MessageMap® Northern Blot

INSTRUCTION MANUAL

BN #775900-12

Revision #023002a

For In Vitro Use Only

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MessageMap® Northern Blot

MATERIALS PROVIDED

Material Provided	Description
MessageMap® northern blot	6 × 8 cm membrane
Miracle*Hyb*™ hybridization solution	25 ml
Miracle*Hyb*™ probe preparation buffer	1 ml
β-actin cDNA control (100 ng purified)	25 ng/μl

STORAGE CONDITIONS

All Components: +4°C

ADDITIONAL MATERIALS REQUIRED

Prime-It® II Random Primer Labeling Kit (Stratagene Catalog #300385)
[α–^{32}P]dCTP (3000 Ci/mmol) or[α–^{32}P]dATP (3000 Ci/mmol)
NucTrap® Probe Purification Columns (Stratagene Catalog #400701 and #400702)

Revision #023002a

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INTRODUCTION

Northern blot analysis of RNA remains the preferred method for assessing the size, number, and relative abundance of gene transcripts. Each lane of the MessageMap® northern blot contains 2 μg of twice selected, high quality poly-A(+) RNA from murine or human tissues and cell lines. The poly-A(+) RNA is size fractionated on a 1% formaldehyde agarose gel and is then transferred to positively charged nylon membranes by positive-pressure alkaline transfer. MessageMap northern blots exhibit high sensitivity and low background signal making them ideal for detection of low abundance messages. MessageMap northern blots are effective when used with traditional radiolabeled probes or with nonradioactive/chemiluminescent protocols. In addition, MessageMap northern blots can be stripped and reprobed for ease of use and increased cost effectiveness.

Membrane and Marker Orientation

Each MessageMap northern blot is configured for clear analysis of signal. The Stratagene logo is located in the lower left hand corner of each membrane. This is the 'front' of the membrane. The RNA molecular weight marker is depicted by a series of dots in the first lane on the left (see Figure 1). Marker sizes are as follows: 9.49 kb, 7.46 kb, 4.4 kb, 2.37 kb. For individual MessageMap blot configurations please refer to the Certificate of Analysis included with your order.

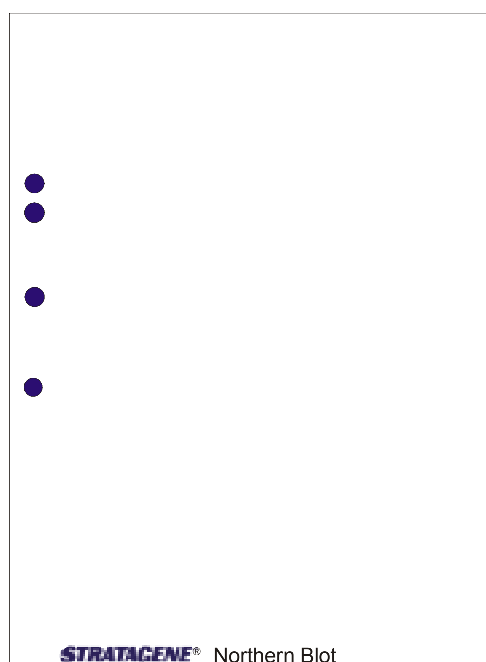

FIGURE 1 Schematic diagram of the MessageMap northern blot

Preprotocol Considerations

β-actin cDNA control

The β-actin control template is provided as a control for hybridization and may also be used for relative quantitation of a mRNA of interest. Due to the abundance of β-actin in most RNA samples, Stratagene recommends probing with the β-actin cDNA control only **after** detection of the mRNA of interest. This will ensure that signal from the β-actin cDNA control does not overwhelm signal from lower abundance sample RNAs. The human cytoplasmic β-actin cDNA control will hybridize to actin mRNA transcripts of ~2.5 kb.

Hybridization

During the hybridization phase of the protocol it is essential that the membrane is not allowed to dry completely. This will lead to irreversible binding of the probe to the membrane, high background, and will prevent successful stripping and reuse of the northern blot.

