximately 3% of whole cell RNA in human cells. By isolating this fraction, more of the relevant RNA can be loaded on the gel and consequently less abundant RNAs can be detected. Another application that calls for fractionation is analysis of the small RNAs. Simple fractionation protocols based on RNA binding columns can be applied to size-fractionate whole cell RNA and thus used to increase the capacity of gels, e.g. for analysis of cellular microRNAs.

Large RNAs (mRNA size) are size-fractionated on agarose gels. The useful range of agarose-concentration is 0.8% (for very large RNAs) to 1.4% (w/v). Outside this range, the gels either become difficult to handle or less efficient in blotting. Small RNAs are size-fractionated on polyacrylamide gels in the range 4–20% acrylamide. The size-fractionation is performed at denaturing conditions. RNA molecules fold into complex structures that involve the formation of double-stranded segments by base pairing. Thus, size-fractionation at native conditions will depend

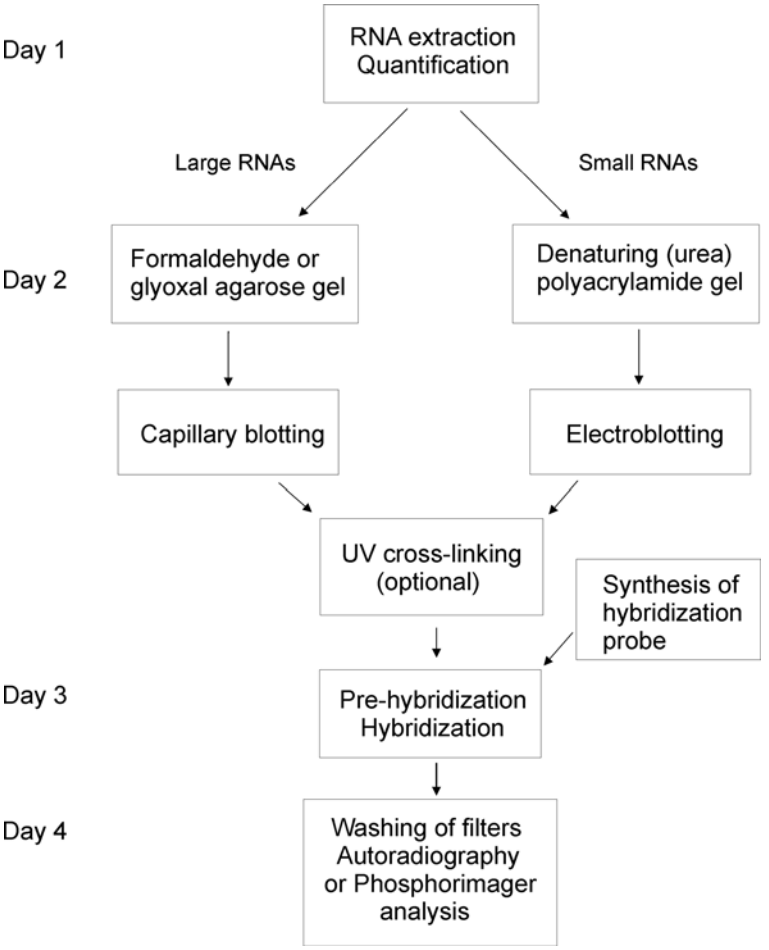

Fig. 7.1. Outline of a northern blotting analysis. The individual steps, except the extraction of RNA, are discussed in the text. A standard experiment takes 3–4 days. The overnight blotting step by upward capillary transfer can be shortened to few hours by application of alternatives discussed in the main text. The overnight hybridization step can similarly be performed in hours using "fast" hybridization solution alternatives.

not only on the length of the RNA chain but also on the folding. The two main methods for denaturing agarose gels are based on formamide/formaldehyde (10) and glyoxal (11), respectively. Formaldehyde is used both in the loading buffer (together with formamide) and in the gel. Formamide denatures the RNA and formaldehyde maintains the denatured state by reacting covalently with the amine groups of adenine, guanine, and cytosine bases. Since these bases are directly involved in formation of the A-U, G-U, and G-C base pairs in RNA, formation of secondary structure during the gel run is prevented. The reaction is reversed by treatment of the filter after blotting to allow the RNA to base pair with the probe. Glyoxal is not used in the gel but only in pre-treatment of the RNA sample. Glyoxal denatures RNA by

reacting covalently with guanine bases with the effect of inhibiting base pair formation similarly to formaldehyde. The glyoxal reaction is also reversed by treatment of the filter prior to hybridization analysis. Glyoxal gels tend to produce sharper bands than formaldehyde gels but care must be taken to prevent pH-dependent reversal of the glyoxalation during the gel run. In northern blotting analysis of small RNAs, the sample is denatured by heating in a loading buffer containing formamide or urea and the gels are made with 50% (approximately 8 M) urea (12).

