Dec 21, 2020

Northern Blot Protocol

Sibylle Mitschka[1], Christine Mayr[1]

[1]Cancer Biology and Genetics Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

1 Works for me dx.doi.org/10.17504/protocols.io.bqqymvxw

Sibylle Mitschka

ABSTRACT

Northern blot is a hybridization technique to visualize and quantify RNAs from cells or tissues using a radio-labeled probe. mRNAs only constitute a small fraction of total RNA in cells. Through enrichment of poly(A)-tailed mRNAs, this protocol provides increased sensitivity for the detection of lowly expressed mRNAs. In addition, RNA denaturation is achieved using glyoxal (in contrast to formaldehyde-based methods), resulting in sharper bands. We have used this protocol extensively to detect and quantify the expression of different 3'UTR isoforms originating from the same gene [1-5].

[1] Mayr, C. and D. P. Bartel (2009). Widespread shortening of 3'UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells. Cell 138(4): 673-684.

[2] Lianoglou, S., V. Garg, J. L. Yang, C. S. Leslie and C. Mayr (2013). Ubiquitously transcribed genes use alternative polyadenylation to achieve tissue-specific expression. Genes Dev 27(21): 2380-2396.

[3] Berkovits, B. D. and C. Mayr (2015). Alternative 3'UTRs act as scaffolds to regulate membrane protein localization. Nature 522(7556): 363-367.

[4] Lee, S. H. and C. Mayr (2019). "Gain of Additional BIRC3 Protein Functions through 3'-UTR-Mediated Protein Complex Formation. Mol Cell 74(4): 701-712 e709.

[5] Mitschka, S. and C. Mayr (2020). Endogenous p53 expression in human and mouse is not regulated by its 3'UTR. bioRxiv: 2020.2011.2023.394197.

DOI

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

PROTOCOL CITATION

Sibylle Mitschka, Christine Mayr 2020. Northern Blot Protocol. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bqqymvxw

CREATED

Dec 14, 2020

LAST MODIFIED

Dec 21, 2020

PROTOCOL INTEGER ID

45560

MATERIALS TEXT

Reagents

- TRIzol Reagent (Thermo Fisher, 15596206)
- Chloroform, molecular biology grade
- Isopropanol, molecular biology grade
- Absolute ethanol, molecular biology grade
- Oligotex mRNA Mini Kit (Qiagen, 70022)
- 3 M sodium acetate solution, pH 5.2, RNase-free
- GlycoBlue Coprecipitant 15 mg/ml (Thermo Fisher/Ambion, AM9515)
- RiboRuler High Range RNA ladder (Thermo Fisher, SM1821)
- SeaKem GTG Agarose (VWR/Lonza, 12001-894)
- TAE buffer, RNase-free
- 20 mM Tris buffer pH 8, RNase-free
- 20x SSC buffer, RNase-free (3 M sodium chloride, 0.3 M sodium citrate, pH 7)
- ULTRAhyb Ultrasensitive hybridization solution, (Thermo Fisher/Ambion, AM8670)
- Gene-specific primer pairs for generating the DNA probes [1]
- QIAquick Gel Extraction Kit (Qiagen, 28706)
- *Taq* DNA polymerase and buffer (NEB, M0273)
- Deoxynucleotide solution set (NEB, 0446S)
- *optional:* Prime-a-Gene Labeling System (Promega, U1100)
- [α-^{32}P] dATP, 250 microCi (Perkin Elmer)
- 0.5 M EDTA stock solution, pH 8.0
- 10% SDS solution
- see additional reagents in the buffers section

[1] Design a primer pair to generate probes against the mRNA targets and blast the product for specificity. Each probe should be between 400-700 nucleotides long. Be aware of alternative splice and 3'UTR variants of the target mRNA. For probe production by PCR, use a cDNA library from cells expressing the gene of interest (step 53).

Equipment

- Tabletop centrifuge
- RNase AWAY Surface Decontaminant (Thermo Fisher, 7000TS1)
- NanoDrop spectrophotometer
- Heat block
- PCR thermocycler
- Small Spin columns (Qiagen, 79523)
- Water bath
- Gel electrophoresis equipment (e.g. Bio-Rad, Wide Mini Sub-Cell GT Cell) including thin gel comb (0.75 mm, Bio-Rad, 17044447) and power supply
- GelDoc or similar instrument for gel visualization
- Nytran SuPerCharge TurboBlotter Kit 110 x 140mm (GE Healthcare/Whatman, 10416304)
- UV Crosslinker (e.g. Stratagene, Stratalinker)
- Hybridization oven with and glass hybridization tubes
- Two rectangular Pyrex dishes
- Orbital shaker
- Plexiglas shield
- Geiger counter
- Long metal forceps
- Storage phosphor screens
- X-ray cassettes
- FLA7000IP Phosphorimager and image eraser device