Stripping the Membrane

MessageMap northern blot membranes can be stripped and reprobed without loss of signal when performed according to the protocol (See *Stripping the Blot for Reuse*).

PROTOCOL

Preparation of Labeled Probe

Stratagene suggests radiolabeling 25–50 ng of sample DNA or RNA. The specific activity of the labeled probe should be >1 × 10^8 cpm/µg when using fresh (less than 1 week old) radiolabel. Stratagene offers the Prime-It® II random primer labeling kit for generating high-specific activity probes. Unincorporated radiolabel must be removed from the probe prior to hybridization. Excess radionucleotides are efficiently removed from labeled probes using Stratagene's NucTrap® probe purification columns.

If using other commercially available labeling or purification kits, please follow the manufacturer's instructions. For additional labeling and purification protocols please consult references 1 and 2.

Hybridization of Radiolabeled Probe to the MessageMap® Blot

1. Generally, 180 µl of the Miracle*Hyb* hybridization solution per cm^2 of the MessageMap blot should be used for standard experiments. More or less Miracle*Hyb* hybridization solution may be used as desired, but it is necessary to ensure that there is sufficient solution to cover the membrane at all times during the prehybridization and hybridization. The table below gives recommended minimum volumes of Miracle*Hyb* hybridization solution for common hybridization containers.

Container*	Minimum volume of Miracle*Hyb*™ hybridization solution
50-ml conical tubes	5 ml
heat-sealable bags or roller bottles	10 ml

2. For **double-stranded probes**, prehybridize the MessageMap blot in Miracle*Hyb* hybridization solution at 68°C for 15 minutes.

   For **oligonucleotide probes and riboprobes**, calculate the melting temperature (T_m) (see *Appendix I: Hybridization and Melting Temperatures* for the mathematical formula). Prehybridize the MessageMap blot in Miracle*Hyb* solution for 15 minutes at 5–10°C below the T_m.

   Note *It is normal for the Miracle*Hyb *hybridization solution to become slightly opaque during the pre-hybridization step, especially at higher incubation temperatures (≥ 50°C).*

* If 50-ml screw cap conical tubes or bottles are used, ensure that the blot is positioned with the Stratagene logo facing inward and not pressed against the walls of the vessel. If processing more than one blot, place each blot in a separate vessel

3. Aliquot into a screw-cap microcentrifuge the correct amount of Miracle*Hyb* probe preparation buffer (50 µl of Miracle*Hyb* probe preparation buffer per 5 ml of prehybridization solution used in Step 2.)

   Note *Always use screw-cap microcentrifuge tubes when boiling radioactive solutions*

4. Add radiolabeled probe to the Miracle*Hyb* probe preparation buffer and mix by pipetting. For double-stranded probes, boil the diluted probe for 2 minutes. Briefly spin in a bench top microcentrifuge. **The boiling step is not necessary for oligonucleotide or RNA probes.**

   For best results, use random-primed radioactive probes with the following concentration and specific activity:

   Suggested Probe Concentration
   1.0×10^6 total counts/ml of hybridization solution

   Specific Activity of the Probe
   10^8 cpm/µg or greater

5. Add the probe/Miracle*Hyb* probe preparation buffer mixture to the prehybridization solution containing the MessageMap blot. **It is important that the probe is added to the prehybridization solution and is not pipetted directly onto the membrane.**

Hybridization Conditions

Carry out the hybridization at 68°C. (When using oligonucleotide probes or other short probes, perform the hybridization at 5–10°C below the T_m.) Probe may be hybridized to the blot (on roller bars or a similar device for gentle agitation) for as few as one to two hours for high-abundance messages or standard Southern blot analysis. For low-abundance messages or when maximum sensitivity is desired, hybridize overnight (≥16 hours).

Washing the Blot

Perform the following membrane washes (gentle agitation is required) for double-stranded probes, oligonucleotide probes and riboprobes:

1. Wash twice for 15 minutes each at room temperature with excess 2× SSC buffer and 0.1% (w/v) SDS wash solution[§].

2. Wash once for 30 minutes at 60°C with excess 0.1× SSC buffer and 0.1% (w/v) SDS wash solution[§] for a high-stringency wash.

