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Multiple Choiceä Northern Blots Applications Guide

Version 2.3

MULTIPLE CHOICEä NORTHERN BLOT
Applications Guide

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1. Package Contents and Storage Conditions

The following components are included:

-- One Multiple Choiceä Northern Blot

-- One vial of human β-actin cDNA to be used as a control probe for hybridization. Vial contains 100 ng of cDNA in TE buffer at 10 ng/ul.

Store Multiple Choiceä Northern Blot in sealed plastic bag between 2 sheets of 3M Whatman paper at 4°C. Store vial of β-actin cDNA at -20°C.

2. Introduction

Northern Blot hybridization is a technique utilized to quantify the amount and size of a specific messenger RNA in a tissue or cell line. Total or poly A+ messenger RNA is separated on a denaturing agarose gel by electrophoresis and then transferred to a membrane such as nitrocellulose or nylon. The size and amount of a specific RNA is then determined by hybridizing the RNA on the membrane to a specific labeled DNA probe.

The Multiple Choiceä Northern Blot enclosed is a charged nylon membrane to which total or poly A+ messenger RNA from a variety of tissues has been transferred. Each lane contains approximately 2 ug of poly A+ or 20 ug total RNA. To ensure equal amounts of RNA per lane, Northern Blots are probed with human β-actin cDNA. Equal amounts of signal are detected for each lane. The specific contents of each lane on the Multiple Choiceä Northern Blot are described on the Certificate of Analysis Sheet included with your order.

The Multiple Choiceä Northern Blot is shipped ready to hybridize with specific-labeled DNA probes including the control β-actin cDNA provided. The Blot can be probed and stripped several times if the suggested protocols are followed. Both radioactive and non-radioactive probes can be used on the Northern Blot with successful results.

The Multiple Choiceä Northern Blots are extremely useful in determining the levels of expression for a specific gene in a variety of tissues. High quality results are then achieved without having to obtain and subsequently isolate messenger RNA from various tissues. It is also possible to determine the size of the messenger RNA transcripts detected by hybridization using the standard RNA markers on the Northern Blot (see section 8B).

3. Total RNA vs. mRNA Northern Blots

Multiple Choiceä Northern Blots are made using either Total RNA or mRNA. Total RNA is isolated directly from tissues using traditional guanidine isothiocyanate methods. Total RNA consists of ribosomal RNAs, transfer RNAs and messenger RNAs (mRNA). mRNA can also be referred to as poly A+ RNA because of the addition of the poly A (polyadenylated) tail to the messenger RNA. Usually, mRNA represents only 1-5% of the total RNA population. The majority of RNAs in total RNA are the 28S and 18S ribosomal RNAs. These can be seen as prominent bands by agarose gel electrophoresis.

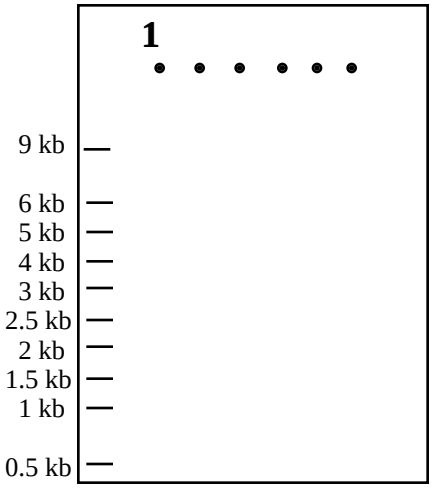
Total RNA Northern Blots are useful for quickly determining where a gene is expressed. In addition, if you had an Expressed Sequence Tag (EST) this would be a useful blot for determining from which tissue library one should attempt to clone the gene. It is important to realize that when screening a Total RNA Northern Blot one must use very stringent wash conditions after hybridization to reduce the possibility of high levels of background. These high levels of background are due to the fact that >95% of the RNA in each lane is not messenger RNA. This increases the signal to noise ratio dramatically. Usually, one can obtain reasonable signals by following the protocols described in this manual.

The production of the mRNA Multiple Choiceä Northern Blots requires that poly A+ RNA is first separated from the remainder of the total RNA, which is largely ribosomal RNA and transfer RNA. Total RNA is denatured to expose the poly A tails. Poly A-containing RNA is then bound to oligo(dT) attached to some type of physical support. The remainder of the RNA which does not bind oligo(dT) can then be washed away. The poly A+ RNA is then eluted off the oligo(dT) and used for Northern Blots.