Buffer recipes:

Citation: Sibylle Mitschka, Christine Mayr (12/21/2020). Northern Blot Protocol. https://dx.doi.org/10.17504/protocols.io.bqqymvxw

10x BPTE Electrophoresis Buffer

- 100 mM PIPES
- 300 mM Bis-Tris
- 10 mM EDTA

The final pH of this 10x buffer is approximately 6.5. The 10x buffer can be made by adding , 6 g of Bis-Tris (free base), and 2 ml of 0.5 M EDTA to 90 ml of distilled water. Treat the solution with DEPC (final concentration 0.1%) for 1 hour at 37°C, and then autoclave.

Glyoxal mix (deionized)

- 6 ml DMSO
- 2 ml deionized glyoxal (see description below)
- 1.2 ml 10x BPTE electrophoresis buffer (see recipe above)
- 0.6 ml 80% glycerol in water
- 0.2 ml ethidium bromide (10 mg/ml in water)

To de-ionize the glyoxal:

1. Mix the glyoxal with an equal volume of mixed bed ion-exchange resin (Bio-Rad AG-510-X8). Alternatively, pass the glyoxal through a small column of mixed bed resin and then proceed to step 3.
2. Separate the de-ionized material from the resin by filtration (e.g. through a Uniflow Plus filter, Schleicher & Schuell).
3. Monitor the pH of the glyoxal by mixing 200 µl of glyoxal with 2 µl of a 10 mg/ml solution of bromocresol green in H_2O, and observing the change in color. Bromocresol green is yellow at pH <4.8 and blue-green at pH >5.2.
4. Repeat the de-ionization process (Steps 1-2) until the pH of the glyoxal is >5.5.

Divide into small aliquots and store at -80°C. Use each aliquot only once and then discard.

10x TAE Recipe

- 48.5 g tris
- 11.4 mL glacial acetic acid
- 20 mL 0.5M EDTA (pH 8.0)
- Fill with sterile water to 1 L

RNA Gel-loading dye

- 95% de-ionized formamide
- 0.025% (w/v) bromophenol blue
- 0.025% (w/v) xylene cyanol FF
- 5 mM EDTA (pH 8.0)
- 0.025% (w/v) SDS

Wash Buffer 1

- 100 mL 20X SSC,
- 10 mL 10% SDS (Sterile)
- Fill with sterile water to 1 L

Wash Buffer 2

- 5 mL 20X SSC,
- 10 mL 10% SDS (Sterile)
- Fill with sterile water to 1 L

SAFETY WARNINGS

This protocol involves the use of radioactive materials. Radioactivity should always be handled with care and

Citation: Sibylle Mitschka, Christine Mayr (12/21/2020). Northern Blot Protocol. https://dx.doi.org/10.17504/protocols.io.bqqymvxw

exposure should be minimized as much as possible. For handling and disposal of radioactive material follow federal, state and local regulations. Wear protective clothing when handling radioactive samples and monitor exposure with a Geiger counter. Whenever possible, work behind a plexiglass shield.

ABSTRACT

Northern blot is a hybridization technique to visualize and quantify RNAs from cells or tissues using a radio-labeled probe. mRNAs only constitute a small fraction of total RNA in cells. Through enrichment of poly(A)-tailed mRNAs, this protocol provides increased sensitivity for the detection of lowly expressed mRNAs. In addition, RNA denaturation is achieved using glyoxal (in contrast to formaldehyde-based methods), resulting in sharper bands. We have used this protocol extensively to detect and quantify the expression of different 3'UTR isoforms originating from the same gene [1-5].

[1] Mayr, C. and D. P. Bartel (2009). Widespread shortening of 3'UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells. Cell 138(4): 673-684.

[2] Lianoglou, S., V. Garg, J. L. Yang, C. S. Leslie and C. Mayr (2013). Ubiquitously transcribed genes use alternative polyadenylation to achieve tissue-specific expression. Genes Dev 27(21): 2380-2396.

[3] Berkovits, B. D. and C. Mayr (2015). Alternative 3'UTRs act as scaffolds to regulate membrane protein localization. Nature 522(7556): 363-367.