One of the most common uses of northern blotting analysis is comparison of RNA levels in different experimental situations represented by different lanes on the gel. Thus, equal loading of RNA samples is critical. When whole cell RNA is used, equal amounts (up to 20 μg) based on spectrophotometry is applied to the gel. This is robust because cytoplasmic ribosomal RNA make up 70–80% of total RNA and together with other stable RNA are unlikely to be affected by the experimental variable. When enrichment by isolation of polyA+ RNA or by other means is used, the RNA of interest can be enriched to different degrees in the samples that are compared. In this case, the RNA levels are normally expressed in relation to an RNA that is detected in parallel by a different probe and known to be unaffected by the experimental variable. Popular examples of standards for comparing mRNA levels are cytoskeletal mRNAs (actin, tubulin), cyclophilin, and the mRNA coding for the glycolytic enzyme glucose-6-phosphate dehydrogenase (GAPDH). However, there are numerous examples of variations in the expression levels of these and the choice of standard should be considered carefully for each type of experiment. It has been suggested that ribosomal RNA would be a convenient general standard based on the arguments presented above. This runs into technical problems in northern blotting analysis because the ribosomal RNAs are so prominently present that they saturate the filter at the position of the ribosomal RNA bands making quantitative hybridization impossible.

Northern blotting analysis of mRNA is very sensitive to even slight degradation of the sample. For this reason, it is important to visually inspect the RNA after gel electrophoresis. RNA is conveniently stained by ethidium bromide similarly to DNA albeit less efficiently because binding is by ionic interaction rather than intercalation. One approach is to include ethidium bromide in the loading buffer for formaldehyde gels. This allows inspection during the gel run. This is not an option with glyoxal gels because ethidium bromide reacts with glyoxal and interferes with its ability to denature the RNA. Alternatively, the gel is stained after the run. Inclusion of ethidium bromide in the gel is not recommended because it migrates towards the cathode creating a front of stain in the gel. An important point in staining of whole cell RNA is that the quality of the RNA can be assessed from

inspection of the ribosomal RNA bands. The large (LSU) and small (SSU) subunit ribosomal RNAs are found in 1:1 ratio in cells. Thus, the differences in size (5,035 nt for LSU (28S) and 1,871 nt for SSU (18S) in humans) which is 2.7:1 should be reflected in the intensities of the two bands in ethidium bromide staining. If the ratio is less, this indicates that the RNA is partially degraded. Binding of ethidium to the RNA may reduce transfer and hybridization efficiency. If this is a problem, a test lane of the RNA should be run in parallel and stained after the gel run.

There are essentially two approaches to the blotting step of northern blotting analysis, capillary blotting and electroblotting. The traditional passive capillary transfer method (similar to the classical Southern blotting method (3)) is beautiful in its simplicity and can be performed with materials that are generally at hand in the laboratory. In essence, a stack of paper towels or similar absorbent material is used to draw a high salt transfer buffer from a reservoir, through the gel and an RNA binding membrane placed on top of the gel. The flow of buffer carries the RNA out of the gel so that it is trapped on the membrane. The setup for such an upward capillary transfer is shown in Fig. 7.2a. Transfer takes several hours and is usually performed over night. A faster and equally simple setup is the downward capillary transfer depicted

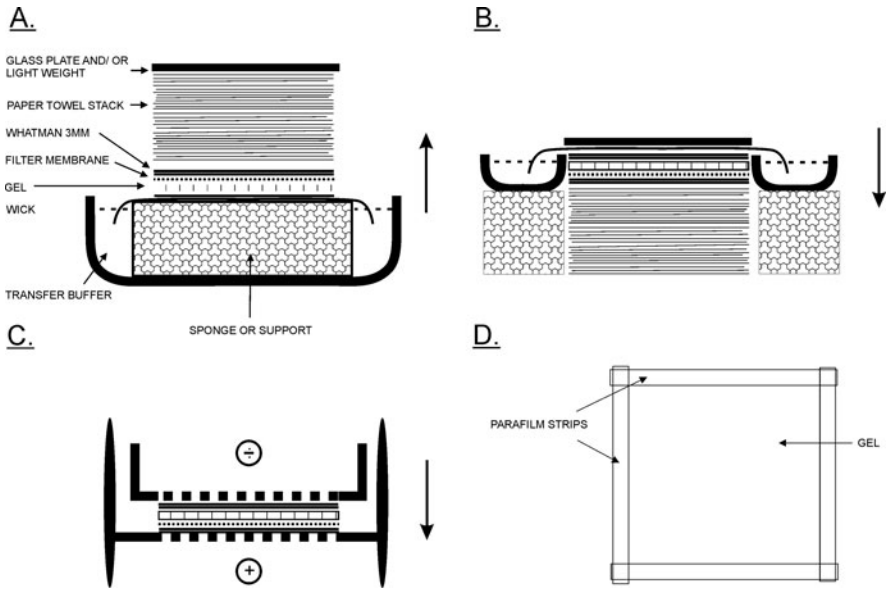

Fig. 7.2. Three different configurations for the gel transfer (blotting) step in northern blotting analysis. **a** Standard upward capillary transfer. This is used with agarose gels and takes several hours (3–6) to overnight to go to completion. **b** Downward capillary transfer. This is used with agarose gels and is completed in 1 h but can be left for longer times. **c** Electroblotting. This is used with polyacrylamide gels and (rarely) with agarose gels. The time depends on the type of gel but is usually complete in 1 h. **d** In all three transfer configurations is the gel lined with overlapping strips of parafilm to avoid short circuiting.

