Chapter 1

Non-Radioactive DNA Hybridization Experiments for the Undergraduate Laboratory: The Southern Blot Analysis

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Reprinted from: Karcher, S. J. 1991. Non-radioactive DNA hybridization experiments for the undergraduate laboratory: the southern blot analysis. Pages 1–31, *in* Tested studies for laboratory teaching. Volume 12. (C. A. Goldman, Editor). Proceedings of the 12th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 218 pages.

Contents

Introduction

The detection of homologous DNA sequences through the method of Southern blotting has made a tremendous contribution to the fields of molecular biology and recombinant DNA technology. Southern blotting is an essential method for basic research problems such as the understanding of gene structure, gene expression and genome organization (Meinkoth and Wahl, 1984). Increasingly, Southern blotting has a role in the diagnosis of heritable diseases and in the detection of microbial and viral pathogens (see Lerman, 1986; and Willey, 1988). Southern blot analysis also has forensic applications (Neufeld and Colman, 1990). The standard radioactively-labeled DNA detection system used in such experiments is expensive, and requires licensing to handle radioactive materials. In addition, special consideration must be given to the safety of handling radioactive materials. The development of a non-radioactive biotinylated nucleic acid detection system has made such DNA hybridization experiments safe and feasible for the undergraduate biology laboratory (Freeman, 1984; Leary et al., 1983).

This chapter presents the protocols to teach a DNA Southern blotting experiment in an undergraduate laboratory. Methods, including isolation of plasmid DNA, agarose gel electrophoresis of DNA, transfer of nucleic acids to nitrocellulose (a Southern blot), biotin-labeling of DNA, and detection of the biotin-labeled DNA through a chromogenic assay are presented in detail. Figure 1.1 outlines the steps used in Southern blot analysis. Figure 1.2 presents a schematic of the preparation of a Southern blot.

This experiment is a part of a molecular genetics laboratory class taught to college juniors and seniors and masters graduate students in the Department of Biological Sciences at Purdue University. Students are delighted to be able to perform the type of Southern blotting experiments that they have read of so often in their lecture classes. The biotinylated-DNA detection system has made these techniques available to the undergraduate.

1 – Isolate DNA
2 – Digest with restriction enzyme (cuts DNA at specific sequences)
3 – Gel electrophoresis (separates DNA fragments on basis of size)
4 – Stain and photograph gel
5 – Denature and neutralize gel, transfer DNA to nitrocellulose filter paper (BLOT)
6 – Hybridize with specific DNA (PROBE)
7 – Detect specific DNA sequence

Figure 1.1. The steps of a Southern blot analysis.

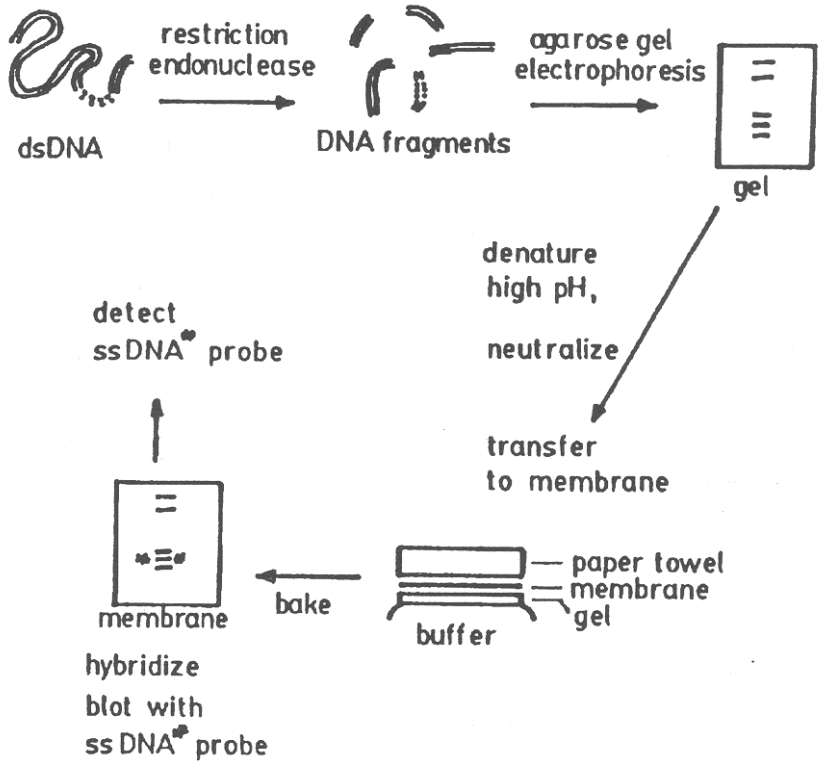

Figure 1.2. The steps in the preparation of a Southern blot experiment.

Southern blotting is a fundamental technique with such wide ranging applications that an understanding of this method should be presented to the beginning undergraduate and perhaps even the advanced high school student.

The experiments given here allow the student to perform all the parts of a Southern blot analysis. However, because of time constraints, it may be necessary for the instructor to do parts of the procedures for the students (with or without help from individual students) outside of the regularly scheduled laboratory class times. Table 1.1 presents a suggested time table for the experiments, as well as suggestions for parts to be done by the instructor.

Table 1.1. Timetable of procedures for a Southern blot analysis.

Procedure*	Laboratories Required (2 hours)	Comments
1 – DNA isolation (Experiments 1 and 2)	1–2	May be done outside of class by instructor or may be purchased.
2 – Restriction enzyme digestion and gel electrophoresis (Experiment 3)	1–2	If step 3 is to be done, prepare gel for step 3 at same time as gel for step 4.
3 – Isolation of DNA fragment from gel for probe (Experiment 4)	1	May be done ahead by instructor. In some hybridizations, entire plasmid can be used as probe.
4 – Southern transfer (Experiment 5)	1	Instructor bakes blots outside of scheduled laboratory.
5 – Label probe DNA with biotin by nick translation (Experiment 6)	1	May be done ahead by instructor or do during step 6 prehybridization.
6 – (a) Prehybridization, (b) Hybridization, (c) Washes (Experiment 7)	1	Hybridization overnight. Washes done outside of scheduled laboratory.
7 – Detection of hybridized probe (a) Blocking in BSA (b) Add SA-AP (c) Add chromogenic substrate (Experiment 8)	1	Begin blocking 1 hour before scheduled laboratory.

* Steps 2, 4, 6, and 7 are the minimum set of experiments for students.

A basic Southern blot experiment is available in kit form from several companies (see Appendix A, item 12). These kits come with all the materials needed except gel electrophoresis equipment and water baths. Such kits may be the method of choice for teaching a Southern blot experiment in the lowest-level classes.

At the present time, many teaching laboratory classes include experiments where DNA is digested with restriction endonucleases and separated by gel electrophoresis. It is important to note that Southern blot analysis is a logical next step in DNA analysis, making use of the gel generated in the previous experiment. With a bit of planning, Southern blot analysis can be done on a gel that has been generated in a previous experiment.

A simple form of the Southern blot is a plasmid-to-plasmid hybridization, where plasmid DNA digested with restriction enzymes is run on a gel and blotted. A piece of the plasmid (a cloned fragment or a fragment isolated from a gel via Experiment 4) is used as a hybridization probe. Such experiments are used to map the location of the fragment and to construct maps of restriction endonuclease sites. Appendix B gives the details of such a plasmid-to-plasmid hybridization. Purified plasmid DNAs can be purchase if the class schedule and instructor's schedule do not allow enough time for plasmid isolation (Experiments 1 and 2).

A more advanced genomic Southern blot analysis could be done in more advanced laboratory classes. In such an experiment, genomic (chromosomal) DNA is isolated from an

organism, cut with restriction endonucleases, run on a gel and blotted. A cloned piece of DNA from the organism or a related organism could then be used as a probe for hybridization. Such experiments give information about the genomic organization of the organism studied. Questions such as the number of copies and the location of sequences homologous to the probe can be answered. For example, Dellaporta et al. (1983) describe a simple method to isolate plant DNA. Plasmids that can be used as probes for high and low copy number plant sequences are available from the author upon request.

The experiments presented here use nick-translation (Experiment 6) to biotin label the probe DNA. Alternative methods to label DNA are mentioned in Appendix A, item 8. Detailed protocols for alternative labeling methods can be found in Forster et al. (1985), Maniatis et al. (1982), and McInnes et al. (1987).

The biotin-labeled DNA is detected because of the tight and specific binding of streptavidin to biotin. The streptavidin has conjugated to it the enzyme alkaline phosphatase. A substrate for alkaline phosphatase is then added. The experiments described in this chapter use a chromogenic (color-generating) substrate for alkaline phosphatase. An alternative is the use of a chemiluminescent substrate for alkaline phosphatase (Bronstein and McGrath, 1989; Carlson et al., 1990; Karcher and Goodner, 1990).

Other non-radioactive DNA detection systems that do not use biotin are listed in Appendix A, items 10 and 11. (Also see Tchen et al. (1984).) Beck (1987) and Kidd (1984) mention advanced uses of non-radioactive DNA detection systems.

Experiment 1:
Plasmid Isolation using Alkaline Lysis and PEG Precipitation

Introduction

The first step in a Southern blot analysis is isolating DNA to be digested with restriction endonucleases and the subsequent electrophoresis of this DNA. This first experiment describes the isolation of plasmid DNA. The plasmid DNA will be used for a plasmid-to-plasmid Southern blot analysis. This procedure is also used to prepare cloned DNA fragments to be used as hybridization probes. The separation of plasmid DNA from *E. coli* chromosomal DNA is the crucial part of this procedure.

