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# Northern blot

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## 5. Purpose

To measure the size and amount of RNA transcribe from a specific gene of interest.

## 6. Theory

Northern blot first uses denaturing gel to separate RNA according to the size. The RNA is then transferred to a nylon membrane while keeping the same distribution in the gel. After fixing the RNA to the membrane, labeled probe complementary to the gene of interest is then added to hybridize to the immobilized RNA. The nonspecifically bound probes are then washed away. The solid membrane with probe specifically bound to RNA of interest is then dried, exposed and analyzed. Since northern blot uses size-dependent separation, this technique can not only determine the abundance but also the sizes of transcript of interest. It can be a very effective way to detect transcript variants of genes. However, if the amount of total RNA for the experiment is limited and expression level of transcript of interest is low, other techniques more sensitive than northern blot, such as quantitative RT-PCR, can be used.

## 7. Equipment

Agarose gel rig

Power supply

Microwave

Centrifuge

PCR machine

Heating block

Vacuum gel transfer system

Nylon membrane

UV crosslinker

Hybridization oven

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Hybridization bottles

G-50 sephadex spincolumn

Scintillation vial

Scintillation counter

Forceps

Pipetor tips

1.5 ml polypropylene tubes

Phosphor screen

Phosphor screen scanning equipment

ImageQuant software (Molecular Dynamics)

## 8. Materials

'Editor's note': The Materials section comprises two parts. The first part is a comprehensive list of chemicals and reagents needed for the experiment.

Example:

Agarose

MOPS

Sodium acetate

Ethylenediaminetetraacetic acid disodium salt dihydrate

NaOH

HCl

Formaldehyde

Glycerol

Ethidium bromide

Bromophenol blue

Xylene cyanol

Orange G

Formamide

Millennium RNA ladder (Ambion)

$MgCl_2$

NTPs: ATP, CTP, GTP, UTP

[$\alpha$-$P^{32}$]-UTP

Salmon sperm DNA

NaCl

Sodium Citrate

Ficoll 400

Polyvinylpyrrolidone

Bovine Serum Albumin

SDS

Sodium heparin

$NaH_2PO_4$

$Na_2HPO_4$

Tris-HCl (pH 8.0)

DTT

Triton X-100

Spermidine, pH7.0

Taq buffer

Enzymes: T7 polymerase, Taq polymerase

## Solutions & buffers

### Step 1

*10* ×MOPS buffer (Store at RT, protected from light)

| Component | Final concentration | Stock | Amount/liter |
|---|---|---|---|
| MOPS | 0.2 M | - | 41.852 g |
| Sodium acetate •$3H_2O$ | 80 mM | - | 10.89 g |
| EDTA | 10 mM | - | 0.372 g |

Adjust pH to 7.0 with NaOH. Add water to 1 liter

Denaturing RNA gel

| Component | Final concentration | Stock | Amount/150 ml |
|---|---|---|---|
| MOPS buffer | 1 × | 10 × | 15 ml |
| Agarose | 1.2% (w/v) | - | 1.8 g |
| $H_2O$ | | | 132ml |

Microwave 3~4 min to dissolve agarose; cool to be able to hold; add formaldehyde 2.8 ml.



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Running buffer

| Component | Final concentration | Stock | Amount/liter |
|---|---|---|---|
| MOPS buffer | 1× | 10 × | 100 ml |
| formaldehyde | 7% | 37% | 19 |

Add water to 1 liter

2× RNA loading buffer

| Component | Final concentration | Stock | Amount/10ml |
|---|---|---|---|
| MOPS buffer | 1× | 10 × | 1 ml |
| glycerol | 20% | 100% | 2 ml |
| formaldehyde | 6.5% | 37% | 1.76 ml |
| Formamide | 50% | 100% | 5 ml |
| Ethidium Bromide | 10 μg/ml | - | 100 μg |
| Bromophenol blue | 0.05% (w/v) | - | 5 mg |
| Xylene cyanol | 0.05% (w/v) | - | 5 mg |

Add water to 10 ml

## Step 2

20× SSC buffer

| Component | Final concentration | Stock | Amount/liter |
|---|---|---|---|
| NaCl | 3 M | - | 175.3 g |
| Sodium Citrate | 300 mM | - | 88.2 g |

Adjust pH to 7.0 with HCl, Add water to 1 L.