in Fig. **7.2b** (13). Transfer in this setup takes around 1 h but can be left for longer periods of time without problems. Other ways to speed up the transfer is to use a vacuum below the gel or to apply a pressure above the gel. Specialized equipments for these configurations are commercially available. Capillary transfer is the method of choice for agarose gels, whereas electroblotting (14) is used for polyacrylamide gels. In this method, the gel and the membrane are placed in a special cassette placed in an electrophoresis cell (*see* Fig. **7.2c**). An electrical field is applied perpendicular to the cassette and the RNA is transferred onto the membrane. This approach is also used for western blotting of proteins and a variety of electroblotting apparatus are available. One particular useful variety is the semi-dry type in which gel and membrane are sandwiched between Whatman 3MM paper wetted with transfer buffer. Electroblotting can be performed in less than an hour in some cases.

The choice of filter membrane is critical in northern blotting analysis. There are several different options, but for most purposes, positively charged nylon membranes are optimal. The positive charge is due to amino groups on the membrane surface and has the dual effect of increasing the affinity for nucleic acid and facilitating the immobilization of the RNA on the membrane. For the very same reasons, nylon membranes tend to produce more background due to unspecific binding of the probe unless care is taken to avoid this. Nylon membranes are characterized by high tensile strength and high binding capacity (4–500 $\mu g/cm^2$). They are produced with different pore sizes (0.22 and 0.45 μm) and some are optimized, e.g. for repeated use or for binding of small RNAs. It is important to notice the manufacturer's recommendations on handling of the membrane. For convenience, it is advisable to use membranes that are charged on both sides.

If the gel was stained with ethidium bromide, successful transfer can be confirmed by inspection of the filter in UV light (or even in daylight). It is also possible to inspect the gel to check that transfer was complete and not biased towards transfer of small RNAs. Alternatively, filter-bound RNA can be stained at later stages by methylene blue (15). RNAs transferred to positively charged nylon membranes at alkaline conditions become covalently bound to the filter. In all other situations, it is important to ensure that the sample RNA is immobilized on the filter in order to prevent loss during subsequent hybridization steps. It is advisable to follow the recommendations of the manufacturer of the membrane, but some general rules apply. The most common method is to use a UV light source to immobilize the RNA (16). UV light activates pyrimidine bases (thymine and uracil) to form covalent bonds with the surface amines of positively charged membranes (17). Wet nylon membranes generally require a dose of 1.6 kJ/m^2, whereas air-dried membranes

require only 0.16 kJ/m^2. This is most conveniently delivered by specialized equipment with a calibrated UV light source (e.g. Stratalinker from Stratagene). These instruments generally have an "auto-crosslink" setting at a dose optimized for damp membranes. Other UV sources can be used, but this may take considerable optimization and care should be taken to avoid extensive radiation at short (254 nm) wavelengths that will damage the RNA. An alternative way to immobilize RNA on nylon membranes is to incubate the filter at 65°C for 1–2 h. The exact principle behind fixation is not known in this case. Some manufacturers claim that the ionic binding to the membrane is sufficient and that there is no need for further steps.

Membranes with immobilized RNA can be stored for extended periods of time and can be used as a record of RNA experiments for later analysis. The next step in the procedure is hybridization analysis. This involves application of a probe to the membrane bound material at conditions that favours specific binding to complementary target molecules on the membrane. The probe is labelled to allow subsequent detection of its complex with the target. The most common approach is radioactive labelling (preferably ^{32}P), but non-isotopic alternatives exist. These probes are generally much less sensitive than ^{32}P-labelled probes. The probes can be DNA or RNA that can be labelled in various ways. The most popular type of probes has been double-stranded plasmids or PCR-products labelled with the random-priming labelling method (18), but asymmetric (single-stranded) PCR-products and RNA probes made by in vitro transcription are more sensitive due to the absence of a competing strand in the probe. Oligonucleotide probes are much less sensitive and are used when discrimination between target molecules of closely related sequences is the issue.

