

DNA LABELING, HYBRIDIZATION, AND DETECTION (Non-Radioactive)

OBJECTIVE:

To hybridize a probe (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:

DNA Labeling:

In order to visualize where the DNA hybrids are forming on the blots, the probe DNA must be labeled using either radioactively labeled (^{32}P) or chemically substituted nucleotides. When radioactivity is used, autoradiography using X-ray film is employed to visualize the hybrid positions. When chemically labeled probes are used, colorimetric reactions are most often used, some relying on antibodies or other chemicals attached to enzymes that can cause a colored precipitate to form from an appropriate substrate.

There are four common ways to label DNA: 1. End-labeling, either at the 3' ends with DNA polymerase or at the 5' end using T4 polynucleotide kinase; 2. Nick translation, using DNA polymerase and a low concentration of DNase I to form the nicks that are filled in by the polymerase); 3. Random primer labeling, where DNA polymerase is used in conjunction with random hexanucleotides which prime the polymerization reactions; and 4. PCR labeling. End-labeling does not result in highly labeled probes but is still used for certain procedures. Nick translation of DNA results in highly labeled probes, but the DNase I concentrations are critical to the proper labeling and utilization of the probe. Too little, and labeling is inefficient. Too much and the DNA is too degraded to use. Even when the concentration is in the proper range, in time the probe still becomes degraded. The third method is the random primed labeling method (Feinberg and Vogelstein, 1983). A mixture of all of hexanucleotides (there are 4^6 , or 4096, different possibilities) is added to the single-stranded DNA (double stranded DNA that has been linearized by cutting with a restriction enzyme and then heat denatured, and quenched on ice), one labeled (with radioactivity or an enzyme) nucleotide, the other three unlabeled nucleotides, and DNA polymerase. The resulting labeled DNA can be just as highly labeled as nick translation labeled DNA but the average size of the DNA is larger. However, if ^{32}P is used, after a time the DNA is broken by the β particle decay, so random primer labeled DNA also is degraded after a while. The fourth method is labeling using PCR. A standard PCR reaction is set up, with the substitution of one or two of the nucleotides with radioactively labeled nucleotides. This method is simple to set up, but the reaction usually takes longer, although fewer cycles can be used if less probe is required. Another problem with radioactive labeling via PCR is that the thermal cycler may become contaminated with radioactivity and thus its use in other experiments might be compromised.

Nonradioactive labeling of the DNA has some advantages over radioactive labeling, but it also has some drawbacks. However, each year better methods for nonradioactive labeling have been developed and some of the disadvantages have

disappeared. The advantages are that there is generally less danger than with radioactive methods. Also, the problems of radioactive disposal are eliminated. Third, the probes can be made in bulk and stored for long periods of time. Some of the current disadvantages are that often higher background and less sensitivity is achieved, but these are being changed, although with some membranes the methods still are unsatisfactory. Procedures for reprobing with other probes is not yet easily achieved using nonradioactive methods, while reprobing with radioactive probes is possible for as many as 50 times.

We will be labeling the probe DNA using a nonradioactive version of the random primed DNA method (Genius, manufactured by Boehringer Mannheim Biochemical Co. - or another method may be substituted). The nucleotide dUTP linked to the steroid hapten digoxigenin will be substituted for dTTP in the DNA polymerization reaction. This probe DNA will then be hybridized to the blotted DNA. An antibody that binds specifically to the steroid hapten, and is conjugated to an enzyme (alkaline phosphatase) which catalyzes a color reaction, is added to the blot, which then binds to the hybridized (and labeled) probe DNA. A substrate for the alkaline phosphatase is then added and the color reaction proceeds, making visible the hybridized bands on the blot.

Hybridization:

The DNA you have on your blots is mostly single-stranded which means that much of the DNA is available for base-pairing with complementary DNA (or RNA) single-strands. Since the DNA is bound to the blot, complementary single-stranded DNA in solution can be used to form base paired lengths of DNA. These lengths are hybrid molecules, hence the name "DNA hybridizations."