[4] Lee, S. H. and C. Mayr (2019). "Gain of Additional BIRC3 Protein Functions through 3'-UTR-Mediated Protein Complex Formation. Mol Cell 74(4): 701-712 e709.

[5] Mitschka, S. and C. Mayr (2020). Endogenous p53 expression in human and mouse is not regulated by its 3'UTR. bioRxiv: 2020.2011.2023.394197.

Total RNA extraction

1

This protocol requires samples that have previously been homogenized in TRIzol. Samples can be stored at -80° C for several months.
High quality RNA is paramount for the success of this procedure. To avoid RNase contamination, only use filter tips when handling RNA-containing samples and clean equipment with water and surface decontaminant.

2 Thaw TRIzol samples at RT.

3 Add 200 µl chloroform per sample and mix vigorously for 15 sec. Then allow to sit for 3 min at RT.

4 Centrifuge 20,000 g/20 min/RT.

Citation: Sibylle Mitschka, Christine Mayr (12/21/2020). Northern Blot Protocol. https://dx.doi.org/10.17504/protocols.io.bqqymvxw

5 Transfer clear upper phase to a new tube and add 500 µl isopropanol. Vortex briefly.

6 To facilitate RNA precipitation, incubate samples for 2 h to overnight at 80 °C.

7 Centrifuge 20,000 g/20 min/4 °C.

8 Pour off the supernatant and add 1 ml 70% ethanol to each sample.

9 Centrifuge 20,000 g/10 min/4 °C.

10 Pour off the supernatant and carefully remove remaining liquid with a small pipet tip. Let the RNA pellet air-dry for 5 min.

11 When RNA pellet appears translucent, add between 10 µl and 60 µl RNase-free water, depending on the size of the pellet. Incubate for 30 min on ice and resuspend the RNA by repeated pipetting.

12 Make a 1:10 dilution of the RNA solution (e.g 0.5 µl RNA plus 4.5 µl water) and measure the RNA concentration using a NanoDrop spectrophotometer. The RNA solution can be stored at -80 °C but repeated freeze-thaw cycles should be avoided.

Enrichment of poly(A) mRNAs with Oligotex beads 2h 23m

13 *Before starting, set a heat block to 70 °C and the water bath to 25 °C.*
Tubes containing the required amount of buffer OEB are pre-warmed in the 70 °C heat block.
Poly(A) mRNA enrichment using Oligotex beads should be performed in batches of not more than 8 samples.

14 Transfer up to 180 µg of total RNA into a new tube and adjust volume to 250 µl with water.

15 Vortex Oligotex bead suspension (Qiagen kit) to homogenize the solution and add 15 µl to each tube.

16 Add 250 µl buffer OBB (Qiagen kit) and mix.

17 Incubate samples in a 70 °C heat block for 5 min.

Citation: Sibylle Mitschka, Christine Mayr (12/21/2020). Northern Blot Protocol. https://dx.doi.org/10.17504/protocols.io.bqqymvxw

18 Then place samples in the 25 °C water bath for 15 min.

19 Centrifuge 20,000 g/2 min/RT.

20 Pipet off the supernatant and resuspend beads in 400 µl OW2 (Qiagen kit).

21 Place a spin column into a 1.5 ml tube and add the Oligotex bead mixture. Centrifuge 20,000 g/1 min/RT.

22 Transfer the spin column to a new tube (the flow-through is discarded) and add another 400 µl OW2. Centrifuge 20,000 g/1 min/RT .

23 Transfer column to a new labeled tube and add 100 µl pre-warmed buffer OEB (Qiagen kit). Resuspend beads by pipetting and place samples in a 70 °C heat block for 1 min.

24 Centrifuge 20,000 g/1 min/RT .

25 Repeat **steps 21-22** to collect another 100 µl of eluate in the tube (total volume 200 µl).

26 Add 20 µl 3 M sodium acetate solution, 1 µl GlycoBlue and 550 µl 100 % Ethanol. Mix and incubate samples for 2 h to overnight at -20 °C.

27 Pour off the supernatant and add 1 mL 70% ethanol to each sample.

28 Centrifuge 20,000 g/10 min/4 °C.

29 Pour off the supernatant and carefully remove remaining liquid with a small pipet tip. Let the RNA pellet air-dry for 3 min.

30 When RNA pellet appears translucent, add 3 µl RNase-free water. Resuspend by incubating on ice and repeated pipetting.