A large variety of hybridization buffers or systems are available and can be used with success for membrane hybridizations. Hybridization theory does not apply to nucleic acids immobilized on a membrane and the success is mainly empirically based. However, some rules of thumb exist. The critical value is the melting temperature (T_m), defined as the temperature at which 50% of all hybrids are dissociated into single strands. The maximum rate of hybridization is typically found around 20°C below the melting temperature. For long probes, the T_m for DNA:RNA hybrids is $T_m = 79.8°C + 18.5 \log[Na^+] + 58.4\ (\%G+C) + 11.8\ (\%G+C)^2 - 0.5\ (\%\ \text{formamide}) - (820/L)$ (19). (%G+C) is the percentage of guanine+cytosine content expressed as a mole fraction, $[Na^+]$ is the molar sodium concentration, and L is the effective length of the probe taking part in base pairing with the target. The constants are slightly different for DNA:DNA and RNA:RNA hybrids. Formamide is used to reduce the hybridization temperature, which is particularly important when using RNA probes

and when filters are to be used for multiple hybridizations (20). For most purposes, a practical approach using "standard" conditions is taken. The temperature of hybridization and the stringency of washing are then varied according to the requirements of the experiment on an empirical basis. The composition of the hybridization buffer is important. Single-stranded DNA of an origin that is unrelated to the experimental samples (mostly salmon sperm DNA) is used to block unspecific nucleic acid binding to the membrane. An additive known as Denhardts solution (Ficoll, polyvinylpyrrolidone, and bovine serum albumin) is frequently used to block the membrane and to increase the probe concentration by reducing the active volume. An important development is hybridization buffers with a high concentration of SDS. Unfortunately, many high-quality hybridization solutions are sold without a declaration of their content and based on the convincing argument that they are optimized for use with the hybridization membranes sold by the same company.

Northern blotting analysis is not the only technique to demonstrate the presence and quantitate the amount of a specific RNA. In nuclease protection experiments, a labelled probe strand is hybridized in solution to the sample RNA. Then, unhybridized single strands are removed by nuclease digestion and the hybrids are analysed on gels. This technique is traditionally considered an order of magnitude more sensitive (detection limit 0.1–1 pg) than classical northern blotting analysis (detection limit 1–10 pg) and more reliable because the hybridization step is in solution. More recent improvements of northern blotting analysis have closed the gap and it is claimed that in some systems as little as 10,000 molecules on a membrane can be detected. This corresponds to a medium abundant mRNA in less than 100 ng of whole cell RNA. Nuclease protection experiments are relatively laborious and require some optimization. RT-PCR based methods are an additional order of magnitude more sensitive than northern blot analysis. The main problem is that they require stringent controls to avoid artefacts and that the quantitative versions of the technique require expensive instrumentation. Thus, the northern blotting analysis remains an important technique that is simple to perform, low-cost, and provide qualitative and quantitative information in the same experiment. In many cases, a northern blot is still required to convince the referees of the validity of an observation.

One final note of caution concerns the interpretation of the result of the experiment. It is becoming increasingly clear that the level of mRNA is correlated with the level of protein for less than 20% of the genes in humans. This observation obviously emphasizes the importance of post-transcriptional regulation. Thus, the quantitation of an mRNA in most cases provides little

information on gene expression in protein sense. On the other hand, the increased interest in RNA makes the observation of the transcript level important in its own right!

2. Materials

Northern blotting analysis is very sensitive to even slight degradation of RNA. For this reason, it is important to wear gloves during all manipulations. Equipment should be free of RNases. One simple precaution is to reserve equipment for RNA work. Alternatively, equipment should be thoroughly cleaned prior to use, especially if it has been in contact with RNases, e.g. during preparation of plasmid DNA. Electrophoresis tanks should be cleaned with detergent, rinsed in water, and dried with ethanol. They are then treated with 3% H_2O_2 for 10 min at RT, followed by rinsing with DEPC-water.

2.1. Formaldehyde Agarose Gel

1. Agarose (Seakem GTG; FMC BioProducts, or similar).
2. 20× MOPS: 0.4 M MOPS ((3-(*N*-morpholino) propanesulfonic acid), 0.1 M sodium acetate, 20 mM EDTA. For 1 L, dissolve 83.6 g of MOPS and 8.2 g of sodium acetate in 800 mL of DEPC-treated water. Adjust the pH to 7.0 with 2 N NaOH. Add 40 mL of 0.5 M EDTA, pH 8.0, and adjust the final volume to 1 L. Autoclave or sterilize by filtration through a 0.2-μm Millipore filter (*see* **Note 1**).
3. 37% formaldehyde (standard 12.3 M solution known as "formalin") (*see* **Note 2**).
4. Loading buffer: 250 μL deionized formamide (*see* **Note 3**), 88 μL 37% formaldehyde, 25 μL MOPS, 2 μL EtBr (3 mg/mL).
5. RNA ladder (commercially available from several companies).
6. Positively charged nylon membrane (Hybond N+ (GE Healthcare Life Sciences), BrightStar Plus (Ambion), Gene Screen Plus (PerkinElmer), or similar).
7. 20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0. For 1 L dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of DEPC-treated water. Adjust the pH to 7.0 with a few drops of 10 N NaOH. Adjust the volume to 1 L with DEPC-treated water and autoclave.
8. 2× SSC made from 20× SSC by dilution with DEPC-treated water.
9. Parafilm.