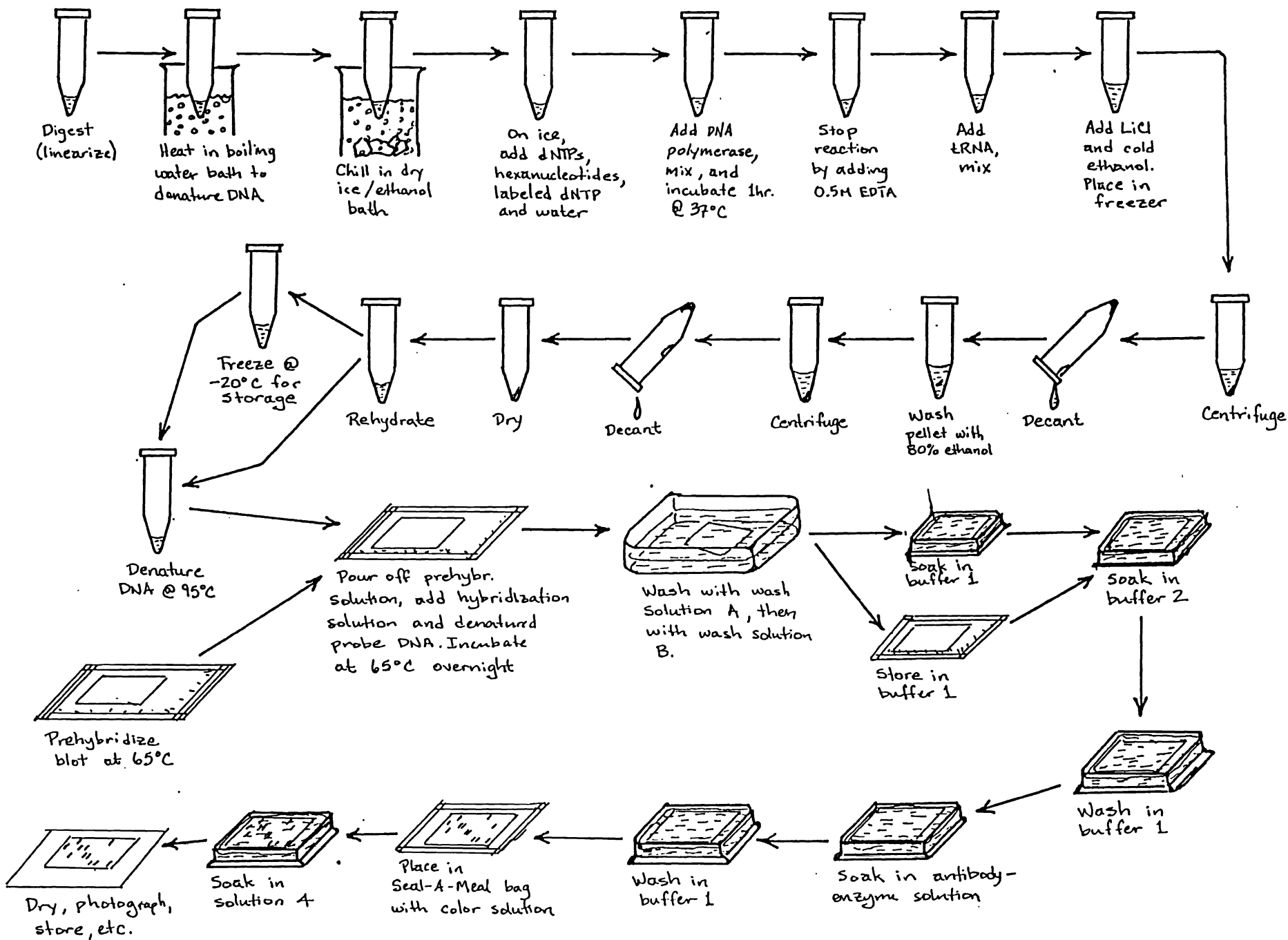
The rate at which hybrid molecules form is a second order reaction, dependent on the concentration of the two single strands involved in the pairing to form the duplex. The reaction then is dependent on the number of molecules attached to the blot (and how available they are for pairing) and the concentration of the probe DNA molecules in solution. A third very important parameter is the temperature at which hybridization is carried out. Each sequence has a particular melting/reannealing temperature, the T_m , which is dependent on the base composition. $T_m = (69.3 + (0.41 \times \%GC \text{ pairs})) ^\circ C$. Since G-C pairs have three possible hydrogen bonds and A-T pairs have only two, G-C rich regions have higher melting temperatures than do A-T rich regions. If a probe is used that came from the same organism whose DNA is to be probed, then the probe is said to be homologous. If it originated from a different organism, then it is a heterologous probe. When using a homologous probe the T_m will always be higher than if a heterologous probe is used. Because of this, one must be careful to choose the conditions for hybridization and washing so that the proper sensitivity and stringency are achieved. Sensitivity is the amount of DNA on the blot that can be detected. Using contemporary methods, the sensitivity is usually around 1 pg (or lower). Stringency has to do with the selectivity of the hybrids formed. Stringency increases as the temperature is increased towards the T_m . As the temperature decreases, more base pair mismatches are allowed since there is insufficient energy to force them apart. The T_m is decreased by about 1 $^\circ C$ for each 1%