The "classical" method of plasmid purification uses a cesium chloride-ethidium bromide density gradient generated by ultracentrifugation. The supercoiled plasmid DNA binds a limited amount of ethidium bromide, has a limited density shift due to the binding of ethidium bromide, and sediments at a lower density in a cesium chloride density gradient than does chromosomal DNA. See Chapter 3 in Rodriguez and Tait (1983).

The procedure described in this chapter is a simpler method to isolate plasmid DNA. It uses lysis of bacterial cells at alkaline pH and centrifugation to separate plasmid from chromosomal DNA. In this alkaline lysis procedure, DNA is denatured by the high pH of Solution II. Solution III returns the lysis mixture to a neutral pH. The supercoiled plasmid DNA renatures relatively rapidly (the two strands of the supercoiled covalently closed circular DNA remain intertwined). The chromosomal DNA fragments do not renature as rapidly and are pelleted from the solution along with denatured proteins while the plasmid DNA remains in solution. For additional information about this procedure see Sambrook et al. (1989).

The procedure described below can be completed in one 3-hour or two 2-hour laboratory periods.

Materials

L-broth

Per liter: 10 g Tryptone

5 g Yeast extract

5 g NaCl

Adjust to pH 7.0 with 1 M NaOH

Solution I: 50 mM Glucose
25 mM Tris, pH 8.0 (Tris[hydroxymethyl] amino-methane, Trizma Base)

10 mM EDTA (Ethylenediamine tetraacetic acid, also called ethylenedinitrilo tetraacetic acid)

Adjust pH 8.0 with NaOH or HCl

Solution II: 0.2 M NaOH
1% SDS (Sodium dodecyl sulfate, a detergent; also called lauryl sulfate)

Make fresh each time, for 100 ml: 10 ml 2 M NaOH, 5 ml 20% SDS

Solution III: 3 M KOAc, pH 5.5 (Potassium acetate)
Adjusted to pH 5.5 with glacial acetic acid

Lysozyme

Phenol:chloroform (1:1)

Use phenol crystals, such as Baker reagent grade phenol crystals. When melted, the phenol should be colorless or have only a slight amount of color. Phenol which is colored deep yellow or deep pink should not be used.

Melt 1 pound phenol at 65°C. (This is about 440 ml.) Add 440 ml of chloroform. Mix. Add 26.4 g NaCl. Mix. Add 440 ml 2 M Tris, pH 7.0. Mix. Remove the aqueous (top) phase. Add 440 ml 50 mM Tris pH 8.0. The solution is now ready for use. Store at 4°C.

PEG (Polyethylene glycol (average molecular weight 8,000) 13% wt/vol); dissolve 1.3 g in H_20,

adjust final volume to 10 ml.

3 M NAOAc (Sodium acetate)

Ethanol

RNaseA (10 mg/ml)

To prepare: Dissolve 1 g RNaseA in 100 ml of 10 mM Tris (pH 8.0), 1 mM EDTA, 10 mM NaCl. Boil solution for 5 minutes. Let cool slowly at room temperature. Aliquot into sterile tubes. Freeze at -20°C.

Centrifuge

Procedure

1. Inoculate 2 ml L-broth with appropriate bacterial strain. Incubate 6–16 hour at 37°C with shaking.
2. Use primary inoculum to inoculate 1 liter of L-broth containing the appropriate antibiotic, incubate 8–16 hours at 37°C with shaking.
3. Harvest the cells by centrifugation at 8,000 rpm for 5 minutes.
4. Re-suspend the cells in 7 ml Solution I.
5. Transfer the suspension to a 40-ml Oak Ridge tube (a screw-capped centrifuge tube). Add 1 ml Solution I with 10 mg lysozyme freshly dissolved in it. Mix, and leave at room temperature for 10 minutes.
6. Add 16 ml of Solution II, freshly prepared, and mix gently by inverting the centrifuge tube.
7. Incubate on ice for 5 minutes.
8. Add 12 ml *cold* Solution III, mix gently by inverting the centrifuge tube.
9. Incubate on ice for at least 15 minutes.
10. Centrifuge at 12,000 rpm for 5 minutes.
11. Transfer the supernatant solution (carefully avoiding the white precipitate) to a 250-ml centrifuge bottle containing 50 µl of RNaseA (10 mg/ml). Incubate at 37°C for 30 minutes.
12. Add 0.6 volumes of cold isopropanol. Centrifuge at 8,000 rpm for 5 minutes.
13. Decant and discard the supernatant solution. Re-suspend the pellet in 5 ml H_2O, and transfer to a 15-ml Corex tube.
14. Add 5 ml phenol:chloroform, mix well, centrifuge to separate the phases. Carefully remove and save the top (aqueous) phase.
15. Repeat the phenol extraction of the aqueous phase 2–3 times until the interface is clear.
16. Add 0.5 ml of 3 M NaOAc and 10 ml of ethanol. Mix.
17. Centrifuge at 10,000 rpm for 10 minutes. Discard supernatant solution.
18. Dissolve the pellet in 367 µl of H_2O. Add 133 µl of 3M NaCl and mix. Add 500 µl of 13% PEG (freshly made) and mix. Incubate in ice water for 1 hour.
19. Spin in microfuge for 10 minutes.
20. Wash the pellet with 70% ethanol. Dry the pellet.
21. Resuspend the pellet (DNA) in appropriate volume of 1 mM Tris-0.1 mM EDTA (pH 7.0)
    - For high copy number plasmids, such as pUC and pBR plasmids, use 0.5 ml.
    - For low copy number plasmids, such as pRK plasmids, use 0.1 ml.

Note: If RNase action has been complete, the PEG treatment can be eliminated. The resulting DNA can be used as is.

The concentration of DNA can be determined spectrophotometrically. Dilute DNA in H_2O or a buffer. (Use the same buffers as a blank for the spectrophotometer.) Read the absorbance at 260 nm and at 320 nm. 50 µg/ml DNA has an absorbance of 1.00 in a 1-cm wide cuvette.

$$\text{Concentration of DNA } (\mu\text{g/ml}) = (A_{260} - A_{320}) \times \frac{50\ \mu\text{g/ml}}{1\ \text{absorbance unit}} \times \text{dilution}$$

Alternatively, the concentration of DNA can be estimated by comparing the intensity of bands of the unknown DNA with similar-sized DNAs of known concentration on a gel stained with ethidium bromide.

Experiment 2:
Alkaline Mini-prep Procedures

Introduction

This protocol provides a rapid way to isolate a small amount of plasmid DNA. It is especially useful when many different plasmids from different *E. coli* strains are to be isolated and then analyzed. It is a rapid way to analyze recombinant DNA plasmids.

This protocol can be used to isolate plasmid DNA to be digested with restriction enzymes, separated by gel electrophoresis, and then analyzed by Southern blot analysis. This procedure can be completed in one 3-hour or two 2-hour laboratory periods.

Materials

For the following materials see Experiment 1:

L-broth
Solution I
Solution II
Solution III
Phenol:chloroform (1:1)

TES: 50 mM Tris-Cl, pH 8.0
20 mM NaCl
5 mM EDTA

RNaseA (10 mg/ml)

37°C shaking water bath
Microfuge
Sterile microfuge tubes
Sterile tips for pipetman
Pipetters
Ethanol
3 M NaOAc

Procedures

1. Grow up 1-ml overnight cultures in L-broth of bacteria to be analyzed.
2. Transfer cultures to microfuge tubes. Centrifuge 30 seconds in a microfuge to pellet bacterial cells.
3. Resuspend cells in 100 µl alkaline extraction Solution I.
4. Immediately add 200 µl of freshly prepared Solution II, mix by inverting the tube (and let tubes sit on ice briefly). Tubes may be kept on ice up to 15 minutes until step 5.
   Prepare fresh Solution II by combining 1 ml 2 M NaOH, 0.5 ml 20% SDS, and 8.5 ml H_2O.
   (Upon addition of Solution II to the bacteria, you will see the solutions begin to clear indicating that the NaOH has lysed the cells.)
5. Add 150 µl of cold solution III to each tube, mix by inverting the tubes, and let tubes sit on ice until you are able to spin in step #6.
6. Centrifuge tubes 5 minutes in microfuge.

(Solution III is added to bring the solution back to a neutral pH. A white precipitate of denatured proteins and cell debris is formed. Much of the chromosomal DNA (being larger) is trapped in this material and is removed from the solution, while the supercoiled plasmid DNA is not. The small RNAs also remain in solution.)

7. Decant the supernatant solution into new microfuge tubes.
8. Add 200 µl phenol:chloroform (1:1) to each tube. (Wear gloves and goggles or glasses and use caution when handling phenol:chloroform.) Vortex samples to mix the phases.
   (The phenol:chloroform step extracts additional proteins from the solution.)
9. Centrifuge samples for 1 minute in microfuge.
10. Using a pipetman or a Pasteur pipet, transfer the upper (aqueous) phase to new microfuge tubes. Be very careful *not* to pick up any of the interface or phenol phase.
11. Add 300 µl cold isopropanol to each tube. Mix thoroughly. (The nucleic acids DNA and RNA are precipitated in this step.)
12. Centrifuge tubes in microfuge for 5 minutes.
13. Decant and drain pellet. (Blotting excess liquid onto clean tissues or paper towels is helpful.)
14. Resuspend pellet in 50 µl TES plus RNaseA (to 1 ml TES add 20 µl 10 mg/ml RNaseA). Incubate for 5 minutes at 37°C. (This brief RNase treatment will remove RNAs from the samples.)
15. Add 5 µl 3M NaOAc and 125 µl cold ethanol to each sample to precipitate DNA. (Samples can be stored at -20°C indefinitely.)
16. Centrifuge samples for 5 minutes in microfuge. Decant ethanol, dry pellets completely. Resuspend pellets in 30 µl 1 mM Tris, 0.1 mM EDTA, pH 7.5. Vortex pellets to aid resuspension.