10× SSC buffer

500 ml 20× SSC buffer, add $H_2O$ to 1 L.

## Step 3

10× Transcription buffer

| Component | Final concentration | Stock | Amount/10ml |
|---|---|---|---|
| Tris-HCl, pH8.0 | 400 mM | 1 M | 4 ml |
| DTT | 50 mM | 1 M | 0.5 ml |
| Triton X-100 | 1% (v/v) | 10% | 1 ml |

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| Component | Final concentration | Stock | Amount/10ml |
|---|---|---|---|
| Spermidine, pH 7.0 | 20 mM | 500 mM | 0.4 ml |
| $MgCl_2$ | 200 mM | 1 M | 2 ml |

Add water to 10 ml

### Sodium phosphate buffer, pH 8.0 (100 mM)

| Component | Stock | Amount/liter |
|---|---|---|
| $NaH_2PO_4$ | 0.2 M | 473.5 ml |
| $Na_2HPO_4$ | 0.2 M | 26.5 ml |

Add water to 1 liter

### Denhardt's Solution 100×

| Component | Final concentration | Stock | Amount/500ml |
|---|---|---|---|
| Ficoll 400 | 0.02 g/ml | - | 10 g |
| Polyvinylpyrrolidone | 0.02 g/ml | - | 10 g |
| Bovine serum albumin | 0.02 g/ml | - | 10 g |

Add water to 500 ml

### Hybridization buffer

| Component | Final concentration | Stock | Amount/250ml |
|---|---|---|---|
| Formamide | 50% | 100% | 125 ml |
| SSC | 3× | 20× | 37.5 ml |
| Denhardt's Solution | 10× | 100× | 25 ml |
| Sodium phosphate buffer, pH 8.0 | 10 mM | 100 mM | 25 ml |
| EDTA | 2 mM | 500 mM | 1 ml |
| SDS | 0.1% | 10% | 2.5 ml |
| Salmon sperm DNA | 200 μg/ml | 5 mg/ml | 10 ml |
| Sodium heparin | 400 U/ml | 5,000 U/ml | 20 ml |

Add water to 500ml.

### Low stringency wash buffer

| Component | Final concentration | Stock | Amount/liter |
|---|---|---|---|
| SSC | 2× | 20× | 100 ml |
| SDS | 0.1% | 10% | 10 ml |

Add water to 1 L.

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High stringency wash buffer

| Component | Final concentration | Stock | Amount/liter |
|---|---|---|---|
| SSC | 0.1× | 20× | 50 ml |
| SDS | 0.1% | 10% | 10 ml |

Add water to 1 L.

## 9. Protocol

### Duration

| Preparation | about 1 day |
|---|---|
| Protocol | about 2 days |

### Preparation

Make all the buffers and autoclave or filter sterilize them.

Generate template by PCR for making probes complementary to sequence of interest. If RNA probe is needed, template used should have a T7 promoter sequence (TAATACGACTCACTATAGGG)

### Caution

RNase-free conditions are important to maintaining the integrity of RNA. Disposable gloves should be worn at all times and changed frequently. All reagents should be autoclaved or filter-sterilized. Consult your institute Radiation Safety Officer for proper ordering, handling, and disposal of radioactive materials.

## Step 1 Separate RNA by a denaturing gel

| | |
|---|---|
| *Overview* | Use denaturing agarose gel with formaldehyde to separate RNA based on the size. |
| *Duration* | 5 hrs |
| 1.1 | Assemble agarose gel rigs and make denaturing RNA gel solution. |
| 1.2 | Pour the RNA gel in hood |
| 1.3 | After gel solidify, equilibrate gel with running buffer for at least 30 min before running. |
| 1.4 | Mix 15 μg RNA sample with equal volume of 2× RNA loading buffer. Dilute 3 μg Millennium RNA Markers in same volume of 2× RNA loading buffer. Incubate @ 65 °C in the heating blocking for 12~15 min and put samples on ice immediately afterwards. |
| 1.5 | Load all the samples to equilibrated gel and leave space between first sample and RNA marker. |
| 1.6 | Run gel at 125 V for about 3 hrs |
| *Tip* | *All the sample volume should not exceed the well volume of the gel comb. If RNA sample is too diluted, salt precipitate the RNA and resuspend with less H2O.* |