base pair mismatch, so using a heterologous probe can have a significant affect. The standard temperatures used for stringent washes is around 60-65 °C in 2X SSC.

STEPS IN THE PROCEDURE:

DNA Labeling (nonradioactive method):

1. Linearize 500 ng of the plasmid (or other) DNA by digestion with restriction enzyme (e.g., *EcoRI*). We will be using the plasmid pBD4 (pSR118 or pSR125, or similar probe), which contains ribosomal RNA genes from the yeast, *Saccharomyces cerevisiae*.
2. Each group will be given 1 µg of pBD4 that has been linearized (by digestion with *EcoRI*). Denature the plasmid by heating in a 95-100 °C water bath for 10 minutes. The water in the DNA solution will evaporate, so either add more water before heating the solution or cap the microfuge tube and spin down the moisture off of the sides of the tube after heating. A screw top centrifuge tube will work best for the latter, since at the high temperatures, enough pressure may be generated inside the tube to blow the cap off. If this happens much of the DNA will also blow out of the tube.
3. Chill the tube rapidly in an ice/alcohol bath.
4. Add the following constituents to the tube containing the DNA (as it sits on ice):
 - a. 2 µl of the hexanucleotide mixture (vial 5)
 - b. 2 µl of the dNTP labeling mixture (vial 6)
 - c. Enough sterile deionized (or double distilled) water to bring the volume to 19 µl.
 - d. 1 µl of the Klenow enzyme (DNA polymerase) solution (vial 7)
5. Mix thoroughly and incubate at 37 °C for at least 60 minutes to overnight.
6. Stop reaction by adding 1 µl 0.5M EDTA.
7. Precipitate the labeled DNA by first mixing in 2.5 µl yeast tRNA (10 µg/µl), then 2.5 µl 4M LiCl and 75 µl cold (-20 °C) ethanol. Mix well.
8. Leave tube for at least 30 minutes to overnight @ -70 °C or for at least 1 hour @ -20 °C.
9. Centrifuge for 15 minutes in microfuge, pour off and discard the supernatant. Wash pellet with 100 µl of 80% cold ethanol.
10. Centrifuge for 5 minutes in microfuge, pour off and discard the supernatant. Dry the pellet under a vacuum (15-30 minutes).
11. Dissolve the pellet in 50 µl of TE.



DNA/DNA hybridization:

1. In a Seal-A-Meal bag, prehybridize the blot in prehybridization solution at 65 °C for at least 2 hours (and up to 12 hours, to reduce background). Use about 20 ml for each 100 cm² blot surface. [For a small blot, 7 X 10 cm, this would be about 15 ml, for a large blot, 16 X 23 cm, this would be 75 ml.]
2. Denature the labeled probe DNA (pBD4) by heating in a 95-100 °C water bath for 10 minutes. Pour off the hybridization solution from the bag containing the blot and replace it with new hybridization solution, containing the denatured labeled probe DNA. For a small blot (7 X 10 cm) use about 50 ng of probe DNA in 2.5 ml of hybridization solution, for large blots (16 X 23 cm) use about 250 ng of probe DNA in 10 ml of hybridization solution.
3. Incubate at 60 °C for at least 6 hours with slight agitation.
4. Pour off the probe/hybridization solution, remove the blots from the bag and place them into a tray containing enough wash solution A to cover the blot (at least 50 ml for the small blots and at least 250 ml for the large blots). Place on a rotary shaker at room temperature for 5 minutes. Replace the solution with new solution A and repeat the shaking. Replace the solution with an equal amount of wash solution B heat in rotating water bath for 15-30 minutes at 65 °C. Repeat this one more time with new wash solution B.