Samples are now ready for restriction endonuclease digestions. Typically, a 10 µl-aliquot is used per restriction enzyme reaction.

Alternate Procedure

Steps 1 through 3 can be eliminated as follows: Have bacterial colonies to be analyzed grown in long streaks or patches on an agar plate. Using a sterile toothpick, remove most of the bacteria from the streak and put them in the 100 µl of solution as described in step 3. Then proceed to step 4.

Experiment 3:
Restriction Endonuclease Digestion and Gel Electrophoresis of DNA Samples

Introduction

In this experiment, DNA is digested with restriction endonucleases. The DNA fragments generated are separated on the basis of size by gel electrophoresis. This procedure takes 1 hour for restriction endonuclease digestion and 1 hour for gel electrophoresis. (See Chapters 4 and 5 in Rodriguez and Tait (1983).)

Cautions

1. Restriction endonucleases should be kept on ice at all times while they are being used. Long term storage of restriction enzymes should be in a -20°C freezer that is *not* a frost-free type.

2. Ethidium bromide should be handled *very carefully*. It is a known mutagen and may be a carcinogen. Wear protective disposable plastic gloves when handling ethidium bromide. See Quillardet et al. (1988) and Appendix E.9 in Sambrook et al. (1989) for a discussion of safety when working with ethidium bromide.

   Note: There is an alternative, far safer method of staining DNA than the use of ethidium bromide. Methylene blue may be used to stain nucleic acids (De Wachter, 1971). This staining takes several hours and may be allowed to go overnight. The gel is then examined under white light and the DNA bands are stained blue. Unfortunately, the methylene blue method to stain DNA is far slower than the ethidium bromide method (3–12 hours for methylene blue, 5–20 minutes for ethidium bromide). In addition, methylene blue staining of DNA is approximately 10 times less sensitive than ethidium bromide staining.

3. The light emitted from a UV transilluminator can burn skin and damage eyes. *Always* wear UV protective glasses, a UV shield for the face, lab coat, and gloves to eliminate or reduce exposure to the UV light.

Materials

DNA to be digested with restriction enzyme
Restriction endonucleases (restriction enzymes)
10X restriction enzyme buffer (salts)
TBE gel buffer
    10X TBE buffer: 0.89 M Tris (Trizma Base), pH 8.0
                    0.89 M Boric acid
                    0.02 M EDTA (free acid)
Stop mix (Stop buffer, gel loading buffer):
                    50% Glycerol
                    0.7% SDS
                    0.1% BPB (bromophenol blue, a dye)
Sterile microfuge tubes
Sterile pipet tips
Pipettors/pipetman
37°C water bath
Microfuge
Gel electrophoresis apparatus: gel box, tray, comb, power supply
UV transilluminator
Agarose
Ethidium bromide

    Stock 10 mg/ml, dissolved in distilled H_20. *Caution:* Wear gloves and dust mask when working with powdered ethidium bromide. Store in refrigerator in a bottle wrapped in aluminum foil.

Procedures

Part 1: Restriction Endonuclease Digestion

1. Set up restriction endonuclease digestion of DNA sample.

   For example, in a microfuge tube, put:

   5 µl (= 0.5 µg) of a plasmid DNA
   (Typically, 0.5 to 1.0 µg of a plasmid DNA or 5 µg of plant chromosomal DNA for a genomic Southern blot analysis would be digested and run on one lane of a gel.)

   1 µl 10X restriction enzyme digestion salts
   (Note the salts are a 10X concentrated stock that must be diluted 10 fold to provide the appropriate salt conditions for optimum restriction endonuclease activity. That is, use 1/10th of final volume of reaction mixture.)

   3 µl distilled H_2O
   (To bring reaction mixture to desired final volume.)

   1 µl restriction enzyme
   (Usually 1 unit of restriction enzyme per mg of DNA is used to cut a plasmid DNA, and 5–10 units are used per mg of DNA for bacterial DNA or eukaryotic genomic DNA.)

2. Allow digestion to proceed at desired temperature. For most restriction enzymes, 37°C for 1 hour.
3. After the digestion time, add 3 µl stop buffer to inactivate the restriction enzyme and to prepare the sample for loading onto a gel.

Part 2: Gel Electrophoresis

1. Prepare agarose gel. Weigh out desired amount of agarose into an Erlenmeyer flask. For 50 ml of a 1% (1 g/100 ml) agarose solution, you need 0.5 g.
2. Add the appropriate volume of 1X TBE buffer.
3. Microwave or boil the agarose solution to dissolve all the agarose. Cool to about 45°C. (Note that many plastic gel casting trays will warp if subjected to higher temperatures.)
4. Pour gel. Allow the agarose to solidify.
5. Put gel in gel box with 1X TBE buffer.
6. Load DNA samples into wells of gel.
7. Subject DNA samples to electrophoresis. Typically for a 1% agarose mini-gel, the conditions are 100 volts for about 1 hour.

Part 3: Visualization of DNA Bands

1. Prepare ethidium bromide (EtBr) staining solution: dilute 100–200 µl of stock 10 mg/ml EtBr into 200 ml of H_2O.
2. Soak gel in EtBr staining solution.
3. Observe DNA bands in gel with UV transilluminator.

Caution: EtBr is a mutagen! Always wear gloves when handling it.

Experiment 4:
Isolation of DNA Fragments by Electroelution

Introduction

This method uses Pharmacia NA agarose to prepare gels for the purification of DNA fragments. The DNA obtained can readily be used in a nick-translation (to label a DNA to use as a probe) or ligation reaction (to clone a piece of DNA).

The method of electroelution allows the isolation of a particular band of DNA from an agarose gel. The agarose (Pharmacia NA) used for electroelution is more highly purified than many agaroses, with lower amounts of sulfated polysaccharides. Polysulfonates can co-purify with DNA and interfere with subsequent enzymatic reactions.

For a discussion of this and other methods of isolation of DNA fragments from gels see Zassenhaus (1982). In this experiment, a fragment of DNA will be isolated to use as a probe in the Southern blot experiment. Time required: 1 hour for restriction endonuclease digestion, 1 hour for gel electrophoresis, 1–2 hours for electroelution.

Materials *(For electroelution)*

NA agarose
Plasmid DNA, digested with appropriate restriction endonuclease
Apparatus for gel electrophoresis: gel box, power supply, ultraviolet transilluminator
Dialysis tubing
Isopropanol or ethanol
1X TBE (Tris borate gel buffer, see Experiment 3)
0.5X TBE (half-strength 1X TBE solution)

Procedures

1. Digest the plasmid DNA with the appropriate restriction enzyme, in a final volume of 100–150 µl.
2. Using Pharmacia NA agarose, make a 1% gel in Tris-borate gel buffer. For a minigel apparatus, use 30–50 ml of gel.
3. After checking that digestion is complete, load the DNA into the broad slot of the Pharmacia minigel, and subject to electrophoresis.
4. Stain the gel in ethidium bromide solution and examine the gel on the UV transilluminator. Cut a strip out of the gel that contains the fragment of interest.
5. Cut a piece of dialysis tubing to enclose the gel strip, rinse it in 0.5X TBE gel buffer. Fill the tubing with buffer, after tying off one end, then slide the excised gel fragment into the tubing. Pour out the excess buffer, and knot the end of the bag, making sure that there are no air bubbles trapped inside.
6. Fill a minigel apparatus with 0.5X TBE gel buffer, and immerse the tubing with the gel fragment in the buffer, arranged perpendicular to the direction of electrophoresis.

7. Electroelute the fragment at 100 mA for 1–2 hours. Inspect the slice with UV light to make sure all the DNA has migrated out of the agarose.
8. Wipe the outside of the dialysis tubing with a tissue, then squeeze the tubing to disperse the electroeluted DNA in the buffer that is in the sack. All these operations can be monitored by looking at the fluorescence under UV light.
9. Cut open one end of the bag. Collect all the liquid into a microfuge tube. One should end up with 200–500 µl of solution. Measure this volume, and add 1/10 volume 3 M NaOAc. Precipitate the DNA by adding either one volume isopropanol or two volumes ethanol. Store in freezer at -20°C overnight. Pellet the DNA by centrifugation. Decant the alcohol. Rinse with 70% ethanol, and dry pellet completely. Resuspend in about 20–50 µl 1 mM Tris-0.1 mM EDTA, pH 7.

Yields should be 90% or greater. There may be some agarose remaining in the DNA sample, but this doesn't interfere with any enzymatic reactions.

Experiment 5:
Preparation of a Southern Blot

Introduction

"Blotting" is the process of transferring nucleic acids or proteins from a gel to a membrane such as nitrocellulose paper. See Dillon et al. (1985), Old and Primrose (1985), and Southern (1975).

To prepare a Southern blot, the double-stranded DNAs must be denatured before transferring because only single stranded nucleic acids bind to nitrocellulose. In addition, the DNA must be single stranded to be able to base-pair (hybridize) with the probe.

By capillary action, the DNA molecules are drawn from the gel to the nitrocellulose membrane, where they bind to the membrane. The baking step fixes the DNA to the membrane. The single-stranded DNA molecules can be thought of as tethered to the membrane, having a portion of the DNA molecule attached to the membrane while the rest of the DNA molecule is free to base pair with a complementary single stranded DNA probe. For a review of the Southern blot technique see Meinkoth and Wahl (1984).