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## Step 2 Transfer RNA from gel to nylon membrane

| | |
|---|---|
| *Overview* | RNA is transferred from gel to nylon membrane using vacuum gel transfer system. |
| *Duration* | 2 hrs |
| 2.1 | Cut a nylon membrane about (or bigger than) the size of the denaturing RNA gel. Cut a filter paper with the same size as the nylon membrane. |
| 2.2 | Rinse the RNA gel with $H_2O$. |
| 2.3 | Fill the wells of the RNA gel with melted agarose. |
| 2.4 | Wet the nylon membrane and filter paper first in $H_2O$ and then 10× SSC buffer |
| 2.5 | Put wet filter paper on the vacuum porous stage and make sure the filter paper is in the area where the cut window of the green plastic gasket is going to be. Place the wetted nylon membrane on top of the filter paper. Make sure there is no air bubble between membrane and filter. |
| 2.6 | Wet the seal o-ring on the base unit with $H_2O$. Place the plastic gasket on top of the membrane/filter paper. Make sure the gasket covers the seal o-ring while the membrane/filter paper overlaps with the window of the gasket. |
| 2.7 | Gently place the gel on top of the gasket with the well-side up. Also make sure the gel overlaps with the gasket by at least 5 mm. Remove all the air bubbles between gel and the nylon membrane. |
| 2.8 | Place the sealing frame on top of the vacuum stage and lock it. |
| 2.9 | Start the vacuum source and adjust the pressure to 5 inches of Hg. Press the gel and along the window gently to apply extra pressure to help the vacuum sealing. |
| 2.10 | Gently pour 1 L of 10× SSC buffer into the reservoir. Place the lid on and transfer for 90 mins at 5 inches of Hg. Occasionally check the buffer level to make sure it is above the gel. |
| 2.11 | When the transfer is over, remove the sealing frame and drain the buffer. Remove the gel and take out the nylon membrane. Dry the nylon membrane between two sheets of filter paper. |
| 2.12 | UV crosslink the membrane twice to fix the RNA on the membrane and use a fine marker to mark the edge of the side with RNA. |
| 2.13 | Clean the gel transfer system thoroughly by rinsing with plenty of $H_2O$. |
| *Tip* | *After the gel transfer, the gel area inside the window of green gasket should be half as thick as the gel outside the window.* |
| *Tip* | *Gel can be illuminated with UV to check whether there is any remaining RNA.* |

## Step 3 Hybridization

| | |
|---|---|
| *Overview* | use in vitro T7 transcription to make radioactively labeled RNA probes complementary to RNA transcript of interest. The probes are then hybridized to the membrane. Nonspecifically bound probes are washed away after hybridization. |
| *Duration* | 18hrs |
| 3.1 | Put cross-linked nylon membrane in the hybridization bottle with the RNA-side up. |
| 3.2 | Add 10 ml hybridization buffer (for small hybridization bottle) to the membrane. Incubate at 68 °C for 1hr to pre-hybridize. |
| 3.3 | During pre-hybridization, start the probe transcription reaction. For a 20 μl gene probe transcription reaction, combine 10–50 pmoles DNA template, 2 μl 10× transcription buffer, 1 μl 10mM ATP, 1 μl 10 mM GTP, 1 μl 10 mM CTP, 5 μl 12.5 mM [$\alpha$-$P^{32}$]-UTP and T7 polymerase (20 U/μl final) in a PCR tube. |
| 3.4 | For a 20 μl transcription reaction for Millennium Marker probe, combine 1 μg DNA template, 2 μl 10× transcription buffer, 1 μl 10mM ATP, 1 μl 10 mM GTP, 1 μl 10 mM CTP, 1 μl 10 mM UTP, 2 μl 12.5 mM [$\alpha$-$P^{32}$]-UTP and T7 polymerase (20 U/μl final) in a PCR tube. |
| 3.5 | Incubate at 37 °C for 2 hours. |
| 3.6 | Add 1 μl DNase I and incubate at 37 °C for 15 min to digest the template. |
| 3.7 | Snap off the bottom of micro G-50 sephadex spin column; open the cap by ¼ circle and put the column in a 1.75 ml tube. Spin at 700g for 1min. Put the column in a new 1.75 ml tube. |
| 3.8 | Load transcription reaction carefully on top of the G-50 column. Spin at 700g for 2 min. |

