HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2017 January 01.

Published in final edited form as:

Methods Mol Biol. 2016 ; 1402: 177–188. doi:10.1007/978-1-4939-3378-5_14.

Detection of Long Non-coding RNA Expression by Non-radioactive Northern Blots

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Abstract

With the advances in sequencing technology and transcriptome analysis, it is estimated that up to 75% of the human genome is transcribed into RNAs. This finding prompted intensive investigations on the biological functions of non-coding RNAs and led to very exciting discoveries of microRNAs as important players in disease pathogenesis and therapeutic applications. Research on long non-coding RNAs (lncRNAs) is in its infancy, yet a broad spectrum of biological regulations has been attributed to lncRNAs. As a novel class of RNA transcripts, the expression level and splicing variants of lncRNAs are various. Northern blot analysis can help us learn about the identity, size, and abundance of lncRNAs. Here we describe how to use northern blot to determine lncRNA abundance and identify different splicing variants of a given lncRNA.

Keywords

long non-coding RNA; RNA expression; Northern blots

1. Introduction

lncRNAs are operationally defined as RNA transcripts larger than 200 nt that do not appear to have coding potential (1–5). Given that up to 75% of the human genome is transcribed to RNA, while only a small portion of the transcripts encodes proteins (6), the number of lncRNA genes can be large. After the initial cloning of functional lncRNAs such as H19 (7,

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[6]If the total volume of sample and dye exceed the capacity of the wells, it is necessary to concentrate the RNA by precipitation and suspend the pellet in smaller volume of water before adding the loading dye.

8) and XIST (9) from cDNA libraries, two independent studies using high-density tiling array reported that the number of lncRNA genes is at least comparable to that of protein-coding genes (10, 11). Recent advances in tiling array (10–13), chromatin signature (14, 15), computational analysis of cDNA libraries (16, 17), and next-generation sequencing (RNA-seq) (18–21) have revealed that thousands of lncRNA genes are abundantly expressed with exquisite cell-type and tissue specificity in human. In fact, the GENCODE consortium within the framework of the ENCODE project recently reported 14,880 manually annotated and evidence-based lncRNA transcripts originating from 9,277 gene loci in human (6, 21), including 9,518 intergenic lncRNAs (also called lincRNAs) and 5362 genic lncRNAs (14, 15, 20). These studies indicate that 1) lncRNAs are independent transcriptional units; 2) lncRNAs are spliced with fewer exons than protein-coding transcripts and utilize the canonical splice sites; 3) lncRNAs are under weaker selective constraints during evolution and many are primate specific; 4) lncRNA transcripts are subjected to typical histone modifications as protein-coding mRNAs, and 5) the expression of lncRNAs is relatively low and strikingly cell-type or tissue-specific.

The discovery of lncRNA has provided an important new perspective on the centrality of RNA in gene expression regulation. lncRNAs can regulate the transcriptional activity of a chromosomal region or a particular gene by recruiting epigenetic modification complexes in either *cis*- or *trans*-regulatory manner. For example, Xist, a 17 kb X-chromosome specific non-coding transcript, initiates X chromosome inactivation by targeting and tethering Polycomb-repressive complexes (PRC) to X chromosome in *cis* (22–24). HOTAIR regulates the HoxD cluster genes in *trans* by serving as a scaffold which enables RNA-mediated assembly of PRC2 and LSD1 and coordinates the binding of PRC2 and LSD1 to chromatin (12, 25). Based on the knowledge obtained from studies on a limited number of lncRNAs, at least two working models have been proposed. First, lncRNAs can function as scaffolds. lncRNAs contain discrete protein-interacting domains that can bring specific protein components into the proximity of each other, resulting in the formation of unique functional complexes (25–27). These RNA mediated complexes can also extend to RNA-DNA and RNA-RNA interactions. Second, lncRNAs can act as guides to recruit proteins (24, 28, 29), such as chromatin modification complexes, to chromosome (24, 29). This may occur through RNA-DNA interactions (29) or through RNA interaction with a DNA-binding protein (24). In addition, lncRNAs have been proposed to serve as decoys that bind to DNA-binding proteins (30), transcriptional factors (31), splicing factors (32–34) or miRNAs (35). Some studies have also identified lncRNAs transcribed from the enhancer regions (36–38) or a neighbor loci (18, 39) of certain genes. Given that their expressions correlated with the activities of the corresponding enhancers, it was proposed that these RNAs (termed enhancer RNA/eRNA (36–38) or ncRNA-activating/ncRNA-a (18, 39)) may regulate gene transcription.

