

Biotinylated Probes in Colony Hybridization

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1. Introduction

Colony hybridization is a procedure that allows the detection of cells containing nucleic acid sequences of interest (1). In this method, microbial colonies grown on, or transferred to, a supporting membrane are lysed and their nucleic acids denatured to single strands and fixed in place on the membrane. The membrane is then exposed to a similarly denatured "probe" sequence, which is identical or homologous to all or part of the target sequence, under conditions favoring reannealing. Probe sequences hybridize to complementary sequences on the membrane. Positive hybridization events are then detected by determining the presence and location of probe sequences on the membrane.

The original colony hybridization method described the use of radiolabeled probes and the detection of positive hybridization events by autoradiography (1). However, because of the high waste disposal costs, short half-lives, long autoradiographic exposures, and potential health hazards associated with radioisotopes, there is interest in alternative methods to detect positive hybridizations.

Nonradioactive technology involves the attachment to the nucleic acid probe of a ligand that can subsequently be detected by chemical or enzymatic methods. The vitamin biotin is one such ligand. Biotin can be covalently

incorporated into nucleic acids in a manner that does not interfere with their ability to hybridize with homologous sequences. This is accomplished by replacing a nucleoside triphosphate with its biotinylated analog in an *in vitro* DNA replication or transcription reaction, generating a biotinylated probe sequence (2,3). Hybridization of such a probe to a homologous sequence immobilized on a membrane results in the retention of biotin at that site. Positive hybridization events can then be detected by assaying for biotin.

Enzymatic reaction schemes that generate insoluble colored products at sites where biotin is bound to the filters have been developed for the purpose of biotin detection in these applications. These detection reactions employ either avidin or streptavidin, two functionally identical proteins that bind to biotin with very high affinities and specificities. These proteins are retained at sites where biotinylated probes have hybridized to homologous sequences. Avidin and streptavidin have multiple biotin binding sites per molecule. They therefore retain biotin binding capability even after binding to probe sequences on the membranes. Incubation with a biotinylated form of an enzyme (e.g., alkaline phosphatase) for which there exists an assay that generates an insoluble, colored product results in the retention of signal enzyme at sites of positive hybridization. These sites are detected by applying the histochemical assay for the signal enzyme.

To facilitate our work on plasmids with no known phenotype, we have developed a method for the use and detection of biotinylated probes in colony hybridization. It is suitable both for the detection of rare positive hybridization events over a background of nonreactive colonies and for the detection of nonhybridizing colonies in a population containing sequences homologous to the probe. The latter capability could be useful in such applications as the detection of cured (i.e., plasmid-free) cells in a bacterial population containing plasmids.

2. Materials

1. Nitrocellulose filters (82-mm diameter, BA 85) are obtained from Schleicher & Schuell (Keene, NH). (Other suppliers may be acceptable.)
2. Formamide is deionized by stirring for 30 min with 10% (w/v) of a mixed-bed ion exchange resin (e.g., Bio-Rad AG 501-X8, 20-50 mesh, Bio-Rad, Richmond, CA), filtering twice through Whatman (Clifton, NJ) no. 1 paper and storing single-use aliquots at -80°C .
3. Bovine serum albumin (BSA, Fraction V, Sigma, St. Louis, MO) is used as obtained. Fatty acid-free albumin gives poor results.
4. Denatured herring sperm DNA is prepared by dissolving in water (10 mg/mL) with stirring at room temperature, shearing by 10 passages through an 18-gage needle, and immersing in boiling water for 10 min.

Aliquots are stored at -20°C . Just prior to use, these are incubated for 10 min in a boiling water bath and chilled in ice water.