10. Whatman 3MM paper.
11. Flat paper towels.
12. Plastic wrap.

2.2. Glyoxal Agarose Gel

1. Agarose (Seakem GTG; FMC BioProducts).
2. 20× MOPS: 0. 4 M MOPS ((3-(*N*-morpholino) propanesulfonic acid), 0.1 M sodium acetate, 20 mM EDTA. For 1 L, dissolve 83.6 g of MOPS and 8.2 g of sodium acetate in 800 mL of DEPC-treated water. Adjust the pH to 7.0 with 2 N NaOH. Add 40 mL of 0.5 M EDTA, pH 8.0 and adjust the final volume to 1 L. Sterilize by filtration through a 0.2-μm Millipore filter (*see* **Note 1**)
3. 6 M glyoxal (40%; freshly deionized) (*see* **Note 4**).
4. DMSO.
5. Loading buffer: 50% glycerol, 1× MOPS, 0.25% bromophenol blue.
6. RNA ladder (commercially available from several companies).
7. Positively charged nylon membrane (Hybond N+ (GE Healthcare Life Sciences), BrightStar Plus (Ambion), Gene Screen Plus (PerkinElmer), or similar).
8. Parafilm.
9. Whatman 3MM paper.
10. Flat paper towels.
11. Plastic wrap.

2.3. Denaturing Polyacrylamide Gel

1. 40% acrylamide (40:1.3): 400 g of acrylamide, 13 g of bisacrylamide. Dissolve in water; adjust to 1 L. Filter through a 3MM filter and store in a dark bottle in the cold (*see* **Note 5**).
2. 10× TBE electrophoresis buffer: 0.9 M Tris, 0.9 M boric acid, 0.02 M EDTA. For 5 L dissolve 544 g of Tris, 278 g of boric acid, and 37.2 g of EDTA. Dissolve in water and adjust to 5 L. Autoclave for long-term storage.
3. 5% UPAG-mix/1× TBE: 125 mL 40% acrylamide-stock (40:1.3), 500 g urea, 100 mL 10× TBE. Add water to dissolve urea; adjust to 1 L. Filtrate through a 3MM filter and store in a dark bottle in the cold.
4. 10% (w/v) ammonium persulfate. Store as frozen aliquots. Once thawed, the solution is stored at 4°C and can be used for a few weeks.
5. TEMED (N, N, N', N'-tetramethylethylenediamine)

6. Loading buffer: 1× TBE, 50% urea, 1 mg/mL bromophenol blue, 1 mg/mL xylene cyanol FF.
7. RNA ladder (commercially available from several companies).
8. Positively charged nylon membrane (Hybond N+ (GE Healthcare Life Sciences), BrightStar Plus (Ambion), Gene Screen Plus (PerkinElmer), or similar).
9. Parafilm.
10. Whatman 3MM paper.

2.4. Hybridization Analysis

1. 20× SSPE: 3 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4. For 1 L dissolve 175.3 g of NaCl and 27.6 g of NaH_2PO_4 and 7.4 g of EDTA in 800 mL of DEPC-treated water. Adjust the pH to 7.4 with NaOH approximately 6.5 mL of a 10 N solution). Adjust the volume to 1 L with DEPC-treated water and autoclave.
2. 20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0. For 1 L dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of DEPC-treated water. Adjust the pH to 7.0 with a few drops of 10 N NaOH. Adjust the volume to 1 L with DEPC-treated water and autoclave.
3. 50× Denhardt's solution: 0.05% (w/v) BSA, 0.05% (w/v) polyvinyl pyrolidone, and 0.05% (w/v) Ficoll 400. For 50 mL dissolve 0.5 g of Ficoll (type 400 Pharmacia), 0.5 g of polyvinylpyrrolidone, and 0.5 g of bovine serum albumine (Fraction V; Sigma) in DEPC-treated water. Filter and store in small aliquots at –20°C.
4. Hybridization buffer: 50% formamide, 5× SSPE, 0.5% SDS, 2× Denhardts solution, 100 μg/mL of denatured carrier DNA. For 10 mL of hybridization buffer, combine 5 mL of deionized formamide (*see* **Note 3**), 4 mL of 20 × SSPE, 0.25 mL of a 20% solution of SDS, 0.2 mL of 50× Denhardts solution, 50 μL of a 20 mg/mL solution of denatured carrier DNA (e.g. salmon sperm DNA) (*see* **Note 6**).
5. Radioactively labelled probe (*see* **Note 7**).
6. Low stringency washing buffer: 2× SSC, 1% sodium pyrophosphate, 0.1% SDS.
7. Medium stringency washing buffer: 0.2× SSC, 1% sodium pyrophosphate, 0.5% SDS.
8. High stringency washing buffer: 0.1× SSC, 1% sodium pyrophosphate, 0.5% SDS.
9. Probe removal buffer: 50% formamide, 2× SSPE.