As a novel class of RNA transcripts, the expression level and splicing variants of lncRNAs are various. Northern blot analysis can help us learn about the identity, size, and abundance of lncRNAs. Here we describe how to use northern blot to determine lncRNA abundance and identify different splicing variants of a given lncRNA.

2. Materials

2.1 Materials for DIG labeled RNA probe synthesis

1. DNA template (see Note 1).
2. Restriction Enzyme.
3. 10x DIG RNA labeling mix (Roche, 11277073910).
4. T7 RNA polymerase (10 U/uL) and 5X transcription buffer (Agilent, 600123).
5. Dnase I (NEB, M0303S, 2000 U/mL).
6. Agarose gel electrophoresis supplies for DNA fragment purification.
7. Quick Spin Columns for radiolabeled RNA purification Sephadex G-50 (Roche, 11274015001).
8. Gel Extraction Kit (Qiagen, 28704).

2.2 Materials for separating RNA by electrophoresis

1. Nucleic acid agarose.
2. 55°C water bath.
3. 10x Denaturing gel buffer (Invitrogen, AM8676).
4. Heat block.
5. Gel electrophoresis apparatus.
6. 3-(N-morpholino) propanesulfonic acid (MOPS).
7. Sodium acetate.
8. 0.5 M EDTA.
9. 10x MOPS buffer: 200 mM MOPS, 50 mM Sodium acetate, 20 mM EDTA, adjust pH to 7.0. To make 1x MOPS gel running buffer, mix one part of 10x MOPS buffer with nine parts of RNase-free water.
10. RNA loading buffer (Invitrogen, AM8552).
11. Ethidium bromide (only if RNA visualization is needed).
12. DIG labeled RNA marker (Roche, 11373099910).

2.3 Materials for transferring RNA to the membrane

1. 20x SSC (Invitrogen, 15557-036).
2. Razor blade.

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[1]The desired DNA template should be a plasmid containing a promoter for in vitro transcription (i. e. T7 or T3) and a target sequence whose 5′end is placed as close as possible to the 3′ end of the promoter. We usually use pBluescript SK(+) and transcribe the target sequence using T7 polymerase. Minimize any unnecessary addition of non-lncRNA sequence into the plasmid to avoid impropriate RNA folding.

3. 3M Filter paper.
4. Positively Charged Nylon Membrane (Roche).
5. Blunt end forceps.
6. Paper towel.
7. RNase-free flat bottomed container as buffer reservoir.
8. Clean glass pasture pipet as roller.
9. Light weights (150–200 g) object serving as weight during transfer.
10. Supports of the reservoir (i. e. a stack of books).
11. Stratalinker® UV Crosslinker.

2.4 Materials for probe-RNA hybridization

1. 20x SSC
2. 10% SDS
3. DIG easy Hyb Granules (Roche, 11796895001).
4. 68°C shaking water bath.
5. Heat block.
6. Hybridization oven.
7. Hybridization bags.
8. Low Stringency Buffer: 2x SSC with 0.1% SDS.
9. High Stringency Buffer: 0.1x SSC with 0.1% SDS.

2.5 Materials for detection of probe-RNA hybrids

1. Washing and Blocking buffer set (Roche, 11585762001).
2. Anti-DIG-alkaline phosphatase antibody (Roche, 11093274910).
3. NBT/BCIP Stock Solution (Roche, 11681451001).
4. CDP-Star, Ready-to-Use (Roche, 12 041 677 001).
5. TE buffer: 10 mM Tris-HCL, 1 mM EDTA, adjust pH to approximately 8.

3. Methods (see Note 2)

lncRNA Northern blot analysis aims to characterize lncRNA expression. The protocol includes five parts:

[2]Northern blot analysis is a golden-standard in RNA detection and analysis. There are many protocols developed by laboratories specialized in RNA research or companies. The protocol described here is adapted from the NorthernMax procedure from Invitrogen and DIG application manual for filter hybridization from Roche. In our hand, this protocol is time efficient and gives satisfying results without using radioactivity.

1. RNA probe synthesis and labeling;
2. RNA sample electrophoresis;
3. RNA transfer;
4. RNA-probe hybridization; and
5. RNA-probe hybrid detection.