5. 20X SSC buffer: 3M Sodium chloride, 0.3M sodium citrate, pH adjusted to 7 with sodium hydroxide. Sterilize by autoclaving, store at room temperature.
6. Proteinase K is obtained from Beckman, Somerset, NJ. Other sources may be acceptable. In using alternate sources, the occurrence of blue backgrounds between colonies, and oversize, blurry signals at colony sites after the final color development step indicates insufficient proteolytic activity. Prepare a solution of 200 $\mu\text{g}/\text{mL}$ in 1X SSC.
7. 50X Denhardt's solution: 1% (w/v) Ficoll, 1% (w/v) polyvinyl pyrrolidone, 1% (w/v) BSA. Filter-sterilize. Store aliquots at -20°C . Do not flame the pipets used to transfer this solution. Denaturation and precipitation of the protein result from the use of hot pipets at this stage.
8. Template DNA for the production of hybridization probes must be pure. Standard methods, such as dye-buoyant density ultracentrifugation, generate acceptable products. Ethidium bromide and cesium chloride are removed prior to use of the DNA (4).
9. Biotin-11-deoxyuridine-5'-triphosphate (BiodUTP) and reagents for its incorporation into DNA by nick translation are obtained commercially. The products from Bethesda Research Laboratories (BRL, Gaithersburg, MD) are acceptable. BRL now provides a prepackaged kit (BioNick) containing necessary supplies and employing biotin-14-dATP as the source of biotin. The concentration of the resulting biotinylated DNA is determined by the histochemical method for biotin (*below*). Adequate instructions are provided with these kits.
10. Prehybridization solution: 50% formamide, 5X SSC, 5X Denhardt's solution, 25 mM sodium phosphate, pH 6.5, 300 $\mu\text{g}/\text{mL}$ freshly denatured sheared herring sperm DNA. Filter through Whatman no. 1 paper on a Buchner funnel, then through a sterile 0.45- μm filter. Store 10-mL aliquots in glass at -20°C . Use only once.
11. Hybridization solution: 45% Formamide, 5X SSC, 5X Denhardt's solution, 20 mM sodium phosphate, pH 6.5, 300 $\mu\text{g}/\text{mL}$ freshly denatured, sheared, herring sperm DNA, 200 ng of biotinylated DNA/mL. Before its addition, the biotinylated probe DNA is denatured by incubating for 10 min in a boiling water bath and quick-chilling in an ice bath. Shearing to reduce size is unnecessary, since the products generated by nick translation are sufficiently small. Filter and store the hybridization solution as was done for the prehybridization solution. Hybridization solution can be recovered after use and stored at -20°C . The solution can be reused at least 10 times over a time-span of at least 5 mo, without noticeable

- reduction in performance. The solution is heat-denatured as described for the herring sperm DNA preparation immediately before each use.
12. Reagents for the detection of filter-bound biotin are obtained from BRL (BlueGene Nonradioactive Nucleic Acid Detection System). Comparable materials are available from Bio-Rad Laboratories.
 13. Special equipment required for this protocol are a vacuum oven, a slab gel dryer, a device for the heat sealing of plastic bags (e.g., Seal-A-Meal, Sears Seal-and-Save), thin rubber sheet (such as dental sheet, A. H. Thomas, Philadelphia, PA), and a filtration device designed for the washing of nitrocellulose filters. The latter was originally described by Grunstein and Hogness (1) and is available from Schleicher and Schuell as the "Screen-It" colony filter hybridization device.
 14. 90% (w/w) Ethanol.
 15. Chloroform: Reagent grade.
 16. Solutions for the posthybridization washing of filters:
 - a. 0.1% (w/v) Sodium dodecylsulfate (SDS) in 2X SSC.
 - b. 0.1% (w/v) SDS in 0.2X SSC.
 - c. 0.1% (w/v) SDS in 0.16X SSC.
 - d. 2X SSC.

3. Methods

3.1. Filter Preparation and Cell Growth

1. Use a soft lead pencil to label nitrocellulose filters with a hash mark and letter or number on one edge to allow subsequent identification and orientation (*see* Note 1).
2. Place the labeled filters between sheets of filter paper, wrap in aluminum foil, and autoclave for 10 min.
3. Seal the packets of sterile filters in an air-tight bag and store at 4°C.
4. To inoculate, place a filter on top of the solidified media in a Petri dish and spread an appropriately diluted bacterial culture over the surface.
5. Incubate the plates until the cells are approx 1–3 mm in diameter (*see* Note 2). Cell densities of approx 800/82 mm diameter filter are compatible with single colony discrimination after hybridization and color assay. If one is attempting to locate positively hybridizing sequences in a generally nonreacting population, and single colony resolution is not required in the first detection step, as many as 10^5 cells can be applied to each filter.

6. Invert a filter and gently lay it onto fresh media just prior to lysis to create a replica of the colony pattern of the filter. (Mark the plate to indicate the orientation of the filter on it.) After an appropriate incubation, this becomes a master plate from which viable analogues of desirable colonies, as identified on the filter after hybridization and processing, can be recovered.