Starting with a stained agarose gel, this procedure will take 2–3 hours to soak the gel in the denaturation and neutralization solutions and set up the Southern transfer. The transfer can be left for up to 24 hours. Sometime the next day, the membrane should be removed. The membrane need not be baked immediately, but it must be baked before hybridization.

There are many different membranes available for blotting procedures. When using a different membrane, follow the supplier's suggested procedures for transfer. (For example: with Nytran 10X, SSC is recommended for the transfer buffer, while 20X SSC is used for nitrocellulose.)

Nitrocellulose must be handled with care because it is rather brittle. Nitrocellulose is recommended in these experiments because it has been found to give lower backgrounds than other membranes in the biotin-strepavidin chromogenic DNA detection system.

Materials

Denaturation solution:0.5 M NaOH, 1.0 M NaCl
Neutralization solution: 0.5 M Tris pH 7.0, 1.5 M NaCl
Transfer buffer (20X SSC–standard sodium citrate–for nitrocellulose membrane):
3 M NaCl, 0.3 M Na_3 citrate pH 7 (adjust to correct pH with HCl)

Whatman chromatography paper (#1)
Nitrocellulose paper
Glass baking dish or plastic box larger than the gel by at least 1 inch in all directions
Rocking platform (optional)

Procedures for "Blotting" a Gel: Preparation of a Southern Blot

Preparing Gel

1. Double-stranded DNA is subjected to electrophoresis through an agarose gel. When DNAs have migrated a sufficient distance, the gel is stained with EtBr (ethidium bromide) at 1 μg/ml, and photographed on a UV light box.
2. To denature the DNA in the gel, soak the gel for 1.5 hour in 1 liter of denaturation solution. The gel can be rocked on a rocking platform during this time (1.5 hour is needed to denature the DNA in a thick, high percentage agarose gel. A shorter soaking time, such as 20–30 minutes, is sufficient for a thin, low percentage gel.)
3. Rinse the gel in deionized H_2O.
4. To neutralize the gel after the base-treatment, rock the gel in 1 liter of neutralization solution for 1.5 hour.
5. The gel is now ready to be blotted.

Blotting Gel

1. Obtain a glass baking dish or plastic box at least 1 inch larger on all sides than the gel, a glass plate large enough to hold the gel, and four (4) bottle caps to support the glass plate. Wear gloves when handling these items. Wrap the glass plate with a piece of Whatman (#1) chromatography paper cut large enough to surround the glass plate completely. Put the glass plate on the supports (bottle caps) in the baking dish. (See Figure 1.3: set-up for Southern blot transfer.)
2. Pour transfer buffer (bottle caps) into baking dish, wetting the filter paper. The level of transfer buffer should not quite reach the glass plate. The filter paper will act as a wick.
3. Place the gel on the wetted filter paper on the glass plate. Mark a gel corner with a small notch to indicate the orientation of the gel. (A horizontal gel should be placed on the Whatman filter paper *upside down* so the nitrocellulose paper will adhere to the bottom of the gel. Because of the way a horizontal gel is cast, the bottom of the gel will be a smoother surface.)
4. Using a very sharp razor blade, cut a piece of nitrocellulose paper to fit the gel *exactly*. Gloves should be worn when handling the nitrocellulose paper. A dull blade will tear the nitrocellulose paper. The nitrocellulose paper can be marked with a pencil.
5. Wet the nitrocellulose paper with distilled H_2O: lay paper on top of a tray of H_2O and allow it to soak; do not submerge paper. When one side is wetted, flip paper over to wet other side. Handle the nitrocellulose paper with a forceps or gloved hands.
6. Once the nitrocellulose is totally wetted, soak paper in transfer buffer.

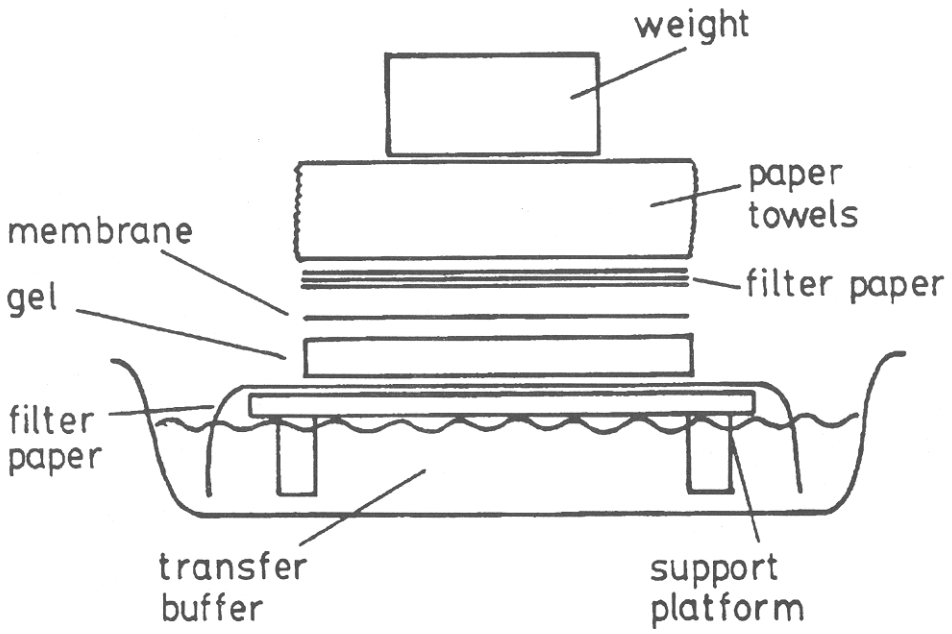

Figure 1.3. How to set up a Southern blot transfer.

7. Place nitrocellulose paper on top of gel, taking care to position the paper precisely. Be especially careful to line up the top of the paper with the top of the gel (sample wells). Also take great care to avoid any air bubbles between the gel and the paper.
8. Cut 4–5 pieces of Whatman filter paper exactly to the size of (or just slightly smaller than) of the nitrocellulose paper. Wet two (2) pieces of filter paper in the transfer buffer and place on top of the nitrocellulose paper. Avoid air bubbles. Then place 2–3 pieces of dry filter paper on top. *Do not* allow paper to hang over edge so that it might act as a wick.
9. Cut a stack of paper towels at least 2 inches high just to fit or be slightly smaller than the nitrocellulose paper. Place a weight, such as another glass plate, on top of the paper towel stack.
10. Allow gel to set overnight, adding more transfer buffer as needed to keep the buffer level just at the bottom of the glass plate.
11. Let the gel blot overnight.
12. Next day, carefully take set-up apart.
13. Mark top of gel on nitrocellulose paper with indelible ink.
14. Wash blot about 30 seconds in 2X SSC. Handle blot with forceps or using gloved hands.
15. Pat blot dry on paper towels.
16. Bake blot in vacuum oven at 80°C for 2–4 hours. This fixes the nucleic acid to the nitrocellulose paper.

Experiment 6:
Labeling Probe DNA by Nick Translation Reaction

Introduction

In this experiment, the DNA to be used as a probe in the Southern hybridization is labeled with biotin. Through an enzymatic reaction, nucleotides to which biotin has been attached will be incorporated into the probe DNA (Fuccillo, 1985).

Double stranded DNA to be labeled is treated with DNaseI and *E. coli* DNA polymerase I. DNaseI makes random nicks (breaks in the phospho-diester backbone) in DNA. *E. coli* DNA polymerase I recognizes such nicks and removes nucleotides one at a time from the 5′ side of the nick and adds nucleotides to the 3′ end of the nick (resulting in movement—translation—of the nick). Also provided in this reaction are the precursors DNA polymerase incorporates into DNA, the deoxy nucleotide triphosphates-dNTPs. In our reaction, dCTP, dGTP, dTTP, and biotin-7-dATP (a dATP analog with biotin attached to the 6-position of the purine base by a 7-atom linker) are provided. By the action of DNA polymerase I, the nucleotides in the DNA are removed and replaced by new ones, including biotin-7-dATP. A biotin-labeled DNA probe is made in this way. Figure 1.4 illustrates the steps of nick translation. Figure 1.5 shows the chemical structure of biotin.

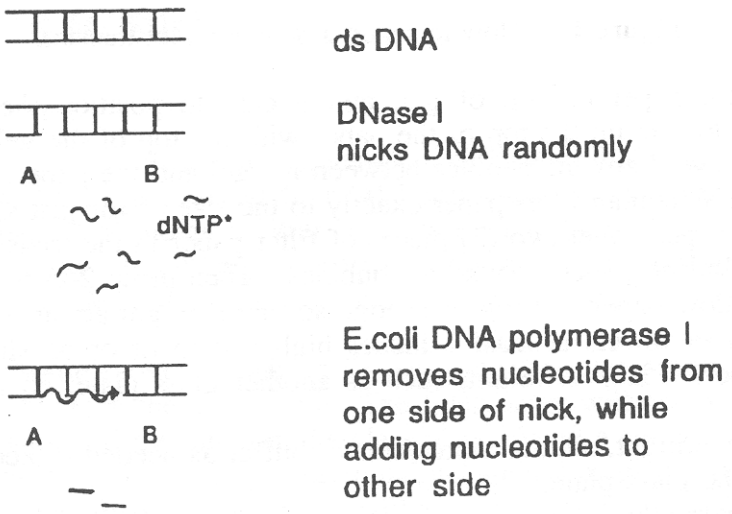

Figure 1.4. Nick translation reaction to label DNA to be used as a probe. Beginning with double stranded (ds) DNA, the enzyme DNase I randomly nicks (breaks the phosphodiester backbone) the DNA. Such a nick is indicated by the gap near A. The labeled deoxynucleotide triphosphates (dNTP*) are represented by a ~. *E. coli* DNA polymerase I removes deoxynucleotides from one side of the nick while adding labeled nucleotides to the other side of the nick. Note: the nick (gap) is now near B, that is, the nick has been translated (moved) by the action of DNA polymerase I. Labeled dNTP*s are now incorporated into the DNA.