3.1 DIG labeled RNA probe synthesis by in vitro transcription

The DIG labeled RNA probe synthesis is very similar to the biotinylated RNA synthesis described in lncRNA pulldown assay. The differences between the two procedures are:

1. Since the probe needs to be complement to the target sequence, the probe RNA is transcribed from the 3′ end of the target sequence. We clone the gene of interest in reverse orientation to make the in vitro transcription template for Northern probes.
2. Use DIG labeling mix in place for the biotin-label mix.

3.2 Separating RNA samples by electrophoresis

3.2.1 Gel setup

1. Wipe the gel rack, tray and combs with RNAZap, rinse with water and let dry.
2. Weight 100 g agarose in a clean glass flask and mix with 90 mL RNase-free water. Melt the agarose completely by heating with a microwave. Put the flask with melted agarose in a 55 °C water bath.
3. In a fume hood (see Note 3), add 10 mL 10x denaturing gel buffer to the gel mix that is equilibrated to 55°C. Mix the gel solution by gentle swirling to avoid generating bubbles. Slowly pour the gel mix into the gel tray, pop any bubbles or push them to the edges of the gel with a clean pipet tip. The thickness of the gel should be about 6 mm. slowly place the comb in the gel. Allow the gel to solidify before removing the comb.
4. Right before RNA electrophoresis, place the gel tray in the electrophoresis chamber with the wells near the negative lead and add 1x MOPS gel running buffer in the chamber until it is 0.5 to 1 cm over the top of the gel (see Note 4).

3.2.2 RNA electrophoresis

1. Mix no more than 30 ug sample RNA with 3 volumes of RNA loading buffer (see Note 5, 21). To destruct any secondary structure of the RNAs, incubate the RNA with loading buffer at 65°C for 15 min using a heat block. Spin briefly to collect samples to the bottom of the tube and put the tubes on ice (see Note 7).

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[3]Always cast the gel in a fume hood as the denaturing solution contains formaldehyde. Solidified gels can be wrapped up and stored at 4°C for overnight.
[4]Do not let gel soaked in running buffer for more than 1 h before loading.
[5]Load no more than 30 ug total RNA in each lane. As the binding capacity of the membrane is limited, more RNA loaded does not guarantee a stronger signal. Overloading can lead to the detection of minor degradation of targeted RNAs.
[7]Use a heat block instead of a water bath to avoid contaminating the samples with water.

2. Carefully draw the RNAs in the tip without trapping any bubbles at the end of pipet tip, place the pipet tip inside of the top of the well, slowly push samples into the well and exit the tip without disturbing the loaded samples. If markers are needed, load one lane with DIG-labeled RNA marker.
3. Run the gel at 5 V/cm (see Note 8).
4. (Optional) Stain the gel with Ethidium bromide and visualize the RNA under UV (see Note 9).

3.3 Transfer RNA from agarose gel to the membrane

3.3.1 Material preparation

1. Use a razor blade to trim the gel by cutting through the wells and discard the unused gel above the wells. For marking the orientation, make a notch at a corner.
2. Cut the membrane to the size slightly larger than the gel. Make a notch at a corner to align the membrane with gel in the same orientation. Handle the membrane with care – only touching the edges with gloved hands or blunt tip forceps.
3. Cut eight pieces of filter paper the same size of the membrane.
4. Cut a stack of paper towels that are 3 cm in height and 1–2 cm wider than the gel.
5. Pour 20x SSC into a flat bottomed container that has bigger dimension of the agarose gel. This serves as the buffer reservoir and can also be used to wet the paper and membrane. Put the reservoir on a support (i.e. a stack of books) so that its bottom is higher than the paper towel stack.
6. Cut three pieces of filter paper that are large enough to cover the gel and long enough reach over to the reservoir. These papers serve as the bridge to transfer buffer from the reservoir to the gel.

3.3.2 Transfer set up

1. Stack paper towel on a clean bench and put three pieces dry filter paper on top.
2. Wet two more pieces of filter paper and put on top of the dry filter paper. Gently roll out any bubbles between the filter paper layers.
3. Carefully put the membrane on top of the wet filter paper. Gently roll out any bubbles between the membrane and the filter papers.
4. Put the trimmed gel onto the center of the membrane with the bottom of the gel touching the membrane (i.e. the gel plane that faces down during electrophoresis

[8]The voltage is decided by the distance between the two electrodes (not the size of the gel). Usually, the run takes about two hours. If the run is longer than three hours, exchange the buffer at the two end chambers to avoid the pH gradient.

