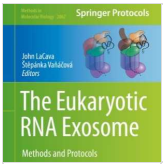

Sep 02, 2021

Hybridization of Random-Primed DNA Probes

Book Chapter

In 1 collection

Jonathan Houseley, Cristina Cruz

1 *Works for me* Share

dx.doi.org/10.17504/protocols.io.bns8mehw

Springer Nature Books

satyavati Kharde

Citation: Jonathan Houseley, Cristina Cruz (09/02/2021). Hybridization of Random-Primed DNA Probes. https://dx.doi.org/10.17504/protocols.io.bns8mehw

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 Although superseded by qPCR and sequencing methods for routine mRNA quantification, northern blotting is the method of choice when complex mixtures of overlapping species are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.bns8mehw

EXTERNAL LINK

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

Citation: Jonathan Houseley, Cristina Cruz (09/02/2021). Hybridization of Random-Primed DNA Probes. https://dx.doi.org/10.17504/protocols.io.bns8mehw

PROTOCOL CITATION

Jonathan Houseley, Cristina Cruz 2021. Hybridization of Random-Primed DNA Probes. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bns8mehw

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

43584

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.

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.

Hybridization of Random Primed DNA Probes

1. DNA probe template—100–1000 bp gel purified DNA fragment.
2. 5 U/µL Klenow (3'-5' exo-) (NEB).
3. 5× Labeling buffer: Mix 33 µg Random 9-mers (NEB) dissolved in 653 µL water, 660 µL NEBuffer 2 (NEB), 2.18 µL each of 100 mM dATP, dGTP, and dTTP. Store frozen in 100 µL aliquots.
4. 3000 Ci/mMol [α-^{32}P] dCTP, 370 MBq/mL.

> ^{32}P sources do not need to be particularly fresh. We use sources up to 6 weeks past the activity date with no problem, or even more for high signals. The exposure time required obviously increases, but the output is similar.

5. Radioactive workroom and protective equipment.
6. mini Quick Spin Columns by Roche or similar**.**
7. Hybridization bottles and oven.
8. ULTRAHyb ultrasensitive hybridization buffer by Ambion (Thermo Fisher Scientific).
9. 6× SSC.
10. 0.2× SSC 0.1% SDS.
11. Typhoon Storage Phosphorimager FLA7000 (GE) or equivalent.
12. Storage phosphor screen and exposure cassette.
13. FLA image eraser (GE) or equivalent.

SAFETY WARNINGS

Citation: Jonathan Houseley, Cristina Cruz (09/02/2021). Hybridization of Random-Primed DNA Probes. https://dx.doi.org/10.17504/protocols.io.bns8mehw

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.

Hybridization of Random-Primed DNA Probes 4h 39m

1 DNA probes lack the strand specificity of RNA or oligonucleotide probes, but are much more sensitive than oligonucleotides and are easier to strip than RNA probes.

2 Ensure that the hybridization bottles and internal seals are in good condition to avoid radioactive buffer leaking during hybridization.

35m

3 Prewarm a bottle of ULTRAHyb to **68 °C** until the SDS fully dissolves (**00:15:00** – **00:20:00**).

4 Wet the membrane with 0.1× SSC 0.1% SDS. Roll it up with the RNA facing inward, drop it into the hybridization bottle and slowly turn the bottle to unroll the membrane against the side of the bottle.

A Stripette can be used to help with this. Push out any obvious air bubbles. Put bottles in the oven so that the visible edge of the membrane is moving with the direction of rotation; if the membrane rolls up into a thin cigar during hybridization or washing then it was the wrong way round. When using a hybridization oven with rotisserie, always be sure to balance the bottles, including the volume of liquid inside. Two membranes can be hybridized in the same bottle, one facing the glass and the other facing the inside of the bottle.

1h

5 

Prehybridize for **01:00:00** at **42 °C** with **7.5 mL ULTRAHyb** .

This is an important step so do not feel tempted to skip it or shorten it. It allows blocking agents in the hybridization buffer to occupy nonspecific binding sites.

6 

Dilute **25 ng DNA template** to **37 µl** with water in a screw-cap tube.

5m

7 Heat at **100 °C** for **00:05:00** .

Citation: Jonathan Houseley, Cristina Cruz (09/02/2021). Hybridization of Random-Primed DNA Probes. https://dx.doi.org/10.17504/protocols.io.bns8mehw

8  5m

Snap-chill on ice water for **00:02:00** – **00:03:00** , centrifuge briefly.

9 Add **10 µl 5× labeling buffer** and **1 µl Klenow (exo-) enzyme** , followed by **2 µl [α-32P] dCTP** .

10  4h

Mix well by pipetting and incubate **01:00:00** – **03:00:00** at **37 °C** .

11 Clean the probe through a column as for an RNA probe (as detailed in the following substeps).

11.1 Flick a mini Quick Spin column inverted and then correct way up to get the sepharose to the bottom, remove top then bottom cap (see product instructions).

11.2 Place the column in a 2 mL microcentrifuge tube, spin at **1000 x g, 00:00:30** , discard tube and place the column in a new 1.5 mL screw cap tube.

11.3 Dilute the probe with water to **50 µl** and then pipette carefully on to the centre of the sepharose matrix in the column.

11.4 Centrifuge at **1000 x g, 00:04:00** .

11.5 Test incorporation by briefly holding the probe and column at equal distances from the Geiger counter. Use tweezers for this. At least 50% of the label should be incorporated, which means the Geiger counter will read the same signal from both column and probe. Discard the column to the radioactive waste.

12 Heat probe at **100 °C** for **00:05:00** . 5m

13  5m

Snap-chill on ice water for **00:02:00** – **00:03:00** , centrifuge briefly.

14 

Citation: Jonathan Houseley, Cristina Cruz (09/02/2021). Hybridization of Random-Primed DNA Probes. https://dx.doi.org/10.17504/protocols.io.bns8mehw

Add the probe to the hybridization bottle while vertical so that it falls directly into the hybridization buffer and incubate **Overnight** at **42 °C** .

15 Pour the probe into a 50 mL tube (Falcon/Corning type) and dispose. Reuse of random primed probes is **not** recommended.

16 See Section "Hybridization of RNA Probes" for washing and exposing directions:

16.1

Perform a single low stringency wash with 6× SSC for **00:10:00** at **42 °C** .

16.2

Perform a single high stringency wash with **preheated** 2× SSC 0.1% SDS at **42 °C** for **00:10:00** .

16.3

Perform one last wash for **00:10:00** with **50 mL 6× SSC** at **Room temperature** .

Washing twice for 30 min each with 2× SSC 0.5% SDS at 42 °C can improve signal-to-noise but can also reduce the signal for some probes. The easiest approach when using a new probe is to follow the low stringency washing protocol, expose the membrane and if background is unacceptable rewash using these more stringent conditions.

Citation: Jonathan Houseley, Cristina Cruz (09/02/2021). Hybridization of Random-Primed DNA Probes. https://dx.doi.org/10.17504/protocols.io.bns8mehw