3. Methods

3.1. Northern Blotting of a Formaldehyde Agarose Gel

The example is a 1.2% agarose gel blotted by traditional upward, passive capillary blotting to a positively charged nylon membrane. This type of gel is used for analysis of small to medium sized mRNA from mammalian cells.

1. Pour 100 mL of dH_2O into a 250-mL Ehrlenmeyer flask. Use a marker pen to indicate the water level on the flask. Remove approximately 10 mL of the water and add 1.2 g of agarose to the flask. Dissolve the agarose by boiling, e.g. in a microwave oven. When the agarose is completely dissolved, cool it to 60°C under running tap water. Add 5 mL of 20× MOPS and 5.36 mL of 37% formaldehyde in a fume hood (*see* **Note 2**). Adjust the volume to the original 100 mL with dH_2O using the mark on the flask. Mix and cast the gel in the tray. Insert the slot former and allow the gel to solidify for at least half an hour.
2. Insert the gel tray into the gel apparatus. Fill up with electrophoresis buffer (1× MOPS) and carefully remove the slot former.
3. Take an aliquot of approximately 10 μg of RNA in 6 μL or less. Add 2.7 vols of loading buffer per μL of RNA to the sample and heat it for 10 min at 70°C. Flush the sample wells with electrophoresis buffer and load the samples. Load a molecular weight marker next to the samples.
4. Run the gel at 2 V/cm for approximately 3 h. The loading buffer contains ethidium bromide allowing the electrophoresis to be followed by inspection of the gel under UV light. Note that not all gel electrophoresis trays are UV transparent.
5. Photograph the gel in the UV transilluminator. Notice the intensity of the two major RNA bands. The upper one (LSU rRNA) should be approximately twice the intensity of the lower (SSU rRNA).
6. Prepare the gel for northern blotting by cutting away those parts of the gel that are not to be transferred (below 5S rRNA, above the wells, and the sides of the gel). Cut off lower left corner of the gel for orientation.
7. Wash out excess formaldehyde and ethidium bromide by soaking the gel in 2× SSC three times 5 min.
8. Optional. Irradiate the gel on a UV transilluminator to introduce random breaks in the RNA backbone. This is used to improve transfer efficiency of large RNA molecules and may improve hybridization efficiency (*see* **Note 8**).

9. Cut a piece of membrane to the size of the gel while leaving the membrane between the two sheets of protective paper. Do not handle the membrane without gloves (*see* **Note 9**).
10. Pre-wet the membrane by floating on the surface of RNase-free water for a few minutes. Once the membrane is wet (usually within less than a minute), inspect the membrane for even wetting (*see* **Note 10**). Submerge the membrane and transfer it to the 10× SSC transfer buffer and equilibrate for 1–2 min.
11. Cut two pieces of Whatman 3MM filter paper to the size of the gel.
12. Make sure that the sponge and the Whatman 3MM filter in the transfer unit is saturated with buffer and that the buffer level in the unit is at least 1 cm from the bottom.
13. Assemble the transfer sandwich: Without trapping air bubbles, place the gel in the transfer unit. Place strips of parafilm along the edges of the gel in order to avoid "short-cutting" of the buffer-flow. Carefully position the filter membrane on top of the gel. Then layer the two pieces of Whatman 3MM filter paper on top of the sandwich, one at a time. Cut a stack of paper towels (5 cm when compressed) and place on top of the sheets of Whatman paper. Finally, put a light weight (e.g. a glass plate) on top of the paper towels to compress the stack. Allow the transfer to proceed for at least 6 h, preferably over night.
14. Disassemble the transfer sandwich. Blot excess liquid using kitchen roll or paper towels and place the filter on top of a sheet of 3MM paper. Do not allow the filter to dry out completely. If the gel was stained with ethidium bromide, successful transfer can be confirmed by inspection of the filter in UV light (or even in daylight). Filter-bound RNA can also be stained at later stages by methylene blue (*see* **Note 11**).
15. Optional. Place the filter in the UV cross-linker with the side of the membrane that was in contact with the gel facing up. Use the "auto-crosslink" setting as a starting point (*see* **Note 12**).

3.2. Northern Blotting of Glyoxalated RNA Separated on an Agarose Gel

The example is a 1% agarose gel blotted by traditional upward, passive capillary blotting to a positively charged nylon membrane. This type of experiment is used for analysis of most mRNA from mammalian cells.

1. Pour 100 mL of dH_2O into a 250-mL Ehrlenmeyer flask. Use a marker pen to indicate the water level on the flask. Remove approximately 10 mL of the water and add 1.0 g

of agarose to the flask. Dissolve the agarose by boiling, e.g. in a microwave oven. When the agarose is completely dissolved, cool it to 60°C under running tap water. Add 5 mL of 20× MOPS. Adjust the volume to the original 100 mL with dH_2O using the mark on the flask. Mix and cast the gel in the tray. Insert the slot former and allow the gel to solidify for at least half an hour (*see* **Note 13**).

