Modern
Biology
Series

A Laboratory Course in Molecular Biology

5. Genetic Engineering

INSTRUCTOR MANUAL

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Plasmid Isolation-Found in green box

Solution I	1 bottle
Solution II	1 bottle
Solution II	1 bottle
Equilibration Buffer	1 bottle
Buffer HB	1 bottle
Wash Buffer	1 bottle
Elution Buffer	1 bottle
HiBind DNA Column	24
Collection Tubes	24
RNase	1 Tube

Appendix 2-PREPARATION OF SOLUTIONS

Electrophoresis Buffer

The electrophoresis buffer is supplied as a 100-fold concentrate. To prepare the working buffer, add 35ml of the buffer concentrate to 3.5 liters of distilled or deionized water. Store the unused buffer in the refrigerator between electrophoretic runs. The buffer should be reused in the electrophoresis chamber for the three electrophoretic runs. However, fresh buffer should be used for the preparation of the agarose gels.

Staining Solution

The staining solution (methylene blue) is supplied as a 1000-fold concentrate. To prepare the working stain, add 1/2ml of the staining concentrate to 500 ml of distilled water. Please note, neither the concentrate nor the working dilution of the staining solution should be pipetted by mouth. Store the unused stain in a tightly capped bottle in the refrigerator.

Ethidium bromide provides a more sensitive method to stain DNA in gels. Ethidium bromide is a mutagen so if this option is chosen gloves should be worn at all times. After electrophoresis, place gels 1ug/ml ethidium bromide in water and view on a UV box after about 20 minutes. Ethidium bromide is available from Modern Biology Inc. (Cat #3-7)

IA PLASMID ISOLATION (Lab Session 3)

1. Add the vial of RNase A to the bottle of Solution I and store at 2-8°C. All other solutions should be stored at room temperature.

2. Add 60 ml of absolute ethanol (96-100%) to the DNA Wash Buffer and mark on the top of the bottle that the ethanol was added.

II. ENZYMES

EcoR1 (Lab Session 1)

The restriction enzyme EcoR1 is provided in a glycerol solution. Immediately before use, add 900µl of restriction nuclease buffer to one of the tubes containing the EcoR1. Mix well the contents of the tube by rotating the tube on its side to ensure that the enzyme comes in contact with the buffer. Place the tube in an upright position in a beaker containing ice chips.

EcoR1 (Lab Session 3)

The restriction enzyme EcoR1 is provided in a glycerol solution. Immediately before use, add 900µl of restriction nuclease buffer to the remaining tube containing EcoR1. Mix well the contents of the tube by rotating the tube on its side to ensure that the enzyme comes in contact with the buffer. Place the tube in an upright position in a beaker with ice chips. 450µl of this enzyme solution will be used to prepare the EcoR1 + Bam H1 mixture described below.

EcoR1 + Bam H1 Mixture (Lab Session 3)

To prepare the enzyme mixture, remove 450µl of the EcoR1 Buffer solution from the tube described above, and add it to the tube containing the Bam H1. Mix the contents of the tube and place it in the beaker with ice.

DNA Ligase (Lab Session 1)

The ligase (15 units) is provided in 15µl or in 7.5µl of a glycerol solution. Immediately befor use, add 500µl of the 2 x ligase buffer to the tube containing the ligase. Mix the contents of the tube and place it in a beaker with ice chips.

III. BACTERIAL CULTURE MEDIA

Xgal-Ampicillin-Agar Plates (Lab Session 2 and 3)

Twenty mg of Xgal, 1ml of Xgal solvent (Dimethyl Formamide), one bottle containing 400ml of nutrient agar plus ampicillin and 20 petri dishes are supplied. Due to the unstable nature of ampicillin, additional ampicillin is also provided and should be added to the nutrient agar plus ampicillin mixture to ensure that sufficient antibiotic is present in the agar plates. The plates must be prepared at least one day before the laboratory session. To prepare the plates:

A. Loosen the cap on the bottle.

B. Place the bottle in a beaker of boiling water over a burner until the agar has liquefied. This should take about 20-25 minutes.

C. Remove the bottle from the bath and let cool at room temperature for about 10 minutes.

D. Pour the entire 1ml of the Xgal solvent into the tube containing Xgal, cap the tube and carefully shake until the Xgal is dissolved.

Note: The Xgal solvent, dimethyl formamide, is toxic at this concentration. The solvent should be handled with caution in a well vented area (a fume hood, if available) and the instructor should wear gloves.

E. Pour the entire 0.6ml of the ampicillin solution and the1ml of Xgal solution into the bottle containing the nutrient agar, replace the cap, and swirl the bottle to mix the contents. The ampicillin, Xgal, and Xgal solvent will be found in 1.5ml tubes along with the other frozen components of this Chemical Package

F. Uncover the petri dishes, one at a time, and pour a thin layer (10-20ml) of agar into the lower half of each dish. Immediately replace the covers. Let the agar harden for about one day at room temperature. If desired, the plates can be stored in the refrigerator in an inverted position for at least two weeks.

Culture Tubes with Nutrient Broth-Ampicillin (Lab Sessions 2 and 3)

200ml of the broth is provided along with twenty-five 20ml sterile culture tubes. A sterile pipet can be used to add about 6ml of media to each tube. Alternatively, place a mark on each tube about 6cm from the bottom and fill the tube to the line with media. Immediately recap the tubes and store them in the refrigerator until needed.

