Troubleshooting Forum

Molecular Biology Techniques Q&A

RNA Methods: Northern blot

This month's question from the Molecular Biology Forums (online at molecularbiology.forums.biotechniques.com) comes from the "RNA Methods" section. Entries have been edited for concision and clarity. Mentions of specific products and manufacturers have been retained from the original posts, but do not represent endorsements by, or the opinions of, *BioTechniques*.

How can I improve signal strength when performing Northern blots? (Thread 23086)

Q My signals are either absent or too weak in my Northern blots. Due to other commitments, I sometimes leave the membranes washing for 30–40 min. Could the long washes reduce the signal?

A Washing is not the problem. You should check the specific activity of your probe. Are you using an RNA or DNA probe? An RNA-RNA interaction is stronger than a DNA-RNA interaction. I have used both DNA and RNA probes with good results, but since you have weak signals, you might want to try an RNA probe.

Check the quality of your RNA and make sure the transfer is complete. About 2–3 h should be sufficient.

Q I am using DNA probes generated by random primer extension and have weak signals even after 3- to 4-day exposures.

Do you think that sealing the blotting tank will improve blotting efficiency? Will the vaporized buffer help with capillary diffusion, like in thin-layer chromatography?

A There is no need to seal the blotting tank. Just make sure the absorbent paper is cut to the same size as the gel and is not touching the wick. You can put strips of X-ray film around the gel to block any contact between the absorbent paper and wick, and add a glass plate on top to increase the weight. As long as the stack of absorbent paper is wet, you should have enough buffer movement to transfer the RNA from the gel to the membrane.

Q I read that using too much template when making the probe results in short probes with low specific activity. Does specific activity mean intensity of the signal?

I tried reducing the probe template significantly, but got no signal at all except with the positive control. A strong positive control signal indicates that the probe works effectively, right?

I don't think transferring causes the problem either, because after blotting, the gels and membranes look clean. I will try increasing the amount of probe and see if that helps.

A Since your positive control works well, the problem may be a low level of messenger RNA in your samples. You can expose the film at -80°C with intensifying screens for a week if you use a radioactive probe and wash the blot well to reduce background.

A Did you restain the gel to check for remaining RNA after your transfer? Sometimes high-molecular weight molecules don't transfer well. Denaturation will remove the ethidium bromide from the gel.

Too much template reduces specific activity simply because more cold probe remains relative to the newly synthesized hot probe. Too little template will lower the amount of synthesized probe. Long templates work better than short ones. As

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the extension length gets shorter, the primers become a larger percentage of the DNA probe, reducing the quantity of hot label.

If you need a hotter probe, consider using gene-specific primers and repeated cycles of primer extension, denaturation, and extension, or asymmetric PCR, which includes a little of the reverse primer. If you have a cyclor that you can use isotope in, that will increase the specific activity because you use less template and make more product that comes mostly from the correct full-length strand.

A Signal from a cloned positive control gene doesn't necessarily indicate a sensitive probe since the copy number is so high. Try using a signal from a housekeeping gene probe to show how well the Northern blots worked. You should use the maximum amount of RNA in the lanes. I usually overload a bit to maximize sensitivity, although it will cause some distortion around the ribosomal RNA (rRNA).

Some other ideas for troubleshooting include using a commercial hybridization mix that is supposed to increase sensitivity or reduce time, or you could try making a new probe. If the probe is too short it may hybridize poorly or form a secondary structure.

Q How much RNA do you usually load on each lane? Is it reasonable to load 30 µg?

A: It depends on your lane size. For a 20 cm × 24 cm gel, I use about 15–20 µg depending on whether I used a 20- or 30-well comb. When the gel is overloaded, the rRNA will push everything near it aside. Eventually the bands might start smearing. I think that 30 µg is a little too high.

Q I dried my pellets in a 60°C oven after washing with 70% ethanol. When I dissolved the pellet with DEPC-water, it formed a transparent, jelly-like mass. My only explanation for this is a possible protein impurity since proteins often become gel-like upon heating. Should I do additional phenol extractions to get rid of protein contamination?

A RNA forms an insoluble pellet if over dried. You should not use an oven to dry the pellets. After the 70% ethanol rinse, decant the ethanol, do a quick spin, and then remove the trace ethanol with a pipet tip. Then air dry your pellet for 15 min at room temperature.

If you have any RNasesecure resuspension solution from Ambion, add enough for a 1× concentration and heat the solution to 60°C for 10 min. This will help dissolve the RNA and inactivate any RNase contamination at the same time. If the pellet doesn't dissolve, phenol extraction will do no good and you will lose your RNA.

A I have encountered jelly-like pellets from some cell types. I think this comes from polysaccharides or other contaminants. Phenol or heating will not solve the problem, but you can avoid it by preparing your RNA with the guanidinium-isothiocyanate/phenol extraction method from Chomczynski or Trizol. You could also try using a larger volume of guanidinium thiocyanate (GSCN) lysis solution or doing the high salt precipitation listed in the Trizol/Invitrogen protocol.

Q When the pellet is dry, should it look white or transparent?

A When it is transparent, it is over dried. The pellet should be white, but with no trace of liquid surrounding it.

Q I tried again and found that an additional wash with 100% ethanol, followed by 5 min at 65°C is enough for drying.

I also solved my Northern blot problem. My probe was only 400 bp long and I had overestimated the concentration of my template. I increased the amount of template and now have completed 8 successful Northern blots in a row.

Selected and edited by Kristie Nybo, Ph.D.

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