This procedure results in a 100-500 fold enrichment of mRNA. Therefore, when probing mRNA Northern Blots, the signal to noise ratio is greatly reduced resulting in stronger hybridization signals as compared to Total RNA Blots. Multiple Choiceä Northern Blots made with mRNA can be used for the same purposes as Total RNA Blots, but will yield stronger signals and higher quality looking results. For publication purposes, we highly recommend Multiple Choiceä Northern Blots made with poly A+ RNA. Another reason to use mRNA Northern Blots is if your gene of interest is expressed at low levels. Genes transcribing mRNAs with a low copy number are much harder to detect in the Total RNA population. Therefore, we suggest using a mRNA Northern Blot to enhance detection of signals from low copy number genes.

4. Description of Multiple Choiceä Northern Blot

The Multiple Choiceä Northern Blot is made with either total RNA or poly A+ RNA. This RNA is isolated and electrophoresed in a 1% denaturing formaldehyde agarose gel. RNA in the gel is then transferred to a positively charged nylon membrane and then crosslinked by UV irradiation. Each lane on the blot contains approximately 20 ug of total RNA or 2 ug of poly A+ RNA. A diagram of a typical blot is shown below. The blot is shown with the RNA side facing up. Markers (RNA Milleniumä Markers; Ambion [catalog# 7150])are indicated by lines on the left side of the blot. Sizes of the markers are shown below and on the Certificate of Analysis accompanying each blot. At the top of the blot, dots are used to indicate the position of the lanes. The number "1" above the first dot serves to ensure proper orientation of the blot.



The Certificate of Analysis which accompanies each Multiple Choiceä Northern Blot will contain detailed information about the contents of each lane on the blot.

5. Additional Required Solutions

The following materials are required but not supplied to perform hybridizations with the Multiple Choiceä Northern Blot.

-- Prehybridization / Hybridization Buffer 1
(for use with oligonucleotide probes)
Mix the following:
25 mls of 20% SDS
125 mls 20X SSPE
50 mls 50X Denhardt's solution
5 mls 10 mg / ml denatured, sheared salmon sperm DNA
295 mls DEPC-treated water
500 mls * Store at -20°C.

-- Prehybridization / Hybridization Buffer 2
(for use with DNA probes)
Mix the following:
5 mls 20% SDS
125 mls 20X SSPE
50 mls 50X Denhardt's solution
5 mls 10 mg / ml denatured, sheared salmon sperm DNA
250 mls 100% formamide (freshly deionized)
100 mls 50% Dextran Sulfate
535 mls

* Store at -20°C.

-- Wash Buffer 1
2X SSC
0.1% SDS

* Store at room temperature (22°C).

-- Wash Buffer 2
0.25X SSC
0.1% SDS

* Store at room temperature (22°C).

-- 20X SSPE
3 M NaCl (175.3 g)
0.2 M $NaH_2PO_4 \cdot H_2O$ (27.6 g)
0.02 M Na_2EDTA (40 mls of 0.5 M stock)
Adjust pH to 7.4 with 10 N NaOH
Add H_2O to 1 Liter.

* Store at room temperature (22°C).

-- 20X SSC
3 M NaCl (175.3 g)
0.3 M $Na_3Citrate \cdot 2H_2O$ (88.2 g)
Adjust pH to 7.0 with 1 M HCl.
Add H_2O to 1 Liter.

* Store at room temperature (22°C).

-- 4X SSC
Mix 200 mls of 20X SSC
800 mls of H_2O

*Store at room temperature (22°C).

-- 50X Denhardt's solution
Ficoll (Type 400, Pharmacia) (5.0 g)
Polyvinylpyrrolidone (Sigma) (5.0 g)
Bovine serum albumin (fraction V, Sigma) (5.0 g)
Add H_2O to 500 ml.

* Store at -20°C.

6. Suggested Protocols for Use with Multiple Choice Northern Blots

The following protocols have been designed for optimum use of Multiple Choiceä Northern Blots. High quality results should be obtained by utilizing the procedures described below. Any modification of these protocols however may reduce the quality of your results. If performing a radioactive hybridization, remember to review all guidelines concerning use and disposal of radioactive materials before beginning these procedures.

A. Hybridization with Oligonucleotide Probes

1. Prewarm Prehybridization / Hybridization Buffer 1 (for use with oligonucleotide probes) by placing in a 40°C waterbath for 30 min.

