

VOLUME 1

Molecular Cloning

A LABORATORY MANUAL

THIRD EDITION

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Front cover (paperback): The gene encoding green fluorescent protein was cloned from *Aequorea victoria*, a jellyfish found in abundance in Puget Sound, Washington State. This picture of a 50-mm medusa was taken on color film by flash photography and shows light reflected from various morphological features of the animal. The small bright roundish blobs in the photograph are symbiotic amphipods living on or in the medusa. The bright ragged area in the center is the jellyfish's mouth.

Bioluminescence from *Aequorea* is emitted only from the margins of the medusae and cannot be seen in this image. Bioluminescence of *Aequorea*, as in most species of jellyfish, does not look like a soft overall glow, but occurs only at the rim of the bell and, given the right viewing conditions, would appear as a string of nearly microscopic fusiform green lights. The primary luminescence produced by *Aequorea* is actually bluish in color and is emitted by the protein aequorin. In a living jellyfish, light is emitted via the coupled green fluorescent protein, which causes the luminescence to appear green to the observer.

The figure and legend were kindly provided by Claudia Mills of the University of Washington, Friday Harbor. For further information, please see Mills, C.E. 1999–2000. Bioluminescence of *Aequorea*, a hydromedusa. Electronic Internet document available at <http://faculty.washington.edu/cemills/Aequorea.html>. Published by the author, web page established June 1999, last updated 23 August 2000.

Back cover (paperback): A portion of a human cDNA array hybridized with a red fluor-tagged experimental sample and a green fluor-tagged reference sample. Please see Appendix 10 for details. (Image provided by Vivek Mittal and Michael Wigler, Cold Spring Harbor Laboratory.)

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Protocol 22

Hybridization of Bacteriophage DNA on Filters

FILTERS CARRYING IMMOBILIZED DNA FROM PLAQUES ARE SCREENED by hybridization in situ with ^{32}P -labeled probes. The technique is extremely robust, highly specific, and very sensitive and allows the identification of a single recombinant among several thousand plaques. The plaque identified as hybridization-positive is then purified by subsequent rounds of screening. The overall sequence of events is presented in Figure 2-15. For further details on the hybridization of DNA immobilized on filters, please see Chapter 6, Protocol 10.

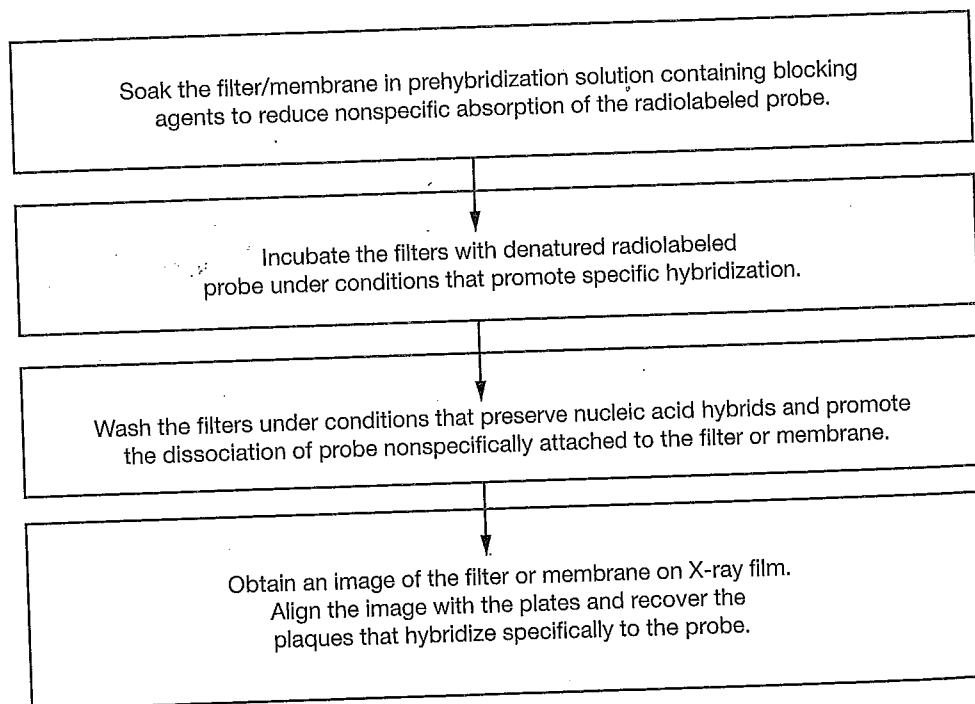


FIGURE 2-15 Flowchart: Sequence of Hybridization Steps

MATERIALS

CAUTION: Please see Appendix 12 for appropriate handling of materials marked with <!>.