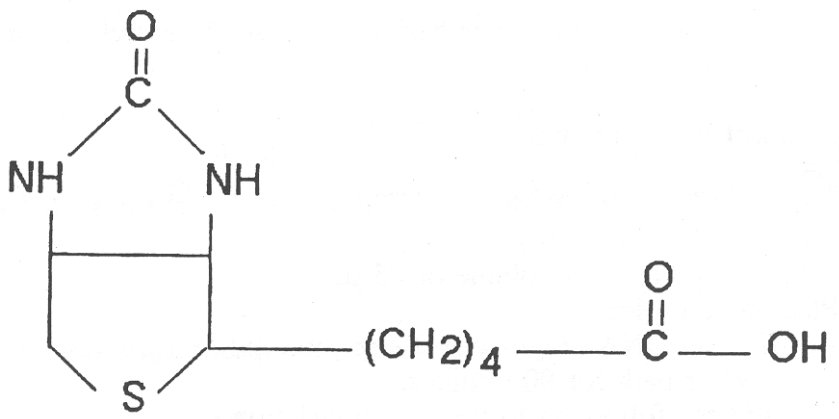

Figure 1.5. The chemical structure of biotin.

At the end of the 90-minute nick translation reaction, the biotin-labeled DNA is separated from the unincorporated biotin-7-dATP by exclusion column chromatography. The column matrix is Sephadex G-100. Large molecules, such as DNA, are excluded from the column and elute from the column first. Smaller molecules, such as the free biotin-7-dATP, enter the channels and pores of the column matrix, are thus retained by the column, and elute from the column later. To monitor the progress of the separation of DNA and nucleotides through the column, a mixture of a large dye (Blue dextran) and a small dye (Orange G) are added with the nick translation reaction to the column. The blue dextran co-migrates with DNA; the Orange G co-migrates with the unincorporated nucleotides. Thus, the blue fraction which elutes from the column first contains the labeled DNA.

This procedure requires 15 minutes to set up the nick translation reaction, 90 minutes for the reaction, and 15 minutes to separate components on the G-100 column.

Materials

Nick Translation System
Labeled nucleotide biotin-7-dATP
DNA to label as probe
5% SDS
Nick translation stop mixture:
- 0.5 M EDTA pH8.0
- 2 mg/ml Blue dextran (average molecular weight of 2×10^6)
- 1 mg/ml Orange G (7-hydroxy-8-phenylazo-1,3-napthalenedisulfonic acid)

Sephadex G-100:
Add 5 g G-100 beads to 150 ml of G-100 column buffer. Autoclave 20 minutes slow exhaust.
G-100 column buffer:
- 0.1 M NaCl, 10 mM Tris pH 7.5
- 2 mM EDTA
- 0.1% SDS

Procedures

This protocol is for use with the components of the Bethesda Research Laboratories (BRL) Nick Translation System (see Appendix A).

1. In a 1.5-ml Eppendorf tube combine:
   1 µg DNA
   5 µl unlabeled dNTPs (dCTP, dGTP, dTTP, 0.2 mM each) provided in kit
   2.5 µl biotin-7-dATP (0.4 mM)
   Sterile distilled H_2O to final volume of 45 µl
   Briefly mix. Place tube on ice.
2. Add 5 µl DNase I/*E. coli* DNA polymerase I enzyme mixture (provided in kit). Mix gently.
3. Incubate in a 15°C water bath for 90 minutes.
4. After 90 minutes, add the following to the nick translation reaction:
   2 µl of 5% SDS (the SDS is to decrease sticking of biotin to tubes)
   100 µl nick translation stop mixture
   Using exclusion chromatography with a Sephadex G-100 column, the biotin-labeled DNA is now separated from the unincorporated biotin-7-dATP.
5. Prepare a Sephadex G-100 column in a sterile Pasteur pipet. Plug bottom of Pasteur pipet with a small piece of glass wool (about 2-mm long plug). Use a thin disposable 1-ml pipet to tap the glass wool into place. Fill the column with Sephadex G-100. Take care to avoid air bubbles along the sides of the column. When the column is packed, lay it horizontally until needed.
6. Load sample onto the Pasteur pipet G-100 column. Elute with G-100 column buffer. Collect the blue fraction that comes off the column first; discard the yellow fraction.
   The biotinylated DNA is stored at 4°C and is stable for up to 1 year.

Experiment 7:
Prehybridization and Hybridization of Blot

Introduction

In this experiment, the biotin-labeled DNA probe prepared in Experiment 6 is hybridized to the Southern blot (nitrocellulose membrane), where the probe will base pair with its complementary sequences.

First, the membrane is incubated in the pre-hybridization solution for 2–4 hours at 42°C. The pre-hybridization solution contains components such as Denhardt's solution and calf thymus DNA that will saturate "non-specific" binding sites on the membrane and thus decrease the background non-specific binding of the probe to the membrane.

Next, the biotin-labeled probe is denatured by boiling. Single-stranded DNA is required to base-pair with complementary sequences.

The denatured probe DNA in the hybridization solution is added to the membrane (Southern blot) and incubated at 42°C overnight. This incubation allows time for the single stranded probe DNA molecules to hybridize (base-pair) to their complementary DNA sequences tethered on the membrane.

Finally, the membrane is subjected to a series of washes that remove any of the DNA probe that is not correctly base-paired to its complementary sequences.

Note: Biotin-labeled DNA should *not* be subjected to phenol extraction, because the biotin may partition into the phenolic phase. Also, biotin-labeled DNA should be boiled and not subjected to high pH, to denature the DNA.

This procedure requires 15–30 minutes to set-up the pre-hybridization; 2–4 hours for the incubation of the blot in pre-hybridization solution; 15–30 minutes to set-up the hybridization overnight for the hybridization incubation; and about 1 hour for washes of the blot.

Materials

Southern blot (prepared in Experiments 5): 8 × 10 cm
Pre-hybridization solution:
- 50% formamide (deionized)
- 5X SSC (defined in Experiment 5)
- 5X Denhardt's solution
- 25 mM sodium phosphate pH 6.5
- 0.5 mg/ml freshly denatured, sheared calf thymus DNA

To make 11 ml prehybridization solution mix:
- 5 ml formamide
- 2.5 ml 20X SSC
- 0.5 ml 100X Denhardt's solution
- 0.5 ml 1 M phosphate buffer (pH 6.8, made by mixing equal volumes 1 M mono- and di-basic sodium phosphate)
- 2.5 ml 2 mg/ml calf thymus DNA, sheared and freshly boiled

100X Denhardt's solution:
- 2% (w/v) BSA (bovine serum albumin)
- 2% (w/v) PVP (polyvinyl pyrrolidone, molecular weight (MW) 4×10^4)
- 2% (w/v) Ficoll (MW 4×10^5, a non-ionic synthetic polymer of sucrose)
- These three components are "non-specific blockers." They help decrease "background" of non-specific binding of probe to nitrocellulose membrane.

Hybridization solution:
- 45% formamide
- 5X SSC
- 1X Denhardt's solution
- 20 mM sodium phosphate pH 6.5
- 0.2 mg/ml freshly denatured, sheared calf thymus DNA

To make 10 ml hybridization solution mix:
- 4.5 ml formamide
- 2.5 ml 20X SSC
- 0.1 ml 100X Denhardt's solution
- 0.4 ml 1 M phosphate buffer
- 1.0 ml 2 mg/ml freshly denatured, sheared calf thymus DNA
- 1.5 ml sterile H_2O
- Biotin-labeled DNA to be used as probe

Wash solutions:
- 2X SSC/0.1% SDS
- 0.25X SCC/0.1% SDS
- 0.16X SSC/0.1% SDS

Plastic box with lid that has a water tight seal, slightly larger than Southern blot membrane (use a microwavable plastic box, such as Rubbermaid Sandwich Keeper)

42°C water bath
50°C water bath

Procedures

Do not touch blot with fingers. Always wear gloves when handling blot. Handle blot only at edges and not with undue pressure. Use forceps to move blot.

1. Prepare 11 ml of prehybridization solution. The amount of prehybridization solution needed is 20–100 μl/cm^2 of surface area of nitrocellulose membrane (from Southern blot).
2. Place membrane in a small plastic box.
3. Add appropriate amount of prehybridization solution.
4. Close box tightly and incubate at 42°C for 2–4 hours.
5. Prepare 10 ml hybridization solution. The amount of hybridization solution needed is 20–100 μl/cm^2 of surface area of blot.
6. Heat denature DNA probe to give final concentration of (0.1–0.5 μg/ml) for 10 minutes in a boiling water bath. After boiling, put probe on ice for 10 minutes. Just before use add probe to hybridization solution, and mix well.
7. Pour the prehybridization solution out of the box and add the hybridization solution.
8. Seal box tightly and incubate at 42°C overnight. (This allows the single stranded DNA probe to hybridize to homologous sequences on the Southern membrane.)
9. The next day, pour off the hybridization solution and immediately wash the membrane as follows. (These series of washes remove any probe that is not correctly base-paired to its complementary sequences.) After each wash, drain off the washing solution and add new washing solution.
   (a) Wash in 250 ml of **2X** SSC/0.1% SDS for 3 minutes at room temperature.
   (b) Repeat step (a).
   (c) Wash in 250 ml **0.25X** SSC/0.1% SDS 3 minutes at room temperature.
   (d) Repeat step (c).
   (e) Wash in 250 ml **0.16X** SSC/0.1% SDS for 15 minutes at 50°C.
   (f) Repeat step (e).
   (g) Rinse in 2X SCC at room temperature for about 30 seconds.