2. Remove Multiple Choiceä Northern Blot from original package with forceps and rehydrate in 50-100 mls of 4X SSC for 10 minutes with shaking. **IMPORTANT:** It is very critical that the blot is reyhdrated before adding to the prehybridization buffer. High background problems can occur if nylon membranes are not rehydrated before use.

3. Place the Multiple Choiceä Northern Blot in a new plastic sealable bag. Add 6 -10 ml of prewarmed Prehybridization / Hybridization Buffer 1 to the bag and seal the ends. Hybridization can also be performed in hybridization bottles in a rotating hybridization oven. **IMPORTANT:** When using hybridization bottles, be sure to place the Northern Blot membrane in the bottle with the RNA side facing up. Placing the membrane in the bottle with the RNA side facing down will potentially result in high backgrounds that are not always easy to remove. This is due to the blot not being properly hydrated and bathed in either the prehybridization or hybridization buffers.

4. Prehybridize the blot for 2 - 4 hours at 42°C in a shaking incubator. *It is important that there be continuous agitation of the solution over the blot for this step as well as all other incubations.*
   NOTE: The desired temperature for prehybridization and subsequent hybridization can be varied between 37° and 42°C using the solutions recommended here to increase detection of signals on the blot.

5. During the prehybridization, prepare a radiolabeled oligonucleotide probe (see protocol in section 7A). Take 5 x 10^6 cpm of the probe / ml of Prehybridization / Hybridization Buffer 1 being used above (6 to 10 ml). Boil the probe for 5 min and add to 6 to 10 mls of fresh Prehybridization / Hybridization Buffer 1. Mix well.

6. Cut open a corner of the bag and remove the prehybridization solution. This is easily done by laying bag on a flat surface and rubbing a 10 ml pipet over the bag.

7. Add the boiled probe in fresh hybridization solution to the bag. Spread the solution over the filter and seal the bag. Seal off any bubbles that may have formed.

8. Hybridize overnight at 42°C (or the same temperature as the prehybridization, if different) with agitation.

9. The next day, cut open the bag and remove the blot. First, wash the blot in Wash Buffer 1 at room temperature three times, each for 5 min shaking continuously. Then proceed to wash the blot in Wash buffer 2 at the hybridization temperature two times, each for 15 min with shaking. **NOTE:** After finishing the washes, examine the blot with a Geiger counter. If the majority of the blot registers around background levels, proceed with exposing the blot to film. If high amounts of radioactivity are detected after the washes, a more stringent wash (i.e. <0.25X SSC or higher temperature) is required.

10. Remove the blot with forceps and place between 2 sheets of plastic wrap. **Important: Do not allow the blot to dry (even partially). If the blot begins to dry, removal of the probe may be difficult.** To mark orientation, use radioactive ink or a luminescent marker.

11. Expose to X-ray film at -70°C with an intensifying screen. Bands are usually visible after one day of exposure. However, this will vary depending on the abundance of the mRNA species and the specific activity of the probe.

B. Hybridization with DNA Probes

1. Prewarm Prehybridization / Hybridization Buffer 2 (for use with DNA probes) by placing in a 40°C waterbath for 30 min.

2. Remove Multiple Choiceä Northern Blot from original package with forceps and rehydrate in 50-100 mls of 4X SSC for 10 minutes with shaking. **IMPORTANT:** It is very critical that the blot is reyhdrated before adding to the prehybridization buffer. High background problems can occur if nylon membranes are not rehydrated before use.

3. Place the Multiple Choiceä Northern Blot in a new plastic sealable bag. Add 6 -10 ml of prewarmed Prehybridization / Hybridization Buffer 2 to the bag and seal the ends. Hybridization can also be performed in hybridization bottles in a rotating hybridization oven. **IMPORTANT:** When using hybridization bottles, be sure to place the Northern Blot membrane in the bottle with the RNA side facing up. Placing the membrane in the bottle with the RNA side facing down will potentially result in high backgrounds that

are not always easy to remove. This is due to the blot not being properly hydrated and bathed in either the prehybridization or hybridization buffers.

4. Prehybridize the blot for 2 - 4 hours at 42°C in a shaking incubator. *It is important that there be continuous agitation of the solution over the blot for this step as well as all other incubations.*

5. During the prehybridization, prepare a radiolabeled DNA probe (see protocol in section 7B). Take 2 x 10^6 cpm of the probe / ml of Prehybridization / Hybridization Buffer 2 being used above (6 to 10 ml). Boil the probe for 5 min and add to 6 to 10 mls of fresh Prehybridization / Hybridization Buffer 2. Mix well.