Buffers and Solutions

Please see Appendix 1 for components of stock solutions, buffers, and reagents. Dilute stock solutions to the appropriate concentrations.

Chloroform <!>

Prehybridization solution <!>

Please see the panel on **PREHYBRIDIZATION AND HYBRIDIZATION SOLUTIONS** in Chapter 1, Protocol 32.

SM

2x SSPE

Depending on the number of filters to be processed, 1 liter or more may be required.

Wash solution 1

2x SSC

0.1% (w/v) SDS

Wash solution 2

1x SSC

0.1% (w/v) SDS

Wash solution 3

0.1x SSC

0.1% (w/v) SDS

Nucleic Acids and Oligonucleotides

Filters immobilized with bacteriophage DNA

Prepared as described in Protocol 21 of this chapter.

Radiolabeled probe <!>

Prepared as described in Chapter 9 or 10.

Special Equipment

Boiling water bath (for denaturing double-stranded probes)

Glass (Pyrex) baking dish or other hybridization chamber

ALTERNATIVE HYBRIDIZATION CHAMBERS

Some investigators prefer to incubate filters in heat-sealable plastic bags (Sears Seal-A-Meal or equivalent) (e.g., please see Chapter 6, Protocol 10) during the prehybridization and hybridization steps, rather than in crystallization dishes. The former method avoids problems with evaporation and, because the sealed bags can be submerged in a water bath, ensures that the temperatures during hybridization and washing are correct. The bags must be opened and resealed when changing buffers. To avoid radioactive contamination of the water bath, the resealed bag containing radioactivity should be sealed inside a second, noncontaminated bag.

If only a small number of filters are subjected to hybridization, then use a glass screw-top bottle that fits the rollers of a hybridization oven in place of a crystallization dish or Seal-A-Meal bag. These bottles and ovens have the advantage that small volumes of hybridization solution can be used and the hybridization temperature can be accurately controlled.

Glue stick, water-soluble (e.g., UHU Stic distributed by FaberCastell)

Incubation chamber preset at the appropriate hybridization temperature

Please see Step 2.

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.

Reusable alternatives to radioactive ink are chemiluminescent markers available from Stratagene (Glogos). The markers can be used multiple times and should be exposed to fluorescent light just prior to a new round of autoradiography.

Whatman 3MM paper

METHOD

1. If the filters are dry, float the baked or cross-linked filters on the surface of 2 $\times$ SSPE until they have become thoroughly wetted from beneath. Submerge the filters for 5 minutes.

Make sure that no air bubbles are trapped under the filters. The filters should change from white to a bluish color as the aqueous solvent penetrates the pores of the filter. Make sure that there are no white spots or patches remaining on the filters before proceeding to Step 2.

2. Transfer the filters to a Pyrex dish or other hybridization chamber containing prehybridization solution. Use 3 ml of prehybridization solution per 82-mm filter or 5 ml per 132-mm filter. Incubate the filters with gentle agitation on a rocking platform for 1–2 hours or more at the appropriate temperature (i.e., 68°C when hybridization is to be carried out in aqueous solution; 42°C when hybridization is to be carried out in 50% formamide).

Whatever type of container is used, the important point is that the filters are completely covered by the prehybridization solution. During prehybridization, sites on the nitrocellulose filter that nonspecifically bind single- or double-stranded DNA become bound by proteins in the blocking solution. Agitation ensures that the filters are continuously bathed in and evenly coated by the prehybridization fluid.

Whether or not to use a prehybridization solution containing formamide is largely a matter of personal preference. Both versions of the recommended 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. Nylon filters are impervious to the deleterious effects of aqueous hybridization at high temperatures.

3. Denature ^{32}P -labeled double-stranded probes by heating for 5 minutes at 100°C. Chill the probe rapidly in ice water. Single-stranded probes need not be denatured.

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 0.1 volume of 1 M Tris-Cl (pH 7.2) and 0.1 volume of 2.5 N HCl. After denaturation, store the probes in ice water until needed. At low temperature, the rate of reassociation of DNA is extremely slow and essentially the probe remains in a single-stranded form.

Between 2×10^5 and 1×10^6 cpm of ^{32}P -labeled probe (specific activity 5×10^7 cpm/ μg) should be used per milliliter of hybridization solution. Using more probe causes the background of nonspecific hybridization to increase; using less reduces the rate of hybridization.

