

DNA LABELING, HYBRIDIZATION, AND DETECTION (Radioactive)

OBJECTIVE:

To hybridize a probe (radioactively-labeled) DNA with DNA immobilized on a blotting membrane, in order to characterize the DNA on the blot with regard to the specific DNA region represented by the probe.

INTRODUCTION:

As was stated in the last section, radioactive labeling generally yields higher sensitivities and lower backgrounds than labeling with other methods. For probing organisms with small to moderate-sized genomes or for probing plasmid DNAs, the nonradioactive methods are acceptable. However, with most higher plant species and many amphibians, which have large genomes, the nonradioactive methods are not currently sensitive enough for detection of low copy number sequences.

For most moderate and high copy number sequences (more than 100 copies or so), either method would generally yield acceptable results. However, when attempting to locate low copy number or "single copy" genes, radioactive labeling is probably the only method which will allow detection of these sequences. For example, ribosomal genes in legumes make up about 0.1% of the genome (one part in 1000, or several thousand copies in a large genome). These sequences are easily detected even when less than 1 μg of total DNA is loaded onto the blot. However, when a short single copy gene is to be probed for, 1 μg is too little total DNA for detection, even with radioactive probes. Often, 10-50 μg needs to be used. Since this amount of DNA is reaching the limits of gel electrophoresis and the binding capacity of the blotting membranes, only radioactive methods can be used. Other methods are not yet sensitive enough for these purposes.

[NOTE: Always wear gloves and a lab coat when working with radioactive isotopes. Also, always have paper and plastic covers over all lab benches that you are working on so that any small spills can be cleaned up easily at the end of the procedure. As many of the following steps as possible should be performed behind a radiation stopping plastic shield. The beta-decay from ^{32}P is very energetic and can easily pass through skin and into the eyes. In addition, the work should be performed under a fume hood, since vapors can be inhaled. During the time you are in the radioactive room or area, check yourself, your work area and the floor frequently (every few minutes) with the Geiger-Mueller counter, or similar device. After using ^{32}P you should always check your hands and any other parts of your body which may have some radioactivity on them. This is especially true if you plan to eat, drink, smoke or grab a door handle in the near future. If you find some radioactivity on your hands, etc. or on the counter tops, etc, WASH IT OFF IMMEDIATELY before you or someone else gets more of an exposure than they want.]

STEPS IN THE PROCEDURE:

Prehybridization:

1. Pour hybridization solution into the Seal-A-Meal bag containing the blot, then seal the bag, attempting to remove most of the air bubbles. [Use 10 ml of solution for small blots and 50 ml for large blots.]
2. Incubate in a 65 °C water bath with agitation for at least 6 hours.

Labeling:

1. Add components of the labeling kit (Random Primed Labeling Kit from BMB, Prima-A-Gene from Promega, Nick Translation Kits from either NEN or Worthington). Each kit has its own components and instructions. Keep the components on ice, and keep the Klenow enzyme (polymerase) at -20 °C until ready to use. I recommend using 10-100 μCi per 200-500 ng DNA for each large blot for random primer labeling and 25-150 μCi per 250-750 ng for nick translation. [Remember, for the first two procedures the DNA must be linearized and denatured, while for the nick translation methods, DNA in any double stranded conformation is acceptable.] "Home made" kits can also be produced. These may save you money in the long run, but you will spend a lot of time trying to optimize the components (from different manufacturers), concentrations and conditions. I recommend buying a prepackaged kit. [Note: PCR can also be used to label with radioactive nucleotides.]

For the Prime-A-Gene system (Promega) add the following:

- a. 10 μl of 5X labeling buffer
- b. 2 μl of the nonlabeled dNTP mix
- c. 2 μl of nuclease-free BSA
- d. 1-10 μl of labeled nucleotide (corresponding to 1-10 μl of ^{32}P dCTP @ 3000 Ci/mmol)
- e. Denatured DNA template and sterile dH_2O to bring total volume to 49 μl
- f. 1 μl Klenow enzyme solution

Incubate reaction for at least one hour at room temperature.