6. Cut open a corner of the bag and remove the prehybridization solution. This is easily done by laying bag on a flat surface and rubbing a 10 ml pipet over the bag.

7. Add the boiled probe in fresh hybridization solution to the bag. Spread the solution over the filter and seal the bag. Seal off any bubbles that may have formed.

8. Hybridize overnight at 42°C with agitation.

9. The next day, cut open the bag and remove the blot. First, wash the blot in Wash Buffer 1 at room temperature three times, each for 5 min shaking continuously. Then proceed to wash the blot in Wash buffer 2 at 65°C two times, each for 30 min with shaking.
   NOTE: After finishing the washes, examine the blot with a Geiger counter. If the majority of the blot registers around background levels, proceed with exposing the blot to film. If high amounts of radioactivity are detected after the washes, a more stringent wash (i.e. <0.25X SSC or higher temperature) is required.

10. Remove the blot with forceps and place between 2 sheets of plastic wrap.
    Important: Do not allow the blot to dry (even partially). If the blot begins to dry, removal of the probe may be difficult.
    To mark orientation, use radioactive ink or a luminescent marker.

11. Expose to X-ray film at -70°C with an intensifying screen. Bands are usually visible after one day of exposure. However, this will vary depending on the abundance of the mRNA species and the specific activity of the probe.

C. Removing Probes from Northern Blots

After a hybridization is complete with one specific gene probe, it may be desirable to examine the expression of another gene on the same blot. To do this, it is necessary to remove the existing probe from the blot. It is also important to remove the probe from the blot before storage for future use.

1. To strip or remove a probe (oligonucleotide or DNA) place the blot in 250 ml of 1%SDS / 1XTE buffer in a glass pyrex dish.

2. Let blot soak for 2 min at room temperature.

3. Cover dish containing blot with plastic wrap and boil solution for 5 min. This can be done on a heated magnetic stirrer or in a designated radioactive microwave.

4. Remove from heat and let glass dish with blot sit for 5 min to cool.

5. Drain off radioactive liquid and dispose of properly.

6. Transfer blot to a clean glass dish and rinse with 4X SSC for 1 min at room temperature.

7. Remove blot and place between 2 sheets of Whatman paper. Transfer this to a clean plastic sealable bag. Seal ends and store at 4^{o}C.

7. Suggested Protocols for Preparing Probes

A. Oligonucleotide Probes

1. For 5' end labeling of oligonucleotides, make the following reaction mix:

3 ul	10X kinase buffer {500 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$, 50 mM Dithiothreitol, 0.5 mg / ml BSA}
150 uCi	[γ-^{32}P]ATP (>3000 Ci / mmol)
10 pmol	oligonucleotide, dephosphorylated
2 units	T4 polynucleotide kinase

Add water to a final volume of 30 ul.

2. Incubate at 37^{o}C for 60 min.

3. Stop reaction by adding 1 ul of 0.5 M EDTA.

4. Separate unincorporated radioactive nucleotides by centrifugation through a spin column containing Sephadex G-50.

5. Estimate the specific activity of the probe by scintillation counting. This number should be > 10^8 dpm/ug.

6. Store the probe at -20°C for no more than 3 days.

B. DNA Probes

To generate DNA probes, use the following protocol for labeling of DNA by random oligonucleotide primed synthesis.

1. Cleave the DNA of interest with an appropriate restriction endonuclease to create either linear DNA or to release a fragment of DNA to be used as the probe.

2. Purify the DNA fragment of interest by gel electrophoresis. (For detailed protocols see Current Protocols in Molecular Biology, 1995).

3. Set up the following reaction mix on ice:

   2.5 ul 0.5 mM dNTP mix containing 0.5 mM each of dATP, dGTP and dTTP but not dCTP.
   2.5 ul 10X Klenow fragment reaction buffer {0.5 M Tris-HCl (pH 7.5), 0.1 M $MgCl_2$, 10 mM Dithiothreitol, 0.5 mg/ml BSA}
   5 ul 3000 Ci/mmol [α-^{32}P]dCTP (50 uCi)
   1 ul Klenow fragment (3 to 8 units)

4. Combine purified DNA fragment (30 to 100 ng) with random hexanucleotide primers (1 to 5 ug) in a total volume of 14 ul. Heat the DNA mixture for 2 to 3 min at 95-100°C and then place on ice.