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| | |
|---|---|
| 3.9 | Add 1 μl of G-50 column purified probe to 5 ml scintillation liquid and check the counts of the probes. |
| 3.10 | For gene probe, add $10^6$ cpm/ml to fresh hybridization buffer; for marker probe, add $0.2 \times 10^6$ cpm/ml. |
| 3.11 | Dump the pre-hybridization solution and add hybridization buffer with probes to the hybridization bottle. Hybridize overnight at 68 °C over night. |
| 3.12 | Pre-warm both the low stringency and high stringency buffer at 68 °C. |
| 3.13 | Wash with low stringency for 5min twice. |
| 3.14 | Wash with high stringency for 15min twice. |
| 3.15 | Take out the membrane and brief dry it on kimwipe. Wrap the membrane with saran wrap and expose to a phosphor screen overnight. |
| *Tip* | *If the gene probe is longer than 400 nt, increase the limiting nucleotide (UTP in this case) concentration to 5–25 μM by adding more non-labeled UTP in the transcription reaction.* |

## References

1. Dorner S, Lum L, Kim M, Paro R, Beachy PA, Green R. A genomewide screen for components of the RNAi pathway in Drosophila cultured cells. Proc Natl Acad Sci U S A. 2006; 103:11880–118855. [PubMed: 16882716]

## 11. TOPICS

'Editor's note': Provide as many keywords as possible to this protocol using the table below as a guideline. Order the keywords based on importance. Not all protocols will necessarily have a keyword in each topic class.

| Topic Class | Keyword |
|---|---|
| **Methods**<br>List the methods used to carry out this protocol (i.e., for each step). | 1 Denaturing agarose gel |
| | 2 *in vitro* transcription |
| | 3 |
| | 4 |
| | 5 |
| **Process**<br>List the biological process(es) addressed in this protocol. | 1 RNA-RNA interaction |
| | 2 |
| | 3 |
| | 4 |
| | 5 |
| **Organisms**<br>List the primary organism used in this protocol. List any other applicable organisms. | 1 |
| | 2 |
| | 3 |
| | 4 |
| | 5 |
| **Pathways**<br>List any signaling, regulatory, or metabolic pathways addressed in this protocol. | 1 |
| | 2 |
| | 3 |

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| Topic Class | Keyword |
|---|---|
| | 4 |
| | 5 |
| **Molecule role/function**<br>List any cellular or molecular functions or activities addressed in this protocol. | 1 |
| | 2 |
| | 3 |
| | 4 |
| | 5 |
| **Phenotype**<br>List any developmental or functional phenotypes addressed in this protocol (organismal or cellular level). | 1 |
| | 2 |
| | 3 |
| | 4 |
| | 5 |
| **Anatomy**<br>List any gross anatomical structures, cellular structures, organelles, or macromolecular complexes pertinent to this protocol. | 1 |
| | 2 |
| | 3 |
| | 4 |
| | 5 |
| **Diseases**<br>List any diseases or disease processes addressed in this protocol. | 1 |
| | 2 |
| | 3 |
| | 4 |
| | 5 |
| **Other**<br>List any other miscellaneous keywords that describe this protocol. | 1 |
| | 2 |
| | 3 |
| | 4 |
| | 5 |

## 12. VIDEO

‘Editor’s note’: Indicate whether any protocol steps or sub-steps would be better illustrated by video (please also provide step or sub-step number e.g. Step 2 or sub-step 2.5).

## 13. IMAGES

It is ok to supply a copy of the figures as a reference within the manuscript in MS Word along with the list of legends. BUT LOW-RES IMAGES INSIDE A WORD DOC ARE NOT REPRO-QUALITY. All text and image files must be submitted via the website, http://emss.elsevier.com. Use the website to post individual files of good-quality images. Login credentials to the website and author guidelines will be provided by Elsevier.