If radiolabeled oligonucleotides are used as probes in this step, please see Chapter 6, Protocol 10, or Chapter 10, Protocol 8 for hybridization and washing conditions.

PURITY OF PROBES

It is essential that probes used to screen bacteriophage λ libraries contain no sequences that will hybridize to DNA sequences in the vector. Several bacteriophage λ vectors, such as $\lambda\text{gt}11$, λORF8 , lambda ZAP, and some of the λ Charon bacteriophages, contain the *E. coli lacZ* gene, either for immunochemical screening or as part of a plasmid integrated into the bacteriophage genome to assist with rescue of cloned sequences, or as part of the central stuffer fragment. Because DNA probes are often prepared from fragments of plasmids that contain all or part of the *lacZ* gene, even a small amount of contamination will cause the probe to hybridize indiscriminately to bacteriophage plaques. To ensure that a DNA fragment does not contain sequences that are complementary to the vector, we recommend (i) purifying the DNA successively through two gels (agarose or polyacrylamide) as described in Chapter 5, before carrying out the radiolabeling reaction, or (ii) preparing the probe by PCR amplification and purification (please see Chapter 8, Protocol 1).

4. Add the denatured probe to the prehybridization solution covering the filters. Incubate the filters for 12–16 hours at the appropriate temperature (please see Chapter 6, Protocol 10).
 During hybridization, keep the containers holding the filters tightly closed to prevent the loss of fluid by evaporation.
 To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6x SSC or 6x SSPE) at a temperature that is 20–25°C below the melting temperature (please see Chapter 1, Protocols 28–30 or Chapter 6, Protocol 10). Both SSPE and SSC work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6x SSPE is preferred because of its greater buffering capacity. For a general discussion of the factors that affect the rate and specificity of hybridization of radioactive probes to nucleic acids immobilized on solid supports, please see Chapter 6, Protocol 10.
5. When the hybridization is completed, *quickly* remove filters from the hybridization solution and *immediately* immerse them in a large volume (300–500 ml) of Wash solution 1 at room temperature. Agitate the filters gently, turning 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 or to stick together. Only a small fraction of the probe forms specific hybrids with the target sequences immobilized on the filters. Therefore, hybridization solutions may be recovered, stored, and reused for a second round of hybridization. However, the intensity of the signal obtained from recycled hybridization solution may be reduced for a variety of reasons: the reduced specific activity of the probe due to radioactive decay, degradation of the probe during incubation, and renaturation of double-stranded probe during the first hybridization step.
6. Wash the filters twice for 1–1.5 hours in 300–500 ml of Wash solution 2 at 68°C.
 With experience, it is possible to use a hand-held monitor to test whether washing is complete. If the background is still too high or if the experiment demands washing at high stringencies, immerse the filters for 60 minutes in 300–500 ml of Wash solution 3 at 68°C.
7. Dry the filters in the air at room temperature on sheets of Whatman 3MM paper or stacks of paper towels. Streak the underside of the filters with a water-soluble glue stick and arrange the filters (numbered side up) on a clean, dry, flat sheet of 3MM paper. Firmly press the filters against the 3MM paper to ensure that they do not move. Apply adhesive labels marked with radioactive ink or chemiluminescent markers to several asymmetric locations on the 3MM paper. These markers serve to align the autoradiograph with the filters. Cover the filters and labels with Saran Wrap/Cling Film. Use tape to secure the wrap to the back of the 3MM paper and stretch the wrap over the paper to remove wrinkles.
8. Expose the filters to X-ray film (Kodak XAR-2, XAR-5, or their equivalent) for 12–16 hours at –70°C with an intensifying screen.
9. Develop the film and align it with the filters using the marks left by the radioactive ink or fluorescent marker. Use a nonradioactive red fiber-tip pen to mark the film with the positions of the asymmetrically located dots on the numbered filters.
10. Identify the positive plaques by aligning the orientation marks with those on the agar plate.
 When duplicate sets of filters are hybridized to the same probe, there is less chance of confusing a background smudge with a positive plaque. Pick only those plaques that yield convincing hybridization signals on both sets of filters for further analysis. When screening a genomic library for a single-copy gene, expect to find no more than one positive clone per 10⁵ plaques screened. When screening cDNA libraries, the number of positives depends on the abundance of the mRNA of interest.
 Filters glued to 3MM paper are readily removed in preparation for stripping (please see Chapter 6, Protocol 10) by placing the 3MM paper with attached membranes in a tub of 2x SSC. The water-soluble glue dissolves in this solution, releasing the filters for transfer to a stripping solution and a round of hybridization to another probe, if desired.