2. While the reaction is incubating (or just before beginning the reaction) set up the mini spin column for separating the DNA from the unincorporated nucleotides as follows:

- a. Place a small plug of glass wool at the bottom of a 1 ml disposable plastic syringe (without needle, or needle removed).

- b. Add some TES to wet the inside of the syringe and the glass wool.
 - c. Slowly add the G-50 slurry (Sephadex G-50 soaked in TES in the cold for at least 24 hours) to the column. Try to avoid bubbles, but don't worry if you get a few, these can be spun out later.
 - d. Place a 1.5 ml screw-capped microfuge tube (without top, for the moment) into the bottom of a 12 or 15 ml conical glass centrifuge tube. Then place the column into the top of the glass centrifuge tube so that the bottom opening of the syringe is inside the top of the microfuge tube.
 - e. Spin in a table top centrifuge at slow to moderate speed for 1 minute. Add more G-50 slurry if needed. There should be about 2-3 cm of free space at the top of the syringe.
 - f. Add more TES and spin for 1 minute. Using a pair of long forceps, pull the microfuge out of the glass centrifuge tube and pour out the TES that is in the microfuge tube. Reassemble the column and microfuge tube in the glass centrifuge tube.
3. Add TES to the labeling reaction to a total volume of about 100 μ l and mix. Then, add this to the top of the syringe column and spin for 2 minutes.
 4. Check the column and the microfuge for any leaking. Remove the column, check the approximate counts per minute with a Geiger-Mueller counter and discard it into the radioactive waste container.
 5. Using a pair of long forceps, carefully remove the microfuge tube from the bottom of the glass centrifuge tube. Screw on the cap of the microfuge tube. Check the approximate counts per minute using the Geiger-Mueller counter. If the number of counts in the microfuge tube is greater than or equal to the number of counts remaining in the column then the labeling was successful. If the number of counts in the microfuge was 20-50% of the total counts (column + microfuge tube) then the labeling is probably sufficient. Any less than 20% (unless you were labeling less than 20 ng of DNA) and the labeling reaction may not be working well, and you should think about what might have gone wrong before you try it again.
 6. Heat the probe in the sealed microfuge tube at 95 °C for 10 minutes to denature the probe. Immediately place on ice until the probe is to be added to the blot.

Hybridization:

1. Cut open the bag containing the prehybridized blot and add the denatured probe DNA.
2. Reseal the bag (twice) and make sure that the probe is well distributed in the hybridization solution by rocking the bag back and forth several times. Watch out for any leaks in the bag!

3. Incubate at 65 °C for at least 6 hours with agitation.

Washing Blots:

[Perform these steps over a baking dish or other container, so that any small spills are contained for easy clean up at the end.]

1. Carefully cut open the bag and pour off the solution into either the radioactive waste container (if the probe is not to be reused) or into a screw-capped tube (if the probe will be reused).

2. Add wash solution I to the bag and mix the blot around in it for 5 minutes. Pour this off down the drain and repeat this with more wash solution I. [Use 40 ml for small blots and 100 ml for large blots.]

3. Transfer blot from the bag into wash solution II in a plastic storage dish (with lid). There should be at least 1-2 cm of this solution covering the blot.

4. Depending on the stringency required, incubate the blot in the wash solution II for 20-30 minutes at the specified temperature. A temperature of around 65 °C is most often used. At the end of the 20-30 minutes, repeat this step one more time with new wash solution II.

5. Pour off the wash solution II and replace it with wash solution III. Place the container with the blot inside on a shaker at room temperature for 20-30 minutes. Repeat this step one more time.

6. Remove the blot from the wash solution III and place the blot onto a clean piece of 3MM paper for a few seconds to remove the excess solution. Do not allow the blot to dry completely, especially if you plan to reprobe the blot.