5. Add 11 ul of the reaction mix from step 3 to the denatured DNA (final volume is 25 ul).

6. Incubate mixture at 37°C for 30 to 40 min.

7. Stop reaction by adding 1 ul of 0.5 M EDTA, 3 ul of 10 mg/ml tRNA and 100 ul TE buffer.

8. Separate the labeled DNA from the unincorporated radioactive nucleotides by chromatography on a Sephadex G-50 spin column.

9. Use immediately or store probe at -20°C for up to 3 days.

8. Analysis of Hybridization Results

A. General Considerations

Hybridization signals generated by probing the Multiple Choiceä Northern Blot will vary depending on the RNA species of interest. Some RNA transcripts may be specific for one tissue type whereas others may be found in several tissues at various levels. It is important to obtain multiple exposures of the blot to film for each hybridization because the amount of your RNA of interest may be quite low and only visible after a few days of exposure. If no signals are detected even after 3 days, there may be a problem with the probe (see Troubleshooting, section 9). Most hybridization signals will appear within 24 hours of exposure to film. These results will identify in which tissues your RNA of interest is most abundant. The signals detected for each tissue can be compared to each other because we have loaded equal amounts of RNA in each lane of the Northern Blot.

B. Transcript Size

To create the Multiple Choiceä Northern Blot, total or poly A+ RNA was isolated from different tissues and separated by size using formaldehyde agarose gel electrophoresis. Because these denaturing gels reduce secondary RNA structure, it is possible to estimate the size of your RNA of interest detected on the Northern Blot. To do so, simply compare your hybridization signal to the markers on the blot. These markers contain RNA transcripts of discrete sizes which can be used to obtain an approximate size of the RNA(s) you have detected.

C. Controls

If you have hybridized the Multiple Choiceä Northern Blot with a DNA probe and have not been able to detect any visible bands, it is possible that the RNA which you are looking for may not be present in any of the tissues on the blot. However, another possibility is that there is a technical problem with the procedure. To aid you in performing a successful Northern Blot, we have included a vial of human β-actin cDNA ready to be radiolabeled as a control probe. This probe is guaranteed to detect an approximately 1.8 kb RNA transcript in every lane of the Multiple Choiceä Northern Blot at equal amounts. In lanes containing RNA from heart and skeletal muscle, a second band approximately 1.4 kb will also be detected. The actin gene is a member of a multi-gene family and therefore generates multiple bands on a Northern Blot. This signal is very strong and should be visualized in several hours of exposure to X-ray film. If you are not able to generate signals with this control probe, please see the Troubleshooting Guide (section 9) or call our technical services department for further assistance.

9. Troubleshooting Guide

PROBLEM	POSSIBLE CAUSE	SOLUTION
High Background	Incomplete washing	Raise stringency of washes (<0.25XSSC or higher temperatures)
		Increase number of washes
	Probe too large	Select smaller DNA fragment, 200-800 nt
	Probe contains free labeled nucleotides	Use Sephadex G-50 columns to remove free nucleotides
	Too much probe	Use less probe, (<5 x 10^6 cpm / ml for oligos, <2 x 10^6 cpm / ml for DNA)
	Membrane dried during hybridization	Use more prehyb / hyb / wash solutions
No or Weak Signal	Probe has low specific activity	Make new probe, specific activity should be >5 x 10^8 dpm / ug
		Requantify DNA fragment or use more to make probe
	Probe not denatured	Boil probe before adding to blot
	Probe is not from same species	If probe is from a different species, reduce final wash stringency
	Blot probed more than 2 times	If RNA of interest is low in abundance, ability to detect it will decrease with each probing of the blot. For highest signals, obtain new blot
Can't Reprobe Blot	Partially Dry Membrane	Blot membrane should not be allowed to dry even partially. Makes removal of probe difficult. Try stripping blot several times.
	High Background	See suggestions above. If still radioactive, store blot at -20°C for 28-42 days, 2 to 3 half lives for ^{32}P.

10. References

Ausubel, F.M. et al, eds. (1995) *Current Protocols in Molecular Biology*. (John Wiley and Sons, Inc., New York City, NY).

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edition. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

11. Related Products

Product

Total RNA from mouse, rat or human tissues

Poly A+ RNA from mouse, rat or human tissues

Multiple Choiceä cDNAs -- First strand cDNAs from rat, mouse and human.

***For more information on any of these products please call our ordering department at 1-888-267-4436