[9]RNA gels that stained with Ethidium bromide are not suitable for Northern blot analysis. Therefore, if a visual examination or photograph of total RNA samples is needed as a reference for the northern blot, we suggest the researchers to either run the same set of samples on a separate gel or stain with Ethidium bromide the gel just for visualization; or de-stain the gel before continuing northern blot analysis. If a gel will be subjected to Northern analysis after UV visualization, avoid prolonged exposure of the gel to UV light.

will be in contact with the membrane), align the notches of the gel and membrane. Roll out bubbles between the membrane and the gel.

5. Place three more pieces pre-wet filter paper on top of the gel and roll out bubbles between filter paper layers.
6. Wet the three pieces of paper bridge and place them with one end on top of the stack and the other end in buffer reservoir. Make sure there is no bubble between any layers of paper (see Note 10).
7. Place a 150 – 200 g object with the size similar to the gel on top of the stack.
8. Transfer the gel for 15–20 min per mm of gel thickness. It usually takes about two hours (see Note 11).

3.3.3—RNA crosslink: disassemble the transfer stack carefully and rinse the member with 1x MOPS gel running buffer to remove residual agarose. Blot off excessive liquid and immediately subject the membrane to crosslink treatment. Cross linking the RNA to the membrane with Stratalinker® UV Crosslinker using the autocrosslink setting (see Note 12). Air-dry the membrane at room temperature. At this point, the membrane can be subjected to hybridization immediately or stored in a sealed bag between two pieces filter paper at 4°C for several month before hybridization.

3.4 Hybridization of DIG-labeled probes to the membrane (see Note 13)

3.4.1 Prehybridization

1. Reconstitute the DIG easy Hyb Granules: add 64 mL RNase-free water into one bottle of the DIG easy Hyb Granules, stir for 5 min at 37°C to complete dissolve the granules. DIG easy Hyb buffer will be used in prehybridization and hybridization. The reconstituted DIG easy Hyb buffer is stable at room temperature for up to one month.
2. For every 100 cm^2 membrane, 10–15 mL Hyb buffer should be used for prehybridization. Measure the appropriate amount of Hyb buffer for prehybridization and place it in a clean tube and pre-warm it in a 68°C water bath (see Note 14).
3. Put the membrane in a hybridization bag, add the pre-warmed Hyb buffer from the previous step, seal the bag properly and incubate the membrane in Hyb buffer at 68°C for at least 30 min with gentle agitation (see Note 15). Prehybridization can be up to several hours as far as the membrane remains wet.

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[10]It is essential to ensure that the only way for the transfer buffer to run from reservoir to the dry paper stack is through the gel. Therefore, extra care is needed to assemble the stack properly to avoid shortcut. The most common shortcut happens between the bridge and the paper beneath the gel. One can cover the edges of the gel with Parafilm to prevent this from happening.

[11]Transfer longer than four hours may cause small RNA hydrolysis and reduce yield.

[12]The autocrosslink Mode of Stratalinker® UV Crosslinker delivers a preset exposure of 1200 microjoules to the membrane and takes about 40 seconds. Other methods of crosslinking RNA to membrane are available and can be used at this step as well.

[13]Once the membrane is wet during prehybridization, it is important to avoid it getting dry during the hybridization and detection process. Dried membrane will have high background. Only if the membrane will not be stripped and reprobed, it can be dried after the last high stringency wash and stored at 4°C for future analysis.

[14]For most northern blot hybridization using DIG Easy Hyb buffer, 68°C is appropriate for both prehybridization and hybridization. In cases of more heterologous RNA probes being used, the prehybridization and hybridization temperature need to be optimized.