Immunological detection:

1. In a tray, wash the blot briefly in a small amount of buffer 1.
2. Incubate the blot for at least 1 hour in buffer 2 (50 ml for small blots, 250 ml for large blots).
3. Wash again briefly in a small amount of buffer 1.
4. Dilute antibody-conjugate (vial 8) to 150 milliunits per milliliter (1:5000 dilution) in buffer 2.
5. Incubate the blots at room temperature for 1 hour in the diluted antibody-conjugate solution. Use about 15 ml for small blots and about 75 ml for large blots. Flip blots after the first 30 minutes.
6. Wash the blots twice (15 minutes each) with buffer 1 (50 ml for small blots and 250 ml for large blots).
7. Transfer the blot into a Seal-A-Meal bag. Soak the blots for at least 2 minute in buffer 3 (15 ml for small blots, 75 ml for large blots).
8. Incubate the blots in the color solution sealed in the bag in the dark (25 ml for a small blots, 50 ml for large blots). The color reaction begins after a few minutes and takes up to several days for completion (usually 16 hours is sufficient. Do not shake or mix the blots while the color is developing.
9. When the desired spots or bands are detected, the reaction can be stopped. Precipitation of

the color also occurs in regions that do not have DNA (or have nonspecific hybridizing DNA), also known as background. The reaction should be stopped before the background becomes too pronounced. To stop the reaction, wash the blot for 5 minutes in buffer 4 (50 ml for small blots, 250 ml for large blots). Store in 2X SSC.

10. The blots may be photocopied or photographed for documentation purposes (since the color may fade over time).

11. Blots can be stored in a Seal-A-Meal bag for long term storage.

SOLUTIONS:

Labeling:

Hexanucleotide mixture (vial 5)

This solution contains the hexanucleotides and buffer

dNTP labeling mixture (vial 6)

1 mM dATP

1 mM dCTP

1 mM dGTP

0.65 mM digoxigenin-dUTP

buffered to pH 6.5

Klenow enzyme (vial 7)

2 U/ μ l Klenow enzyme (DNA polymerase, purified away from the exonuclease portion of the holoenzyme)

0.5 M EDTA

(see previous exercises)

4 M LiCl

Hybridization:

Prehybridization solution

5 X SSC

1.0% (w/v) blocking reagent (vial 11)

0.1% N-laurylsarcosine (sarkosyl)

1.0% SDS

Hybridization solution

5 X SSC

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0.1% (w/v) N-lauroylsarcosine (sarkosyl)

1.0% (w/v) SDS

Wash solution A

2 X SSC

0.1% (w/v) SDS

Wash solution B

0.1 X SSC

0.1% (w/v) SDS

Immunological detection:

Buffer 1

100 mM Tris (pH 7.5)

150 mM NaCl

Buffer 2

1.0% (w/v) blocking reagent (vial 11) in buffer 1

Buffer 3

100 mM Tris (pH 9.5)

100 mM NaCl

50 mM MgCl₂

Buffer 4 (TE)

10 mM Tris (pH 8.0)

1mM EDTA (pH 8.0)

Color solution (freshly made), for each 10 ml

45 µl nitroblue tetrazolium (NBT) solution (vial 9,
which is 75 mg/ml NBT in 70% dimethylformamide)

35 µl X-phosphate solution (50 mg/ml toluidinium salt
of 5-bromo-4-chloro-3-indolyl phosphate in
dimethylformamide)

10 ml buffer 3

[ALL solutions will be provided for you.]

Alternative solution:

Buffer 1

100 mM maleic acid (pH 7.5)

150 mM NaCl

REFERENCES:

A.P. Feinberg and B. Vogelstein, 1983, Anal. Bioch. 132:6