Citation: Sibylle Mitschka, Christine Mayr (12/21/2020). Northern Blot Protocol. https://dx.doi.org/10.17504/protocols.io.bqqymvxw

31 Make a 1:10 dilution of the RNA solution (e.g. 0.3 µl RNA plus 2.7 µl water) and measure its concentration using a NanoDrop spectrophotometer. It is best to use the mRNA immediately but it can be stored at -80 °C.

Final sample preparation and running the gel 1h 2m

32

> *Make required buffers and reagents ahead of time. Thaw the glyoxal mix and RiboRuler on ice. The glyoxal mix should be handled with care because it contains ethidium bromide.*
> *Set a heat block to 55 °C. Clean all equipment with water and RNase Away surface decontaminant.*

33 For each sample, transfer an equal amount of mRNA to a new tube (e.g. 2 µg) and adjust volume to 3 µl with water. Keep samples on ice. Prepare an additional tube for the marker RNA containing 3 µl RiboRuler and process it in parallel with the other samples.

34 Add 10 µl glyoxal mix to each tube and incubate samples in a 55 °C heat block for 1 h.

35 In the meantime, prepare a 1% agarose gel by mixing 1 g GTG Seakam Agarose with 100 mL 1x TAE buffer (RNase-free). Bring the agarose to boil in a microwave (about 2 min). Let the melted agarose cool a bit and pour the mixture into a clean agarose gel tray. Make sure that the surface is very even and add a thin gel comb Let the gel solidify at RT.

36 After one hour, briefly spin down RNA samples and add 2 µl RNA-loading dye to each tube.

37 Load entire sample volume of 15 µl into wells of prepared agarose gel.

38 Run the agarose gel in 1x BPTE buffer at 4 °C for 1.5 h at 120 V. The darker dye front should have migrated approximately 8 cm (3.1 in) from the well.

RNA blotting 1h 2m

39 After the run is complete, take a picture of the gel using the GelDoc imaging system. In intact RNA samples, the ribosomal RNA bands should be sharply defined with 28S/18S rRNA intensity ratios of about 2:1.

40 In a clean Pyrex dish, soak the gel, membrane and some of the filter papers in 20x SSC for 5 min.

41 Assemble the blot according to the manufacturer's instructions. Be sure to avoid any air bubbles between membrane and gel. Fill the outer reservoir of the chamber with 20x SSC and transfer overnight. Do not put any weights on the blot

Citation: Sibylle Mitschka, Christine Mayr (12/21/2020). Northern Blot Protocol. https://dx.doi.org/10.17504/protocols.io.bqqymvxw

stack.

42 The next morning, record another picture of the agarose gel. There should be a substantial and even loss of RNA signal in the gel relative to the pre-blot picture from **step 39**.

43 Put the membrane into a Pyrex dish filled with 200 mL 2x SSC and shake it on an orbital shaker for 5 min.

44 Transfer the membrane into a new Pyrex dish containing 200 mL 20 mM Tris pH 8.0 and incubate for 25 min. This step reverses the glyoxal cross-linking reaction.

45 Dry the membrane on a piece of filter paper.

46 Crosslink the RNA to the membrane by exposing it to 1,000 J in a UV crosslinker.

47 Afterwards, place blot in the hybridization oven at 80 °C for 30 min.

48 Mark the gel orientation on the membrane with a pencil. The blot can be cut using scissors to enable simultaneous staining with different DNA probes.

Pre-hybridization and probe preparation 1h 2m

49

Set the hybridization oven to 42 °C and pre-warm the ULTRA-Hyb buffer in the oven for an hour or until the entire contents of the bottle are liquid and warm.

50 Place blot in a glass cylinder with the top side facing inside and add 17 mL of the pre-warmed ULTRA-Hyb buffer to each hybridization tube. Close the hybridization tube securely and make sure that the gasket is inside the cap. Roll the closed tube with the buffer inside until the blot sticks to the inside of the tube. Avoid air bubbles between glass and membrane. Use long forceps to adjust the membrane if necessary.

51 Place the hybridization tube into the 42 °C oven and rotate on a low speed setting. Pre-hybridize the membrane for at least 1 h.

DNA Probe labeling 2h 10m

52

Citation: Sibylle Mitschka, Christine Mayr (12/21/2020). Northern Blot Protocol. https://dx.doi.org/10.17504/protocols.io.bqqymvxw

Purify the product and generate a single-stranded radio-labeled probe in a single primer PCR reaction (step 54). Alternatively, the second step of probe labeling can be carried out using a commercial kit (Prime-A-Gene Labeling System from Promega, U1100).