2. Insert the gel tray into the gel apparatus. Fill up with electrophoresis buffer (1× MOPS) and carefully remove the slot former.
3. Mix in a RNA quality microfuge tube 5.4 μL of 6 M glyoxal, 16.0 μL of DMSO, 1.5 μL of 20× MOPS, and 7.1 μL of RNA (up to 10 μg). Place the sample at 50°C for 1 h to denature. Remember to treat the RNA ladder in parallel.
4. Cool the RNA sample on ice, add 4 μL of loading buffer, and immediately load the samples into the wells of the gel.
5. Run the gel while ensuring that no pH-gradient is formed during the run. Glyoxal dissociates from RNA at pH>8.0.
6. At the end of the run (typically when the bromophenol blue has migrated 8 cm), the lane(s) containing the RNA ladder can by cut out and stained. Alternatively, proceed to the blotting steps.
7. Transfer the RNA to a positively charged nylon membrane as described in **Section 3.1**, **Steps 6, and 8–15.**
8. Optional. After immobilization, remove glyoxal from RNA by washing the filter for 15 min at 65°C in 20 mM Tris-Cl, pH 8. Alternatively, the glyoxal will be removed during incubation with hybridization buffer in the pre-hybridization step.

3.3. Denaturing Polyacrylamide Gel

The example is a denaturing (urea) 5% polyacrylamide gel. The effective separation range is 50–500 nt and the migration of the marker dyes are approximately 130 nt (xylene cyanol FF) and 35 nt (bromophenol blue), respectively. A Semi-Phor Blotter (Hoefer Instruments) was used for semi-dry transfer of the RNA. This type experiment is used for analysis of small RNA molecules, such as snRNA or snoRNA.

1. Cast the gel. For a 5% UPAG pour 35 mL of gel-mix into a beaker, add 135 μL of 10% ammonium persulfate and 70 μL of TEMED. Cast the gel in the pre-assembled sandwich of glass-plates. Allow to polymerize for 45 min.
2. Remove the slot former. Mount the gel in the electrophoresis unit. Pre-run the gel for 10–30 min.
3. Add one vol of loading buffer to the sample RNA. Heat-denature the RNA at 70°C for 2 min. Meanwhile flush

the slots with 1× TBE running buffer. Load and run the gel.

4. After gel electrophoresis: stain the gel with ethidium bromide and place it on a quartz plate. Take a photo and cut the gel to appropriate size. Measure its size with a ruler, and note the position of the wells. Do not remove the gel from the quartz plate, but cover it with plastic wrap to ensure that it does not dry out.
5. Cut out a piece of nylon membrane and three pieces of Whatman 3MM paper to the size of the gel to be blotted (*see* **Note 9**).
6. Pour a small amount of 1× TBE in a wash tray and wet the membrane (cut to the exact size of the gel) by floating it carefully on the buffer surface before immersing it. This will avoid trapping of air bubbles in the pores of the membrane. Do not allow the membrane to dry at any time prior to the completion of the transfer procedure.
7. Remove the plastic foil from the gel and soak the surface of it with 1× TBE. Lay the wet membrane carefully on the gel. Align it with two diagonal corners, and then gently roll the membrane down onto the gel. Remove any trapped bubbles by gently pushing them to the side or rolling them out with a pipette. Flood the surface of the membrane with additional 1× TBE.
8. Wet one of the Whatman 3MM papers (cut to exact size of the gel) with 1× TBE and place it over the membrane. Flood the lower grating of the electroblotter with 1× TBE. Now take the quartz plate with the gel, membrane, and Whatman paper between your hands and invert it. When the gel has slipped off the quartz plate, lay the sandwich, paper down, on the grating. Flood the surface of the gel with 1× TBE.
9. Wet the two remaining pieces of 3MM paper in 1× TBE and place them, one at a time, precisely on top of the gel. Carefully remove all air bubbles. Remove buffer around the gel with paper towels and align pieces of parafilm along the edges of the gel. The buffer must not bypass the gel, as it will result in inefficient and uneven transfer of the RNA.
10. Flood the surface of the 3MM paper with 1× TBE and remove any excess of buffer from the parafilm. Place the lid of the electroblotter on top of the paper and blot for 30 min at 150 mA.
11. After transfer is complete, dismantle the electroblotter. Be sure that you are wearing gloves. Take out the sandwich of paper, membrane and gel and place it inverted (membrane

over the gel) on a piece of plastic foil. Remove the upper paper and cut off the lower left corner of the membrane (this will correspond to the lower right corner of the gel). Mark the membrane with a pencil to allow identification of tracks.

12. Lay the membrane, RNA side up, on plastic wrap (cut-off corner at lower right). If an effective RNA transfer has occurred, this will show up as ethidium bromide fluorescence under a UV lamp. Fix the RNA covalently to the membrane by UV irradiation in the UV crosslinker. The membrane is now ready for hybridization analysis (*see* **Note 12**).