3.4.2 Hybridization

1. For every 100 cm^2 membrane, 3.5 mL Hyb buffer is needed for hybridization. Measure the appropriate amount of Hyb buffer for hybridization and place it in a clean tube and pre-warm it in a 68°C water bath (see Note 14).
2. Determine the amount of RNA probe needed (see Note 16) and place it into a microcentrifuge tube with 50 uL RNase-free water. Denature the probe by heating the tube at 85°C for 5 min and chill on ice immediately.
3. Mix the denatured probe with pre-warmed Hyb buffer by inversion.
4. Remove prehybridization buffer from the membrane and immediately replace with the pre-warmed hybridization buffer containing the probe.
5. Seal the bag properly and incubate the membrane in probe-containing Hyb buffer at 68°C overnight with gentle agitation (see Note 15).
6. The next day, pre-warm the High Stringency Buffer to 68°C and pour Low Stringency Buffer in an RNase-free container at room temperature and make sure it is enough to cover the membrane.
7. Cut open the hybridization bag, remove the Hyb buffer, and immediately submerge the membrane in the Low Stringency Buffer.
8. Wash the membrane twice in Low Stringency Buffer at room temperature for 5 min each time with shaking.
9. Wash the membrane twice in High Stringency Buffer at 68°C for 5 min each time with shaking (see Note 17).

3.5 Detection of DIG-probe/target RNA hybrids

3.5.1 Localizing the probe-target hybrid with anti-DIG antibody

1. Transfer the membrane from the last wash in High stringency buffer to a plastic container with 100 mL Washing buffer. Incubate for 2 min at room temperature and discard the Washing buffer.
2. Add 100 mL Blocking buffer onto the membrane and incubate for more than 30 min (up to 3 h) with shaking at room temperature.
3. Dilute anti-DIG-alkaline phosphatase antibody at the ratio of 1:5000 in Blocking buffer and incubate the membrane in 20 mL diluted antibody for 30 min at room temperature with shaking.
4. Wash membrane twice with 100 mL of Washing buffer for 15 min each time at room temperature.

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[15]Prehybridization/hybridization can be performed in containers other than bags, as far as it can be tightly sealed. Sealing the hybridization container can prevent the release of NH4, which changes the pH of the buffer, during incubation.
[16]For RNA probe synthesized by in vitro transcription, it is recommended that the probe concentration should be 100 ng per mL Hyb buffer.
[17]If the probe is less than 80% homologous to the target RNA, the high stringency wash should be performed at a lower temperature, which needs to be empirically determined.

3.5.2 Visualizing probe-target hybrids using chromogenic or chemiluminescent method (see Note 18)

1. Equilibrate the membrane in 20 mL Detection buffer for 3 min at room temperature. If using the chromogenic method, prepare the color substrate solution while equilibrating the membrane.
2. For chromogenic detection:
   a. Put the membrane with the RNA side facing up in a container and incubate in 10 mL color substrate solution in the dark without shaking.
   b. When the desired intensity for the band is observed, discard the color substrate solution and rinse the membrane in 50 mL of TE buffer for 5 min (see Note 19).
   c. Document the result by photographing the membrane (see Note 20).
3. For chemiluminescent detection:
   a. Put the membrane with the RNA side facing up on a plastic sheet (i.e. cut out of a hybridization bag) and add 20 drops of CDP-Star, Ready-to-Use reagent.
   b. Immediately cover the membrane with another sheet to evenly distribute the reagent without creating any bubbles.
   c. Incubate for 5 min at room temperature.
   d. Squeeze out excess reagent and seal the bag.
   e. Develop the membrane with an X-ray film in a dark room (see Note 20).

Acknowledgments

This work was supported, in whole or in part, by the Basser Research Center for BRCA, the NIH (R01CA142776, R01CA190415, P50CA083638, P50CA174523), the Ovarian Cancer Research Fund (XH), the Breast Cancer Alliance, Foundation for Women's Cancer (XH), and the Marsha Rivkin Center for Ovarian Cancer Research.

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[18]The DIG probe-target RNA hybrids can be detected in two ways. One uses chemiluminescent method, whereas the other uses chromogenic method. The chemiluminescent method is sensitive and fast, but it requires the usage of the films and the accessibility of a darkroom. The chromogenic method requires no film or dark room and different targets can be detected simultaneously using different colored substrate. However, the chromogenic method may not be sensitive enough for low-abundant targets.
[19]At this step, if there are multiple membranes, process one at a time. Depending on the abundance of target RNAs, the band may appear as quickly as a few minutes after adding the chromogenic agents. The reaction can be stopped when the band reaches a desired intensity.
[20]If reprobing is needed, photograph the result while the membrane is wet and proceed to stripping and reprobing. If no reprobing is needed, dry the membrane, document the result by photograph and store the dried membrane in a clean bag at room temperature.

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