   Dry blot or continue with blocking.

Experiment 8:
Detection of Biotin-labeled Probes

Introduction

In this experiment the biotin-labeled DNA is detected and thus the bands where the probe hybridized are visualized. The basis of biotin detection is the interaction between biotin and streptavidin. Figure 1.6 shows the steps of the biotin detection system. Streptavidin binds very

tightly and specifically to biotin. An enzyme that is easy to assay is co-valently linked to streptavidin. In this system alkaline phosphatase is conjugated to streptavidin (called SA-AP, streptavidin-alkaline phosphatase). A substrate for alkaline-phosphatase is then added. Our experiment uses a chromogenic (color-generating) substrate, 5-bromo-4-chloro-3-indoyl phosphate (BCIP). The dye nitro-blue-tetrazalium (NBT) is also added. When alkaline phosphatase cleaves the phosphate group from BCIP, the NBT-BCIP solution which was pale yellow forms a blue precipitate. Thus, the biotin-labeled DNA is visualized as a blue band on the membrane.

There are other substrates for alkaline phosphatase that can be used. For example, if a chemiluminescent substrate is used, the biotin-labeled DNA can be detected on X-ray film because of the light emitted. (See the discussion of alternative non-radioactive DNA detection systems in Appendix A.)

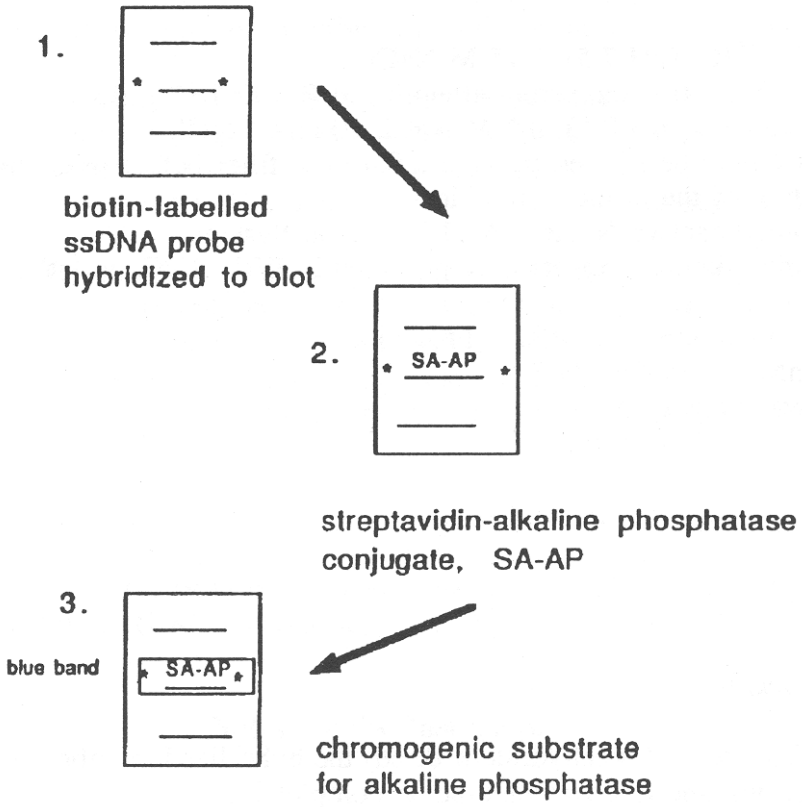

Figure 1.6. The biotin detection system. (1) The nitrocellulose membrane (Southern blot) is hybridized with a biotin-labeled single stranded (ss) DNA probe. The probe DNA binds to its complimentary sequences tethered to the membrane (indicated by **). (2) The hybridized membrane is incubated with streptavidin (SA) covalently coupled to the enzyme alkaline phosphatase (AP). The streptavidin binds tightly to the biotin part of the labeled DNA. (3) A chromogenic (color-generating) substrate for alkaline phosphatase is added. The alkaline phosphatase acts on the substrate, producing a blue precipitate (blue band) where the biotin-labeled DNA binds.

To summarize the steps:

1. The DNA probe is labeled with biotin.
2. The biotin-labeled DNA probe hybridizes with its complementary sequences on the Southern blot.

3. The blot is incubated with SA-AP (streptavidin-alkaline phosphatase). The SA moiety of SA-AP binds tightly to the biotin of the labeled DNA.
4. A chromogenic substrate (BCIP and NBT) for alkaline phosphatase (AP) is added to detect the biotin-labeled DNA. A blue precipitate is observed.

This procedure requires 1.5 hours for membrane blocking and 1–2 hours for binding of SA-AP and addition of substrate.

Materials

Buffer 1: 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl
Buffer 2: 3% (w/v) BSA (bovine serum albumin) in Buffer 1 (3 g BSA/100 ml Buffer 1)
Buffer 3: 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$
[Buffers 1, 2, and 3 may be filtered through a 0.45 μm filter before use. This reduces background (non-specific binding of the probe to the blot).]
BRL BluGene Nonradioactive Nucleic Acid Detection System
SA-AP (Streptavidin-alkaline phosphatase; provided in BRL BluGene kit)
BCIP (5-bromo-4-chlor-3-indoyl phosphate) – in BRL BluGene kit
NBT (nitro-blue-tetrazolium) – in BRL BluGene kit
20 mM Tris/0.5 mM Na_2EDTA pH 7.5
Plastic box with water-tight lid
Polypropylene tube, 3 ml or 10 ml
Platform rocker
65°C water bath

Procedures

Membrane Blocking

1. In the plastic box, wash (or rehydrate if dried) the hybridized membrane for 1 minute in Buffer 1. Use enough volume to cover membrane completely.
2. Incubate membrane for 1 hour at 65°C in Buffer 2 (pre-warmed to 65°C) in the sealed box. (Note: Buffer 2 contains the protein BSA. Soaking the membrane in BSA saturates non-specific protein binding sites on the membrane. This step minimizes the non-specific binding of SA-AP to the membrane.)
3. The membrane may be dried at this point (air dried or dried in a vacuum oven 80°C 10–20 minutes) and stored for months, or continue with the procedure.

Binding of Streptavidin-alkaline Phosphatase Conjugate

1. Again, use a plastic box. If membrane is dry, soak membrane in Buffer 2–10 minutes to rehydrate completely.
2. In a polypropylene tube, *immediately before use*, add 7 μl SA-AP conjugate (1 mg/ml) to 7.0 μl of Buffer 1. Incubate membrane in diluted SA-AP conjugate for 10 minutes. During this time occasionally pipet the solution over the membrane. Pour off the solution.

3. Wash membrane with 250 ml Buffer 1. Gently rock for 15 minutes. Pour off Buffer 1.
4. Wash membrane with an additional 250 ml of Buffer 1.
5. Wash membrane in 250 ml of Buffer 3 for 10 minutes. (Note: Buffer 3, pH 9.5, provides the appropriate conditions for alkaline phosphatase enzyme activity.)

Addition of Chromogenic Substrate to Visualize DNA (prepare dyes immediately before their use)

1. In a polypropylene tube, add 33 µl NBT solution to 7.5 ml of Buffer 3. Mix gently by inverting the tube. Add 25 µl BCIP solution, mix gently. (The use of a polypropylene tube rather than a polystyrene tube is recommended because biotin has a tendency to stick to polystyrene, but less so to polypropylene.) *Use caution:* The dye solutions contain dimethylformamide, which is harmful if inhaled, swallowed, or absorbed through the skin. Wear gloves and wash hands thoroughly after use.
2. Incubate the membrane in dye solution in the small plastic box, with the lid on. Let the color development proceed in the dark or in very low light. Check for color development every 5–10 minutes. DNA bands should be visible within 10 minutes to 3 hours. (Longer incubations may result in increased background.) The bands will be clearer on one side of the membrane than the other.
3. To stop color development reaction, wash membrane in 20 mM Tris pH 7.5/0.5 mM Na_2EDTA. Let membrane air dry. Store membranes away from strong light to prevent color fading. To some extent, wetting the membrane in water helps bring out the color again. These membranes cannot be reprobed because NBT and BCIP cannot be removed from the nitrocellulose. However, it is possible to reprobe with a different sequence and detect additional bands.

Results

Figure 1.7 shows an example of typical student results. The biotin-labeled probe was detected using a chromogenic substrate (BRL's BluGene).

Notes for the Instructor

Experiments 1 and 2

For convenience, *E. coli* cells to be used in plasmid DNA isolation can be harvested by centrifugation, the media drained off, and the pellet of *E. coli* cells stored frozen at -20°C until needed. Frozen cell pellets can be stored for at least 2 weeks. When beginning the plasmid DNA isolation procedure, be sure the pellet is completely resuspended in Solution I.

If the laboratory time is not long enough to allow the completion of the plasmid DNA isolation in one session, good stopping points are when the samples are in alcohol. After adding isopropanol or ethanol, samples may be stored at -20°C until the next laboratory session when the procedure is resumed.

