prehybridization, sites on the nitrocellulose filter that nonspecifically bind single- or double-stranded DNA become blocked by proteins in the BLOTTO.

When ^{32}P-labeled cDNA or RNA is used as a probe, poly(A) at a concentration of 1 μg/ml should be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

Whether or not to use a prehybridization solution containing formamide is largely a matter of personal preference. Both versions of these solutions give excellent results and neither has clear-cut advantages over the other. However, hybridization in 50% formamide at 42°C is less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. Offsetting this advantage is the two- to threefold slower rate of hybridization in solutions containing formamide.

To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6× SSC or 6× SSPE) at a temperature that is 20–25°C below T_m (see Chapter 9, pages 9.50–9.51). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6× SSPE is preferred because of its greater buffering power.

Prehybridization solution

Either
50% formamide
6× SSC (or 6× SSPE)
0.05× BLOTTO
or
6× SSC (or 6× SSPE)
0.05× BLOTTO

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

1× BLOTTO: Bovine Lacto Transfer Technique Optimizer (Johnson et al. 1984) is 5% nonfat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C. 1× BLOTTO is as effective a blocking agent as Denhardt's reagent, but much less expensive. It should not be used in combination with high concentrations of SDS, which will cause milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent when radiolabeled RNA is used as a hybridization probe, because of the possibility that the dried milk may contain significant amounts of RNAase activity.

Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

4. Denature ^{32}P-labeled double-stranded DNA probe by heating for 5 minutes to 100°C. Chill the probe rapidly in ice water. Single-stranded probe need not be denatured. Add the probe to the prehybridization solution covering the filters. Incubate at the appropriate temperature until $1-3 \times C_o t_{1/2}$ is achieved (see Chapter 9, page 9.48). During the hybridization, the containers holding the filters should be tightly closed to prevent loss of fluid by evaporation.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris · Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   Between 2×10^5 and 1×10^6 cpm of ^{32}P-labeled probe (sp. act. $\geq 5 \times 10^7$ cpm/μg) should be used per milliliter of prehybridization solution. Using more probe will cause the background of nonspecific hybridization to increase; using less will reduce the rate of hybridization.

5. When the hybridization is completed, remove the hybridization solution and immediately immerse the filters in a large volume (300–500 ml) of a solution of 2 × SSC and 0.1% SDS at room temperature. Agitate the filters gently, and turn them over at least once during washing. After 5 minutes, transfer the filters to a fresh batch of wash solution and continue to agitate them gently. Repeat the washing procedure twice more. At no stage during the washing procedure should the filters be allowed to dry.

   Hybridization mixtures containing radiolabeled single-stranded probes may be stored at 4°C for several days and reused without further treatment. However, hybridization mixtures containing complementary strands of DNA should be discarded, since there is no satisfactory way to denature the double-stranded DNA that forms during the first round of hybridization.

6. Wash the filters twice for 1–1.5 hours in 300–500 ml of a solution of 1 × SSC and 0.1% SDS at 68°C. At this point, the background is usually low enough to put the filters on film. If the background is still high or if the experiment demands washing at high stringencies, immerse the filters for 60 minutes in 300–500 ml of a solution of 0.2 × SSC and 0.1% SDS at 68°C.

7. Dry the filters in the air on paper towels at room temperature. Arrange the filters (numbered side up) on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filters. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with radioactive ink.

   Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot (>2000 cps on a hand-held minimonitor), hot (>500 cps on a hand-held minimonitor), and cool (>50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

8. Cover the filters with a second sheet of Saran Wrap. Expose the filters to X-ray film (Kodak XAR or equivalent) for 12–16 hours at −70°C with an intensifying screen (see Appendix E).

9. Develop the film and align it with the filters using the marks left by the radioactive ink. Use a nonradioactive fiber-tip pen to mark the film with the positions of the asymmetrically located dots on the numbered filters. Tape a piece of clear Mylar or other firm transparent sheet to the film. Mark on the clear sheet the positions of positive hybridization signals. Also mark (in a different color) the positions of the asymmetrically located dots. Remove the clear sheet from the film. Identify the positive plaques by aligning the dots on the clear sheet with those on the agar plate.

   Some batches of nitrocellulose filters swell and distort during hybridization and subsequent drying, so that it becomes difficult to align the two sets of dots. This problem can be alleviated to some extent by autoclaving the dry filters between pieces of damp Whatman 3MM paper before use (10 lb/sq. in. for 10 minutes on liquid cycle). Nylon membranes do not suffer from this problem.

10. Each positive plaque should be picked as described on page 2.63 and placed in 1 ml of SM containing a drop of chloroform. Often, the alignment of the filters with the plate does not permit identification of an individual hybridizing plaque. In this case, an agar plug containing several plaques should be picked. An aliquot (usually 50 μl of a 10^{-2} dilution) of the bacteriophages that elute from the agar plug is replated so as to obtain approximately 500 plaques on a 90-mm plate. These plaques are then screened a second time by hybridization. A single, well-isolated positive plaque should be picked from the secondary screen and used to make a plate stock (see page 2.65 or 2.66).