

Anti-RNA blotting with chemiluminescent detection

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April 16, 2015

This protocol is for northern blotting/dot-blotting to detect specific RNAs via chemiluminescence, using biotinylated oligo-dT probes. Much of it is adapted from ULTRAhyb-Oligo and Brightstar BioDetect protocols.

Dot Blot

1. Blot 10uL of each RNA sample on positively charged nylon membrane under vacuum.

Northern Blotting

1. Put 1uL (or other measured amount) of each RNA sample into total 10uL of 1X RNA loading buffer (NEB). Heat the tubes at 90-95°C for 4 min to denature RNA and then immediately place the tubes on ice.
2. Set up a Bio-Rad TBE-Urea 5% acrylamide gel with 1X TBE buffer, wash wells. Load samples on the gel along with 5uL RNA ladder/markers. Run the gel in 1X TBE buffer at 200 V for 90min, or until the first bromophenol blue dye reaches the bottom of the gel.
3. Turn off the current, remove the gel, and transfer the RNA onto a positively-charged nylon membrane using a standard Western blotting apparatus with 0.5X TBE buffer at 1 amp for 2 h. Trim off the portions of the gel that do not contain the samples to decrease the size of the membrane and use of smaller amounts of reagents in subsequent steps.
4. Use a pencil to mark the *sample* side of the membrane. Crosslink RNA to the membrane by UV (in a UV-crosslinker, about 3kJ total; keep the membrane wet during crosslinking) and dry the membrane; sample side faces *up* throughout.

Hybridization

Ingredients:

- ULTRAhyb-Oligo® buffer (Life technologies AM8663)
- oligo-dT probes: 5' biotinylated, 36-45nt long, 60-75°C hybridization temperature, 45-65% GC-content, in 10µM working solution.
- Wash buffer: 2X SSC (300mM NaCl, 30mM sodium citrate, pH 7.0), 0.5% SDS. Make from 20X stock.
- BrightStar® BioDetect™ Nonisotopic Detection Kit (Life technologies AM1930)
- Positively Charged nylon membrane (e.g. BrightStar™ -Plus Positively Charged Nylon Membrane, or Roche 11-209-299-001)

Steps:

5. Preheat ULTRAhyb-Oligo to 42-68°C to dissolve all precipitated material.
6. Prehybridize the blot for 30 min at 42°C. In the Drummond lab, use a yeast incubator at 42°C, 40rpm shaking for this. Use 1 mL ULTRAhyb-Oligo Hybridization Buffer per 10 cm² of membrane. Be certain that enough solution is present to keep the membrane uniformly wet.
7. Add 5nM of the end labeled oligonucleotide (0.5µL of 10µM working solution per mL of buffer). Since it is important that undiluted probe solution does not touch the membrane, pour the ULTRAhyb-Oligo solution from the prehybridization into a 50 mL conical tube, add the labeled probe, mix by swirling, and then immediately pour the solution back into the container with the blot.

8. Hybridize overnight (14-24 hr) at 42°C with gentle agitation.
9. Wash the blot 2x30 min at 42°C. Immediately pour at least 50 mL wash buffer onto the blot and incubate at 42°C for 30 min with gentle agitation. Repeat with fresh wash buffer.

Detection

10. Measure the surface area of your membrane to determine the amounts of the washing and assay buffers needed. Dissolve the precipitate in the 5X washing buffer at 37-65°C. Dilute the 5X washing buffer and 10X assay buffer to 1X working buffers before the experiment, prepare enough for one experiment only (discard the unused diluted buffer after each experiment). For example, for a 10 x 10 cm membrane (100 cm²), 100 mL of washing buffer and 30 mL of assay buffer are needed (the volumes below are given for a 100 cm² membrane). Place the membrane sample side up in a plastic box of similar size. Do not allow the membrane to dry throughout the entire procedure. The procedure is performed at room temperature on a rocking shaker at medium speed (10-20 rockings/min).
11. Wash the membrane 2 times, 5 min each, in 20 mL 1X washing buffer.
12. Wash the membrane 2 times, 5 min each, in 10 mL 1X blocking buffer.
13. Wash the membrane once for 30 min in 20 mL 1X blocking buffer.
14. Incubate the membrane for 30 min in 10 mL of the conjugate solution (10 mL blocking buffer plus 1 μ L of streptavidin-alkaline phosphatase conjugate).
15. Wash the membrane once for 15 min in 20 mL 1X blocking buffer.
16. Wash the membrane 3 times, 15 min each, in 20 mL 1X washing buffer.
17. Wash the membrane 2 times, 2 min each, in 15 mL 1X assay buffer.
18. Incubate the membrane in 5 mL CDP-star solution for 5 min. Make sure that the solution evenly covers the entire membrane, by rotating the box by hand.
19. Take out the membrane with forceps. Let the excess of CDP-star solution drip off, place the membrane in a transparent plastic bag, remove all the air, and heat-seal the bag.
20. Image the membrane on the Chemi-doc or other digital imager. Usually 1 min to 2h exposure is sufficient; the CDP-star reaches peak light emission in 2-4 h, and the light emission persists at the high level for several days. The image can also be obtained and quantified using Kodak Digital Science Image Station.