11. Pick each positive plaque as described in Protocol 2 and store in 1 ml of SM containing a drop (50 μ l) of chloroform.

If the alignment of the filters with the plate does not permit identification of an individual hybridizing plaque, an agar plug containing several plaques in the area of interest should be cored using the large end of a sterile Pasteur pipette. Use a fresh pipette to core each hybridization-positive area.

12. To purify a hybridization-positive plaque, plate an aliquot (usually 50 μ l of a 10^{-2} dilution) of the bacteriophages that are recovered from the cored agar plug and proceed with subsequent rounds of screening by hybridization.

Ideally, in a second round plating, there should be ~300 plaques on a 100-mm plate. These plaques are then screened a second time by hybridization. Pick a single, well-isolated positive plaque from the secondary screen and subject it to additional rounds of screening until the stock is genetically pure and every plaque hybridizes to the probe of interest. Use a plaque derived from the final round of screening to make a stock as described in Protocol 3 or 4.

ADDITIONAL PROTOCOL: STRIPPING PROBES FROM MEMBRANES

Although in many cases, it is possible to remove probes from membranes after an image has been recorded and to reprobe the membrane with different probes, the following are some of the problems associated with this procedure.

- **Irreversible binding of the probe to the membrane.** Probes become irreversibly bound when nitrocellulose membranes and nylon membranes are allowed to dry for extended periods of time. If a given membrane is to be hybridized with more than one probe, every effort should be made to ensure that the solid support remains wet at all stages during hybridization, washing, and exposure to X-ray film or phosphor-imager cassettes.
- **Fragility of membranes.** Nylon membranes containing either genomic DNA or RNA can be stripped and rehybridized five to ten times. Nitrocellulose membranes, however, are more fragile and generally do not survive more than two or three cycles of hybridization and stripping.
- **Leaching of nucleic acids from the membranes.** A fraction of the DNA or RNA immobilized on the membrane leaches away during each cycle of hybridization and stripping so that the strength of the signal decreases progressively with each use. Nitrocellulose membranes are the worst offenders in this respect.

Method**Removal of a Hybridized Probe from a Nitrocellulose Membrane**

1. Prepare elution buffer by heating several hundred milliliters of 0.05x SSC, 0.01 M EDTA (pH 8.0) to boiling. Remove the fluid from the heat and add SDS to a final concentration of 0.1% (w/v).
2. Immerse the membrane in the hot elution buffer for 15 minutes. Rock or rotate the container during this time period.
3. Repeat Step 2 with a fresh batch of boiling elution buffer.
 ▲ **IMPORTANT** Do not allow the membrane to dry when transferring it between batches of hot elution buffer.
4. Rinse the membrane briefly in 0.01x SSC at room temperature. Blot most of the liquid from the membrane by placing it on a pad of paper towels, sandwich the damp membrane between two sheets of Saran Wrap, and apply it to X-ray film to check that all of the probe has been removed.
5. Dry the membrane, wrap loosely in aluminum foil or between sheets of blotting paper, and store at room temperature — preferably under vacuum — until needed. To rehybridize the membrane, place it in prehybridization solution and continue with Step 2 of Protocol 10.

Removal of a Hybridized Probe from a Charged or Neutral Nylon Membrane

Most manufacturers of nylon membranes provide instructions describing how various types of probes may best be stripped from their particular type of membrane. It is advisable to follow these instructions. Alternatively, treat the hybridized membrane with one of the three stripping solutions described in the table below.

1. Prepare several hundred milliliters of one of the recipes for stripping solution given in the table below, immerse the membrane, and treat as described to remove the probe and to wash the membrane.

Stripping solution	Treatment	Washing solution
1 M Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0), 0.1x Denhardt's reagent	2 hours at 75°C	0.1x SSPE at room temperature
50% formamide <1>, 2x SSPE	1 hour at 65°C	0.1x SSPE at room temperature
0.4 M NaOH <1>	30 minutes at 42°C	0.1x SSC, 0.1% SDS, 0.2 M Tris-Cl (pH 7.6) for 30 minutes at 42°C

2. Blot most of the liquid from the membrane by placing it on a pad of paper towels, sandwich the damp membrane between two sheets of Saran Wrap, and apply it to X-ray film to ensure that all of the probe has been removed.
3. Dry the membrane, wrap loosely in aluminum foil or between sheets of blotting paper, and store at room temperature — preferably under vacuum — until needed. To rehybridize the membrane, place it in prehybridization solution and continue with Step 2 of Protocol 10.