3.4. Hybridization Analysis

The example describes a standard hybridization aimed at detection of mRNA in whole cell RNA and using a formamide containing hybridization buffer to avoid extended incubations at high temperatures that leads to RNA degradation.

1. Place the hybridization membrane in a hybridization bottle. Prehybridize the membrane for 1–2 h in 10 mL of hybridization solution at 42°C.
2. 5×10^6 cpm of probe/mL of hybridization buffer is adjusted to 500 μL with hybridization solution and denatured by boiling in a boiling water bath for 2 min. The probe is added to the hybridization solution in the bottle without spotting it directly on the membrane.
3. Hybridize over night at 45°C.
4. Remove the membrane from the bottle using forceps and submerge immediately in low stringency washing buffer.
5. Wash the membrane 2 × 10 min in low stringency washing buffer at RT. Follow the progress of the washing steps using a Geiger-Müller tube monitor.
6. Wash the membrane 2 × 20 min in medium stringency washing buffer at 65°C.
7. (Optional). Wash the membrane 2 × 20 min in high stringency washing buffer at 65°C.
8. Autoradiography or phosphorimager analysis of the membrane.
9. (Optional). The filter can be stripped of the probe by incubation for 1 h at 65°C in 50% formamide, 2× SSPE. This is followed by a brief rinsing in 0.1× SSPE and blotting of excess liquid. The filter should be kept damp until further hybridization steps. It is extremely difficult to completely remove probe from a filter that has been allowed to dry out and to re-hybridize a filter that been dried in the presence of SDS.

4. Notes

1. The MOPS-buffer turns yellow with age if exposed by light or by autoclaving. Straw-coloured buffers can be used, but darker coloured buffers should be discarded.
2. Formaldehyde is supplied as a 37% (12.3 M) solution, containing 10–15% methanol. It should be stored in tight bottles out of direct sunlight. The pH should be checked before use (should be grater than 4.0). Formaldehyde is carcinogenic.
3. Many batches of formamide are sufficiently pure to be used directly. If a yellow colour is present, the formamide must be deionized by batch treatment with a mixed-bed resin (e.g. Dowex XG8) prior to use. The formamide is stirred with the resin for 30–60 min and then filtered through a filter paper. Aliquots are stored at –70°C under nitrogen. Formamide is a suspected teratogen and should not be handled by expectant mothers.
4. Glyoxal is obtained as a 6 M (40%) solution. It is readily oxidized in air and the oxidation products (glyoxylic acid) will cause fragmentation of the RNA sample. For this reason, glyoxal must be deionized before use. This can be done by passage through a mixed-bed resin (Bio-Rad AG 501-X8) until its pH is greater than 5.0. The deionized glyoxal is stored in small aliquots at –20°C in tightly capped tubes. Each aliquot is only use once. Glyoxal is a mutagen.
5. Acrylamide is a very potent neurotoxin that is readily absorbed through the skin. A mask and gloves should be worn when handling unpolymerized acrylamide and gloves should also be used when handling gels. Handling of acrylamide gels, e.g. for staining is difficult, and it is advisable to seek assistance from more experienced persons.
6. If the formamide is left out of the hybridization buffer, standard hybridization is performed at 68°C.
7. Many different kind of probes can be used, including double-stranded DNA labelled according to the random priming labelling method, radioactive in vitro transcripts, and asymmetric PCR-product. The two latter are preferred because of the single-stranded nature of the probe. The probes should be labelled to a specific activity exceeding 10^9 cpm/μg.
8. The efficiency of transfer can also be improved by slight alkaline treatment of the gel prior to transfer. The gel is placed in a tray with 50 mM NaOH, 0.1 M NaCl for

20 min with gentle shaking. Extensive alkaline treatment will fragment the RNA to the extent that it is no longer hybridization competent. Before transfer, the gel is neutralized in 0.1 M Tris-HCl, pH 7.6, for 10–15 min.

9. Membranes should be handled carefully. Finger grease on the membrane will reduce its performance and contamination with finger RNases are detrimental. Membranes are supplied in a sandwich between two sheets of protective paper. It is a good idea to keep the protective paper in place while cutting out a gel-sized piece of membrane.
10. A wetted membrane appears gray. Patches of white indicate areas that have been damaged. If these are in critical parts, the membrane should be discarded.
11. RNA on nylon membranes can be stained by the non-toxic methylene blue. The staining solution is 0.02% methylene blue in 0.3 M sodium acetate, pH 5.5. The RNA will stain in a matter of few minutes. De-staining is by incubation in 1× SSPE (10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl and 1 mM EDTA).
12. Filters that have not been used for hybridization experiments are stored dry between sheets of Whatman 3MM paper. Filters that are stored after a hybridization experiments are stored damp in vacuo or frozen to avoid opportunistic growth. Storage can be for several months.
13. In formaldehyde gels, RNases are inhibited due to the presence of formaldehyde in the gel. In glyoxal gels, inhibition of RNases can be achieved by addition of solid sodium iodoacetate to 10 mM to the melted agarose.

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