7. Place a sheet of Saran Wrap longer than twice the length (and over one blot width) on the bench. Tape the top ends to the bench, slightly stretching the plastic wrap. Tape the edges of the middle of the plastic wrap to the bench, again stretching it to remove most of the wrinkles.

8. Carefully place one edge of the blot face down on the plastic wrap and allow the rest of the blot to roll onto the plastic wrap. This will form a smooth seal of liquid between the blot and the plastic wrap. Fold and stretch the free end of the wrap over the top of the blot until it is about 1 cm above the blot, then press one corner of the wrap onto the blot. The rest of the wrap will then adhere to the blot without forming many air bubbles.

9. Trim the edges of the wrap with scissors to about 1 cm from the edge of the blot. Then, fold the edges over so that the liquid cannot squeeze out of the wrap (this will damage the X-ray film, later). Tape the wrapped blot onto a piece of paper that will fit into one of the X-ray exposure holders.

Autoradiography:

1. Place the paper, with attached blot into an X-ray exposure holder (8 X 10, for standard blots). Get one sheet of 8 X 10 XAR-2 film (in protective envelope) and an intensification screen, if needed, and go into a darkroom with a safelight.
2. Load the holder in the following sequence: a) Place blot (taped to paper) face up into the holder, b) tear open the film envelope and place the film on top of the blot, c) place intensification screen over film, if needed (intensification screens cost around \$30-50 each, treat them with care!), d) place another piece of paper over the intensification screen. Close the holder and place a piece of plastic over the front and back and clip them in place. Next, put this inside a ziplock bag and place it into a -70 °C freezer.
3. If the counts on the blot were low (less than 5-10 cpm from the G-M meter) expose for 24 hours or longer. If there were bands that had over several hundred counts, either expose for only an hour or do not use the intensification screen and expose for several hours.
4. At the end of the exposure period, remove the bag from the freezer and allow it to sit at room temperature for 15 minutes before opening, to minimize the amount of condensation forming on the exposure holder and its contents.
5. In the darkroom with safelight, develop the film as follows:
 - a. Place the film (and only the film) into a tray with GBX developer for 3 minutes with gentle agitation. The bands should begin appearing in 10-30 seconds.
 - b. Transfer the film into tray of acidified water (water with about 20 ml of acetic acid per tray mixed into it) for 30 seconds. This will stop the development of the exposed silver grains.
 - c. Transfer the film into Rapid Fix for at least 5 minutes, with gentle agitation. This will remove all of the unexposed silver grains remaining on the film. After this step the room lights may be turned on, if desired.
 - d. Wash the film in cold running water for 30 minutes and then hand to dry (for at least 1 hour).
 - e. While the film is washing, pour the GBX developer and the Rapid Fix fixer back into their respective containers. Both are reusable. The Rapid Fix should last for 6 months or more, while the GBX can probably be used for 1-3 months before it looks like bad coffee and should be thrown away at this stage.

SOLUTIONS:

10 X TES (aka, TNE and STE)

100 mM Tris (pH 8.0)
10 mM EDTA
1 M NaCl

Sephadex G-50 slurry

Place TES and G-50 into a bottle (TES volume should be 4-5 X more than the G-50 volume in the bottle) for at least 24 hours (and up to several months, as long as no microorganism growth is noted).

Hybridization Solution

1 M NaCl
1% (w/v) SDS (a higher percentage of SDS can be used, but lower than 1% will increase background on the blot)

20% SDS

20 g SDS
water up to 100 ml

Wash Solution I

2 X SSC (dilute 20 X SSC, see Southern Transfer exercise)

Wash Solution II

2 X SSC (dilute 20 X SSC)
1% SDS (dilute 20% SDS, see above)

[Note: To increase stringency, decrease SSC concentration, and vice versa.]

Wash Solution III

0.1 X SSC (dilute 20 X SSC)