3.2. Cell Lysis

All operations are conducted at room temperature unless otherwise noted. After Steps 1–3, gentle suction is applied to the filters (*see* Note 3). Steps 2–4 are conducted in glass Petri dishes, one filter per dish. It has not been determined if these steps can be done batchwise. It is difficult to process more than 12 filters at a time.

To achieve lysis, incubate the filters in the following fashion (*see* Note 4):

1. 7 min, colony-side up, on filter paper sheets stacked to a thickness of 4 mm and saturated with fresh 0.5M NaOH.
2. 5 min in 1.5M sodium chloride, 0.5M Tris-HCl, pH 7.4, 30 mL/filter.
3. 1 h in prewarmed proteinase K in 1X SSC, 30 mL/filter, 37°C.
4. 2 × 2 min in 90% (w/w) ethanol, 30 mL/filter (*see* Note 5).
5. Air-dry, 20 min.
6. Wash each filter with 100 mL of chloroform using the Screen-It colony hybridization device. A single sheet of filter paper is used as an underfilter.
7. Air-dry (approx 15 min).
8. Sandwich the filters individually between filter paper, wrap loosely in aluminum foil, and bake at 80°C *in vacuo* for 2 h.
9. Store the filters in a vacuum desiccator at room temperature.

3.3. Prehybridization, Hybridization, and Detection of Hybridization

1. For prehybridization, place pairs of filters containing lysed, fixed colonies back to back in sealable plastic bags. Add 20 mL of prehybridization solution, seal the bag, seal it within a second bag, and incubate at 42°C for 2 h. Maintain the proper temperature by submersion in a water bath.
2. After prehybridization, replace the liquid with 20 mL of hybridization solution, exclude air bubbles, reseal the bags, and immerse in the water bath. Brief incubations (1 h) are sufficient for the detection of relatively abundant sequences, such as unamplified plasmid pBR322 in *E. coli*. More extensive incubations (45 h) may be necessary to detect less abundant sequences.

3. Following hybridization, wash the filters sequentially:
 - a. Twice in 250 mL of 0.1% (w/v) SDS in 2X SSC, 3 min per wash, room temperature;
 - b. Twice in 250 mL of 0.1% (w/v) SDS, 0.2X SSC, 3 min per wash, room temperature;
 - c. Twice in 250 mL of 0.1% (w/v) SDS, 0.16X SSC, 15 min per wash, 50°C; and
 - d. Briefly in 2X SSC at room temperature.
4. Detection of the sites of hybridization-dependent binding of biotinylated probe to the filters is most readily conducted with commercially available kits. Favorable results have been obtained with the BluGene Nonradioactive Nucleic Acid Detection System from BRL. Follow the manufacturer's instructions when carrying out the following steps. After washing, sequentially expose the filters to streptavidin and biotinylated alkaline phosphatase (or to a conjugate of these two proteins). This causes the immobilization of alkaline phosphatase at sites of positive hybridization.
5. Incubate the filters with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT). Indoxyl generated from BCIP by the action of alkaline phosphatase condenses to form indigo (blue). Indigo then reacts with NBT to form insoluble diformazan (purple).
6. Terminate the reaction when reacting colonies are intensely purple (*see* Note 6) by replacing the dye solution with 20 mM Tris-HCl, 5 mM EDTA, pH 7.5. Nonreactive colonies should be light blue on a white background.
7. Store the moist filters in sealed bags. The elapsed time from the end of hybridization to the termination of color development is approx 3 h. Figure 1 illustrates typical results obtained with this method.

4. Notes

1. Cellulose filters give unacceptably diffuse colony patterns after lysis and should not be used. Nylon filters should be acceptable, although we have not examined their suitability.
2. The lysis of colonies larger than specified above is generally acceptable. However, with relatively mucoid strains, such as *Xanthomonas*, the lysis of oversize colonies results in smeared colony patterns. The researcher should investigate the performance of younger cells if such behavior is experienced.