Isolated plasmid DNA may be stored at 4°C. For long-term storage, aliquots of DNA may be frozen. Frequent thawing and refreezing of DNA samples should be avoided.

Experiment 3

An agarose gel may be prepared ahead of time and stored either at room temperature under 1X TBE running buffer or wrapped in plastic wrap and refrigerated. Gels may be stored in this manner for at least a few days.

Once a gel is run, it should be stained with ethidium bromide immediately. A stained gel should first be rinsed in distilled water, and may then be wrapped in plastic wrap and stored in the refrigerator for 1 day. If finished gels are stored for longer periods of time, the bands of DNA will gradually diffuse and not be as sharp as when the gel was first run.

Experiment 4

For an alternative method to isolate DNA fragments see Peloquin and Platzer (1991).

Experiment 5

It is best to begin a Southern blot immediately after the gel has been run. However, a gel to be blotted can be run the day before, stained with ethidium bromide and stored, wrapped in plastic wrap, in the refrigerator overnight. A gel can be stored for longer period, but there may be extensive diffusion of the DNA bands over longer periods. *Note:* Nitrocellulose must be baked in a vacuum oven because it has a flash point of, 200°C. After transfer and baking, the blot can be stored dry at room temperature indefinitely.

Experiment 6

Alternate methods to label DNA are listed in Appendix A, item 8.

Experiment 7

Following the hybridization of probe with the Southern blot and washes to remove excess probe (step 9), the blot may be dried and stored at room temperature until the time to begin Experiment 8.

Experiment 8

If a dried blot is used, allow extra time (about 5 minutes) in step 1 to rehydrate the blot completely before proceeding with the blocking step (Buffer 2). Do not heat Buffer 2 above 65°C. If heated to high, the BSA in Buffer 2 can gel. This gel sometimes sticks to a blot in large pieces that can be difficult to remove.

Acknowledgements

I gratefully acknowledge Ms. Wilma Foust for preparation of the manuscript, Dr. S. B. Gelvin for careful reading of the manuscript, graduate students B. W. Goodner and K. Igo, who have served as teaching assistants, and the students of Purdue University's Biology 542, who have tested these experiments through the years.

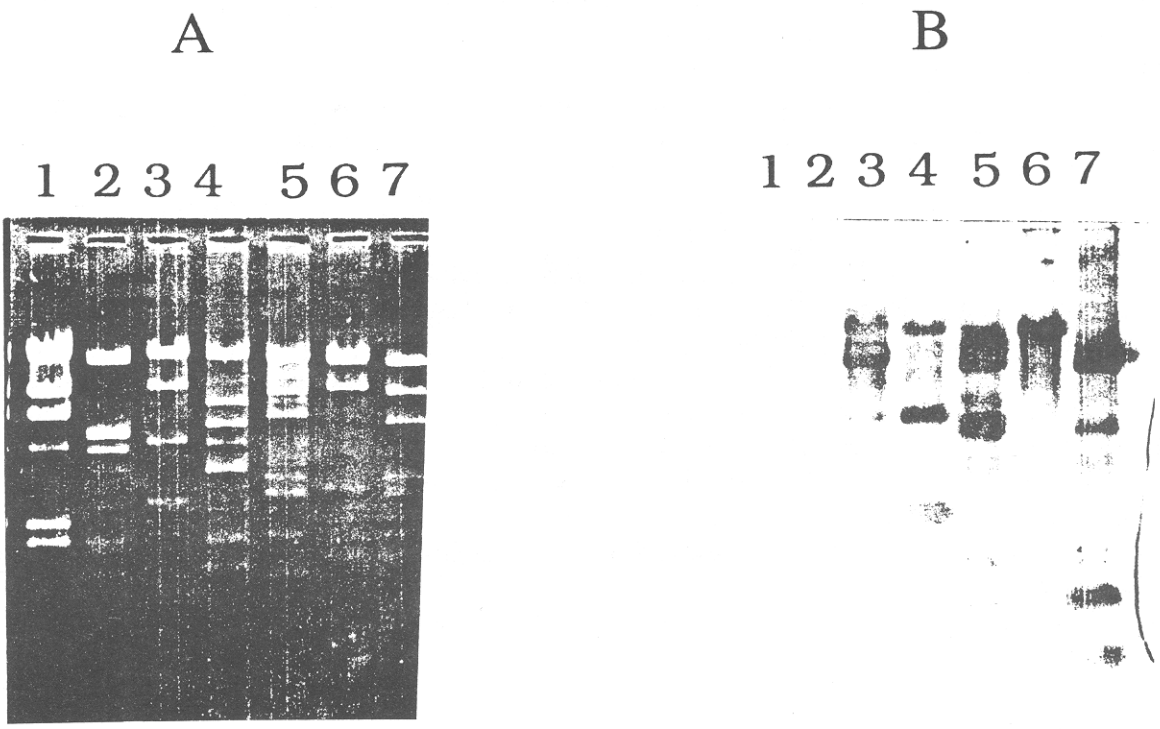

Figure 1.7. An example of a Southern blot using a chromogenic biotin detection system.
(A) Agarose gel stained with ethidium bromide. (B) Southern blot made from the agarose gel in A.
Lane 1: Phage λ DNA, cut with the restriction enzyme HindIII, used as a size standard.
Lane 2: Phage λ DNA, digested with HindIII and EcoRI, used as a size standard.
Lanes 3–7: A large plasmid to be studied, cut with different restriction endonucleases in each lane.
The blot (B) was hybridized to one piece of the total large plasmid contained in the gel. The probe was labeled with biotin, hybridized to the blot, and the biotin-labeled DNA detected by a chromogenic detection system (BluGene from BRL). The bands of hybridization which were blue appear gray in this black and white photograph of the blot.
Note: Lanes 1 and 2 show no hybridization because these lanes contain phage λ DNA, not the plasmid DNA. There is no sequence homology between λ DNA and the probe. In lanes 3–7, various bands show hybridization to the probe DNA. This information can be analyzed to map restriction endonuclease sites in the plasmid.

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APPENDIX A
Sources of Equipment and Materials

The following list gives sources for purchasing equipment and materials. It is not a complete list of all vendors in these areas. Inclusion in this list should not be interpreted as an endorsement of the company or product. Prices indicated are in US funds.

1. Restriction endonucleases
   - Example: EcoRI 5,000 units $ 25.00

   Bethesda Research Laboratories (BRL/Life Technologies, Inc.), P.O. Box 6009, Gaithersburg, MD 20877, (800) 638-8992.

   Fisher Scientific, Fisher Biotech Headquarters, 50 Fadem Rd., Springfield, NJ 07081, (800) 331-7341.

2. Gel electrophoresis equipment

   (a) Construction of gel electrophoresis

   Home-made laboratory equipment (make your own gel box). See pages 166–169 *in* P. B. Hackett, J. A. Fuchs, and J. W. Messing (An introduction to recombinant DNA techniques, 1984, Benjamin/Cummings, [ISBN 0-8053-3672-9]).

   (b) Gel boxes

   Mini-size:
   Grau-Hall Scientific, 6501 Elvas Ave., Sacramento, CA 95819, (800) 331-4728.
   - Cat. # 775-0000 $90.00

   Mini horizontal:
   Hoeffer Scientific, 654 Minnesota St., Box 77387, San Francisco, CA 94107, (800) 227-4750.
   - Cat. # HE 33B $165.00

   (c) Electrophoresis power supply

   Grau-Hall Scientific, 6501 Elvas Ave., Sacramento, CA 95819, (800) 331-4728.
   - ST-11 Cat. # 775-0001 (can run up to 3 gel boxes at the same time). $150.00

   Modern Biology, Inc., P.O. Box 97, Dayton, IN 47941-0097, (800) 733-6544.
   - A simple power supply $195.00

3. DNA visualization system

   Hoeffer Scientific, 654 Minnesota St., Box 77387, San Francisco, CA 94107, (800) 227-4750.
   - Hoeffer Scientific Mighty Bright UV transilluminator $650.00
   - Cat. # UV TM-10

4. Membranes for blotting

   Schleicher and Schuell, 10 Optical Ave., Keene, NH 03431, (800) 245-4024.
   - Nitrocellulose: 33-cm × 3-m roll ($50.00 for five 20-cm × 20-cm sheets) $130.00
   - Nytran (nylon membrane, less fragile, blot can be reused) for same price as nitrocellulose

5. DNA

   Bethesda Research Laboratories, P.O. Box 6009, Gaithersburg, MD 20877, (800) 638-8992.
   - Example: phage λ DNA, 500 mg $50.00

6. Labeled nucleotide (for labeling probe DNA)

   Bethesda Research Laboratories, P.O. Box 6009, Gaithersburg, MD 20877, (800) 638-8992.
   - Biotin-7-dATP (enough to label up to 50 μg of DNA)
   - Cat. # 9509SA $75.00

7. Nick translation kit to label DNA probe

   Bethesda Research Laboratories, P.O. Box 6009, Gaithersburg, MD 20877, (800) 638-8992.
   - 50 reactions $99.00

8. Alternatives to nick translation to label DNA probe

   Amersham, 2636 South Clearbrook, Arlington Hts., IL 60005, (800) 341-7543.
   - Multiprime labeling system (30 reactions) $125.00

   Boehringer Mannheim Biochemical, 9115 Hague Rd., P.O. Box 50100, Indianapolis, IN 46250, (800) 262-1640.
   - Random-primed DNA labeling kit (50 reactions) $165.00

   Bethesda Research Laboratories, P.O. Box 6009, Gaithersburg, MD 20877, (800) 638-8992.
   - Photobiotin (50 reactions) $105.00

9. Biotin detection system (with formation of insoluble colored precipitates)

   Bethesda Research Laboratories, P.O. Box 6009, Gaithersburg, MD 20877, (800) 638-8992.
   - BluGene (40 reactions) $ 95.00

   Zymed Laboratories, Inc., Suite 3, 52 S. Linden Ave., South San Francisco, CA 94080, (800) 874-4494.
   - DNAtect Kit (20 reactions) $ 65.00

10. Other non-radioactive DNA detection systems

    FMC Corp Bioproducts, 191 Thomaston, Rockland, ME 04841, (800) 341-1574.
    - Chemiprobe (labeling and detection kit to label 4 mg DNA and detect 20 blots) $210.00

    This kit uses sulfonation of DNA to be used as probe. One then detects this hybridized probe with a monoclonal antibody specific for modified DNA, and visualizes the bound monoclonal antibody with antibody-AP conjugate and a chromogenic substrate.