§ See *Preparation of Media and Reagents*.

Detection

Using forceps, grasp the MessageMap blot by one corner and lift it out of the wash solution. Hold it vertically so that the excess liquid is allowed to drain off. Remove the last traces of excess liquid by touching the bottom corner of the membrane to a clean paper towel or tissue. Wrap the blot in plastic wrap. Expose the wrapped MessageMap blot to autoradiography film with an intensifying screen at –80°C for 2 hours–overnight. For very low-abundance messages or increased signal intensity, film exposure may be extended up to one week without greatly increasing background.

Stripping the Blot for Reuse

1. Heat the 0.1× SSC buffer and 0.1% (w/v) SDS wash solution to boiling.

2. In a glass dish, pour the 0.1× SSC buffer and 0.1% (w/v) SDS wash solution over the MessageMap blot and wash the blot twice for 15 minutes each.

Proceed with the prehybridization step for the next hybridization. If the MessageMap blot will not be used immediately for a second round of hybridization, remove the excess liquid from the membrane by draining (see *Detection* above), then store the MessageMap blot in plastic wrap, desiccated.

β-actin cDNA Control

Use 25 ng of the β-actin cDNA control when preparing the control probe using random primer labeling protocols. Probes suitable for this experiment should have a specific activity of $>1 \times 10^8$ cpm/μg. The hybridization solution for the control experiment should contain 1×10^6 cpm/ml β-actin probe. A sharp band ~2.5 kb* should be detected on the film after 1–2 hours at –80°C with an intensifying screen.

* In some cases the β-actin control might also hybridize to α-actin, causing two bands to appear on the blot.

APPENDIX I: HYBRIDIZATION AND MELTING TEMPERATURES

Hybridization Temperature

The hybridization temperature for oligonucleotide probes equals 5–10°C below the T_m.

Calculation of the Melting Temperature

Note *The first equation below overestimates the* T_m *of hybrids involving longer oligonucleotides. The second formula works only for monovalent cation concentrations of ≤1 M. The molar concentration of monovalent cations in Miracle*Hyb *solution is 0.5 M.*

Oligonucleotides Shorter than 18 Bases

$T_m = 2°C(A + T) + 4°C(G + C)$

Oligonucleotides 14 Bases and Longer (up to 60–70 Nucleotides)

$T_m = 81.5 + 16.6(\log_{10}[MC]) + 0.41(\%G + C) – (600/N)$

where $[MC]$ is the molar concentration of monovalent cations and N is the chain length.

More discussion regarding calculation of melting temperature can be found in references 1 and 2.

TROUBLESHOOTING

Observation	Suggestion
Low hybridization signal	Check the specific activity of the labeled probe to ensure that it is $>1\times10^8$ cpm/µg
	Ensure that the concentration of the probe in solution is $>1\times10^6$ cpm/ml of Miracle*Hyb* hybridization solution
	Increase exposure time of blot to X-ray film
High background	Remove unincorporated nucleotides from the probe prior to hybridization
	Repeat the wash sequences
	Reduce exposure time
	Ensure that the correct amount of DNA was used in the labeling reaction

Preparation of Media and Reagents

20× SSC Stock Solution	**2× SSC Buffer and 0.1% (w/v) SDS Wash Solution**
175.3 g of NaCl (3 M final concentration) 88.2 g of sodium citrate-trisodium salt (300 mM final concentration) Adjust the pH to 7.0 with HCl Add distilled water (dH_2O) to 1 liter	100 ml of 20× SSC buffer 5 ml of 20% (w/v) SDS dH_2O to 1 liter
20% (w/v) SDS Stock Solution	**0.1× SSC Buffer and 0.1% (w/v) SDS Wash Solution**
Dissolve 20 g of SDS in 90 ml of distilled water (dH_2O) Mix well and heat to 68°C if necessary Add dH_2O to a final volume of 100 ml	5 ml of 20× SSC buffer 5 ml of 20% (w/v) SDS dH_2O to 1 liter

REFERENCES

1. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G. *et al.* (1987). *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.
2. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

ENDNOTES

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