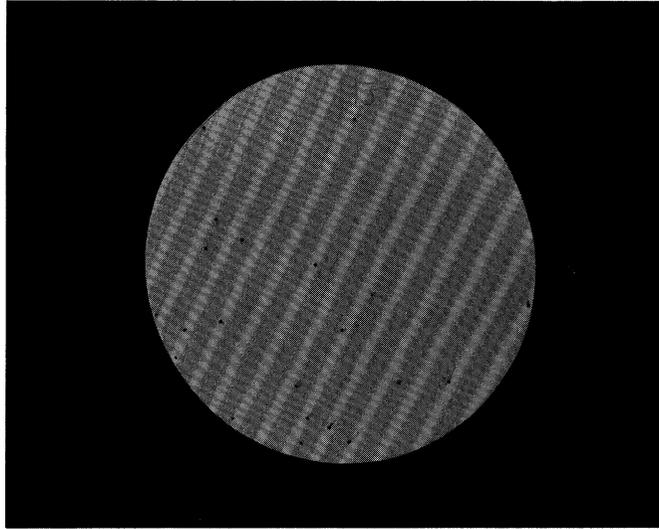


Fig. 1. Specific identification of *E. coli* containing plasmid pBR322. Approximately 225 colonies, consisting of a 10:1 mixture of plasmid-free and plasmid-containing cells, was grown on a nitrocellulose filter. The filter was subjected to the lysis protocol described here, followed by a hybridization with biotinylated pBR322. Sites of positive hybridization were detected by means of streptavidin and alkaline phosphatase. The dark sites correspond to colonies harboring pBR322. Plasmid-free cells give the faint signals present at numerous sites on the filter.

3. The application of gentle suction to the filters following Steps 1–3 of the lysis protocol reduces the dispersion of cells from their sites, promoting tighter patterns and stronger signals, and reducing the interference of signals from adjacent colonies with one another. Suction is applied by means of a slab gel dryer and a gentle vacuum source. A single sheet of filter paper serves as an underfilter. On this sheet is placed a template made by cutting into a sheet of flexible rubber holes slightly smaller than the nitrocellulose filters. The filters are placed over these holes and vacuum is applied. A brief suction suffices to remove excess moisture from the filters and to pull lysed colonies down onto them. Six filters can be treated at a time with a standard commercially available gel dryer with an 18 by 34 cm suction surface.
4. In our initial studies, the filters were swirled in the lysis solutions in an attempt to ensure lysis. After hybridization and application of the color assay, it was found that positively reacting colonies had “tails” extending away from them in a circular pattern across the filters. These tails

obscured the signals of adjacent colonies. Tailing was eliminated by omitting the swirling action during lysis. This omission did not noticeably reduce the efficiency or sensitivity of the detection reaction.

5. The ethanol concentration in Step 4 of the lysis protocol is a w/w concentration. Ethanol solutions made up v/v, or otherwise in excess of 90% w/w, exceed the ethanol tolerance limits of some batches of nitrocellulose. Filters washed in such solutions may become brittle and be reduced nearly to powder by the end of the hybridization-color assay procedure. The appropriate solution can be made from 100% ethanol.
6. The final color development step must be conducted under dim light (i.e., incubated in a drawer) since the reagents are light sensitive. Examine the filters at frequent intervals (10 min) during this incubation. Stop the reaction when the color of positively reacting colonies is deep purple. Further incubation past this point allows the color of nonreacting colonies to darken to such a degree that they are mistaken for positives. Overdevelopment is the greatest single factor contributing to the appearance of false-positive signals.
7. The minimal probe concentration necessary for efficient detection of target sequences has not been determined. It has been noted, however, that probe concentrations of 10 to 20 ng/mL, when coupled with overnight hybridizations, are too low to give strong signals for nonreiterated target DNAs 3 Mdalton or larger in size. Maas (5) has reported a simple modification of the Grunstein-Hogness protocol (1), which is reported to increase the sensitivity of the colony hybridization method by 100-fold. This could increase the ability to detect single copy sequences.
8. Nucleic acids can also be biotinylated by nonenzymatic methods with Photobiotin, a photoactivatable biotin analog (6), which can be commercially obtained from BRL, Sigma Chemical Co., and other commercial sources. We have not compared the suitability of this method of biotin incorporation with that reported here, but expect that the method would be fully acceptable. FMC (Rockland, ME) markets an alternate nonradioactive sequence detection kit known as Chemiprobe. The basis of this system is a chemical modification of cytosine residues in the probe DNA. After hybridization, the probe is detected by means of a monoclonal antibody that specifically recognizes the sulfonated DNA. Detection of the bound monoclonal antibody is achieved by means of an alkaline phosphatase conjugated second antibody.

References

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