    Boehringer Mannheim Biochemical, 9115 Hague Rd., P.O. Box 50100, Indianapolis, IN 46250.
    - Genius Detection Kit (25 labeling reactions and 50 detections) $330.00

    This system may have less background and blots may be reprobed. Digoxigenin-11-dUTP is used to make digoxigenin labeled DNA probe, which is detected by antibody to the modified DNA; this is visualized using Anti-digoxigenin-alkaline phosphatase conjugated antibodies and chromogenic substrates.

11. Non-radioactive chemiluminescent detection systems

    These systems use a chemiluminescent substrate to detect the hybridized DNA probe. The light emitted is detected on a piece of X-ray film.

    Advantages: Blots used with this system can readily be used with different probes because there is no insoluble precipitate formed. It is easy to detect weak and strong hybridizing bands by varying the length of exposure time of the X-ray film.

    Possible disadvantage: This system does not result in immediate visualization of hybridizing bands. X-ray films must be developed before the hybridization can be detected.

    Bethesda Research Laboratories, P.O. Box 6009, Gaithersburg, MD 20877, (800) 638-8992.
    - PhotoGene detection kit $177.00

    Amersham, 2636 S. Clearbrook Dr., Arlington Heights, IL 60005, (800) 323-9750.

• ECL (Enhanced Chemiluminescence) Gene Detection System $295.00
Includes labeling and detection kits. They recommend using special film and membranes.

Tropix, 47 Wiggins Ave., Bedford, MA 01730, (617) 271-0045.
• Southern-Light Plus (signal detected by X-ray or instant film) $325.00
Includes labeling and detection kits.

Boehringer Mannheim Biochemicals, 9115 Hague Rd., P.O. Box 50100, Indianapolis, IN 46250.
• Lumi-Phos 530: Chemiluminescence for the Genius System (100 ml of solution) $260.00
Based on the Genius System, uses digoxigenin labeled DNA with a chemiluminescent substrate instead of a chromogenic substrate (see Genius Detection Kit, item 10).

12. Packaged kits and systems for teaching

Fotodyne, Inc., 16700 W. Victor Rd., New Berlin, WI 53151, (800) 362-3686.
• DNA mini-gel system
Foto/Phoresis I, complete: gel box, power supply, camera, UV transilluminator. $1,650.00

Modern Biology, Inc., P.O. Box 97, Dayton, IN 47941-0097, (800) 733-6544.
• DNA Hybridization Analysis $ 95.00
Comes as a **kit** with DNA provided for 16 students; uses biotinylated DNA, and horseradish peroxidase detection system.

EDVOTEK, Inc., P.O. Box 1232, West Bethesda, MD 20817, (800) 338-6835.
• Gel electrophoresis box and power supply $270.00
Mini Lab Station contains a mini-gel electrophoresis apparatus and minipower pack.

• DNA fingerprinting kit $185.00
Cat. #311 comes as a **kit** to demonstrate blotting—uses prelabeled DNA so a hybridization is not done. Includes enough agarose, DNAs, and membranes for four gels.

13. Chemicals

BSA (bovine serum albumin): Sigma (cat. # A9647)
BCIP (5-bromo-4-chlor-3-indoyl phosphate): provided in BluGene kit from BRL
BPB (bromophenol blue): Sigma (cat. # B8026)
Blue dextran (average molecular weight of 2×10^6): Sigma
EDTA (Ethylenediamine tetraacetic acid, also called ethylenedinitrilo tetraacetic acid): Mallinckrodt
Labeled nucleotide biotin-7-dATP: BRL
NA agarose: Pharmacia (cat. #17-0554-01/02/03 for 10/100/1000 g)
NBT (nitro-blue-tetrazolium): provided in BluGene kit from BRL
Orange G (7-hydroxy-8-phenylazo-1,3-napthalenedisulfonic acid): Sigma
PEG (Polyethylene glycol): Sigma
SDS (Sodium dodecyl sulfate, also called lauryl sulfate): Sigma (cat. # L4390)
Sephadex G-100: Pharmacia
Tris (Tris[hydroxymethyl] amino-methane): Sigma (sold as trizma base or trizma hydrochloride)
Tryptone: Difco (cat. #0123-07)
Yeast extract: Difco (cat. #0127-01-7)

Bethesda Research Laboratories, P.O. Box 6009, Gaithersburg, MD 20877, (800) 638-8992.
Difco Laboratories, P.O. Box 1058A, Detroit, MI 48232, (800) 521-0851.
Mallinckrodt, P.O. Box M, Paris, KY 40361, (800) 354-2050.
Pharmacia, P.O. Box 1327, Piscataway, NJ 08855-1327, (800) 558-7110.
Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178, (800) 325-8070.

APPENDIX B
Example of a Simple Southern Blot Experiment

A simple plasmid-to-plasmid Southern blot can be performed using a well-characterized plasmid, pBR325. (Contact the author for an *E. coli* strain containing this plasmid.) Plasmid DNA may be isolated by the student or instructor (Experiment 1) or pBR325 DNA may be purchased. (Plasmid pBR325, from BRL/Life Technologies, Inc., P.O. Box 6009, Gaithersburg, MD 20877, 800-828-6686, 15 μg for $50 US.)

A hybridization probe may be made by digesting pBR325 DNA with restriction enzymes and then isolating a particular restriction endonuclease fragment from the gel (Experiment 4). Figure 1.8 shows a map of the plasmid pBR325 with key genes and selected restriction endonuclease sites indicated. The nucleotide locations of the restriction sites are given in the figure legend. For example, if pBR325 is digested with PstI and Bam HI, a 3.2 kbp and a 2.8 kbp fragment are generated. The 2.8 kbp fragment can be used as a probe against pBR325 DNA digested with various restriction enzymes, run on a gel and blotted. The students can predict the results expected based

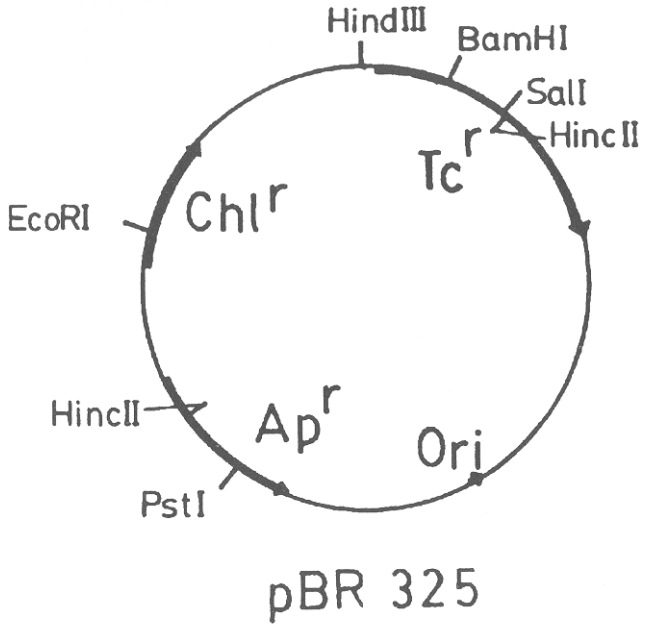

Figure 1.8. Map of the plasmid pBR325, showing key genes and selected restriction endonuclease sites. Indicated on the map are: Ori, origin of DNA replication; Ap^r, ampicillin resistance gene; Chl^r, chloramphenicol resistance gene; and Tc^r, tetracycline resistance gene. Numbers indicate the nucleotide location of the restriction endonuclease site on the map of pBR325: pBR325 (5996 bp), HindIII (29), BamHI (375), HincII (651) and (3,907), SalI (651), PstI (3,609), and EcoRI (4,779).

Problem

1. Predict the sizes of all DNA fragments generated when pBR325 is digested with the following restriction enzymes: (a) EcoRI and HindIII, and (b) EcoRI, HindIII and PstI.
2. Indicate which fragments will hybridize to the 2.8 kbp BamHI-PstI fragment used as a probe.
3. How did this compare with your experimental results?

Answer

(a) EcoRI and HindIII

1. Two fragments: 4.75 kbp and 1.25 kbp.
2. Both fragments will hybridize to the probe.
3. Were any differences in signal intensity observed? Because the probe is homologous to only 0.35 kbp of the 4.75 kbp fragment, that fragment may show a less intense signal.

(b) EcoRI, HindIII, and PstI

1. Three fragments: 3.6 kbp, 1.25 kbp, and 1.2 kbp.
2. All three fragments will hybridize to the probe.
3. However, the 1.25 kbp and 1.2 kbp fragments may not separate on an agarose gel, so that only two hybridizing bands are detected.