53 Amplify the target region from cDNA with gene-specific primers using standard PCR procedures. Run the whole reaction on an agarose gel and perform gel extraction using the QIAquick Gel Extraction kit according to the manufacturer's instructions. Measure the concentration of the template DNA using the NanoDrop.

5m

54 Set up the labeling reaction (single primer PCR) behind a plexiglass shield:
- 2 µl 10x *Taq* buffer
- 2 µl 10x dNTP mix without dATP (i.e. dTTP, dCTP, dGTP, 2 mM each)
- 1 µl reverse primer (10 µM)
- 30 ng purified DNA template from **step 53**
- 5 µl [α-^{32}P] dATP
- 1 µl *Taq* DNA polymerase
- add sterile water to a total volume of 20 µl

55 Run the following program in a PCR thermocycler:
1. Initial denaturation 95 °C for 2 min
2. denature at 95 °C for 30 sec
3. Anneal at 45 °C for 2 min
4. Anneal at 72°C for 30 sec
5. Repeat steps 2-4 35 times

56 Add 5 µl of 0.2 M EDTA to the PCR reaction (the total volume is now 25 µl) and heat to 95 °C for 5 min. Afterwards, place the probe immediately on ice.

57 Add the entire probe volume to the hybridization tube containing the blot membrane.
Continue to incubate the blot rotating at 42 °C overnight.

Washing the blots and exposure 1h 30m

58

This protocol describes the use of the phosphorimaging system for signal detection, as it provides a greater dynamic range and increased sensitivity. However, X-ray autoradiographic films can be used as an alternative (step 64).

59 The next day, pour off the UltraHyb + probe into a falcon tube (if you are going to re-use the probe), or into the liquid radioactive waste container. If you are saving the buffer/probe in a falcon tube, transfer it carefully and store the falcon tube in a radiation-resistent plexiglass box at 4 °C. Use within a week.

60 Add 30 mL of Wash Buffer 1 to the hybridization tube and wash for 10 min in the hybridization oven, rotating at fairly high speed.

Citation: Sibylle Mitschka, Christine Mayr (12/21/2020). Northern Blot Protocol. https://dx.doi.org/10.17504/protocols.io.bqqymvxw

61 Pour off Wash Buffer 1 and repeat **step 60**.

62 Pour off Wash Buffer 1 and add 30 mL Wash Buffer 2. Wash in Wash Buffer 2 for 20 min.

63 Pour off Wash Buffer 2. Use forceps to take the blots out of the tubes and place them into a clean plastic sleeve for exposure.

64 For a first estimate of the signal, expose the blot for 1 h in a cassette (put down blots in their sleeves, then put a storage phosphor screen over the blot and close cassette, noting the date and time). After an hour, take a picture of the blot in the Phosphorimager and re-expose for the desired time (up to several days).

Stripping of blot membranes 2m 50s

65

Blot membranes can be stripped for re-probing several times with the protocol described below. It is strongly suggested to begin by probing the blot against the mRNA with the lowest expression, as each stripping reduces the signal strength. Furthermore, very strong hybridization signal cannot be completely removed by stripping. This can cause specific "shadow" band to appear in subsequent exposures. Always note the bands of all prior hybridizations.
With the decay of the radioactivity, the probe signal is also disappearing naturally over time (phosphor 32 has a half-life of about 14 days). If stored at -20 °C, the membrane can be re-probed over several months.

66 Warm 250 mL 0.5% SDS (50 ml 10% SDS in 1 L water) to a boil in a microwave. For 250 mL, this takes about 2:30 minutes. Use a large glass beaker as the solution has very low surface tension leading to overflow when boiling.

67 Allow the 0.5% SDS solution to cool for 20 sec until all the bubbles have settled.

68 Put the blot into a Pyrex dish on the shaker and carefully add on the 0.5% SDS (not directly on the blot).

69 Shake for at least 20 minutes at RT.

70 Pour off the 0.5% SDS.

Citation: Sibylle Mitschka, Christine Mayr (12/21/2020). Northern Blot Protocol. https://dx.doi.org/10.17504/protocols.io.bqqymvxw

71 Repeat steps 1-5 for at least another 2 times.
More washes remove more of the radio-labeled probe but also diminish the remaining signal on the blot.

72 Remove the blot from the dish and let it dry for 15 min on dry Whatman paper. Store the blot in a plastic sleeves or prepare for hybridization. Alternatively, expose the blot for 1 hour to see how well the stripping worked.

Citation: Sibylle Mitschka, Christine Mayr (12/21/2020). Northern Blot Protocol. https://dx.doi.org/10.17504/protocols.io.bqqymvxw