IV - HYBRIDIZATION (LAB SESSIONS 4, 5, and 6)

A. Nylon Membranes

One sheet of nylon membrane (10cm x 20cm) is provided with this program. We recommend that the instructor cut the sheet to the desired sizes prior to the lab session. Note that the nylon should be handled with gloves and cut with a razor blade

or sharp scissors. For gels that are 5cm x 7 1/2cm, four small sheets of 5cm x 5 3/4cm should be prepared. With other gel sizes, the nylon should be cut so that the four individual sheets are 1-2cm shorter than the distance between the sample wells and the end of the gel.

B. Solutions for Hybridization

In addition to the solutions provided, you will need distilled water, sodium chloride and sodium hydroxide.

5M NaCl - To prepare the solution, place 292 grams of NaCl in a 1 liter flask and bring the solution to a final volume of 1 liter with water. Stir the solution well until the crystals are dissolved.

Hybridization Buffer - The solution should be warmed to ~37°C before use as the SDS precipates in the cold.

1/4 x Hybridization Buffer - Combine 55ml of Hybridization Buffer with 165ml of water.

Transfer Buffer - 0.5M NaCl - 10mM Tris, HCl pH8.0. Combine 30ml 1M Tris-HCl, pH 8.0 with 300ml 5M NaCl and 2.7 liters of water.

Denaturing Solution - 0.4M NaOH,-0.5M NaCl. Combine 4.8 grams of NaOH with 30ml of 5M NaCl and bring the volume up to 300ml with water.

Neutralization Buffer - 0.5M NaCl - 0.3M tris-HCl, pH 8.0. Combine 15ml of 5M NaCl, 45ml 1M Tris-HCl, pH 8.0 and 90ml of water.

Tris-Buffer Saline (TBS) - 0.15M NaCl-20mM Tris-HCl, pH 8.0. Combine 21ml 5M NaCl, 14ml Tris-HCl, pH 8.0 and 665ml of water.

TBS-NP40 - TBS - 0.05% NP40. The 1ml of Nonidet-P40(NP40) is provided as a 10% solution. Add the contents of this tube to 200ml of TBS.

TBS-Gelatin - TBS-1% Gelatin. To prepare the solution, add 2g of the gelatin to 200ml of boiling TBS and stir until the gelatin is dissolved. Cool to room temperature before use. This solution may be stored for 1-2 days at room temperature or up to two weeks in the refrigerator. It will solidfy upon refrigeration and should be allowed to warm to room temperature before use.

Color Development Buffer - 50mM Tris-HCl, pH 8.0. Combine 15ml 1M Tris-HCl, pH 8.0 with 285ml H_2O.

Development Solution - Color Development Buffer, 4-chloronapthol, hydrogen peroxide. This solution is prepared by adding the 5ml of 4 chloronapthol to 100ml of color devleopment buffer. The solution may turn cloudy. 200μl of hydrogen perooxide is then added and the solution mixed thoroughly and filtered. NOTE: The solution should be prepared immediatley before use.

APPENDIX 3 - EXPERIMENTAL RESULTS

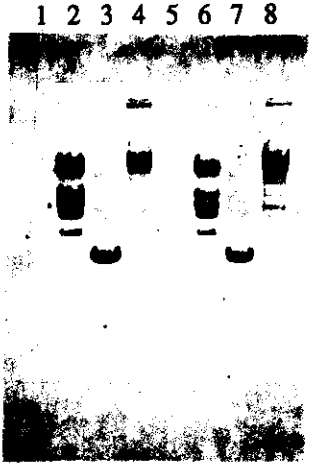
Typical results of this program are given below.

Laboratory Session 2 : Electrophoretic Analysis

Lanes 2,6 ("λ-E") - Six DNA bands should be observed. (See Figure on next page.)

Lanes 3,7 (pUC18-E") - One band containing 2686 base-pairs should be present.

Lanes 4,8 ("L-E") - A smear of slow migrating DNA fragments should be the major species. Most of these species are circular molecules and circular molecules migrate slow on agarose gels because of their shape.



Laboratory Session 2: Number of Transformants

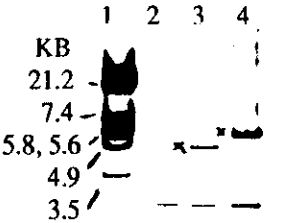
50-100 Blue

10 -20 White

Laboratory Session 3: Electrophoretic Analysis

The gel shows results of an EcoR1 digestion

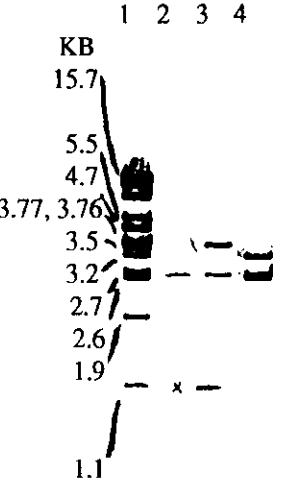
Lane Number		
1	λ	
2	Plasmid from a blue colony	
3	Plasmid from a white colony (W1)	
4	Plasmid from a white colony (W2)	



Laboratory Session 4: Electrophoretic Analysis

The gel shows the results of an EcoR1 + Bam H1 digestion.

Lane Number		
1	λ	
2	Plasmid from a blue colony	
3	Plasmid from a white colony (W1)	
4	Plasmid from a white colony (W2)	

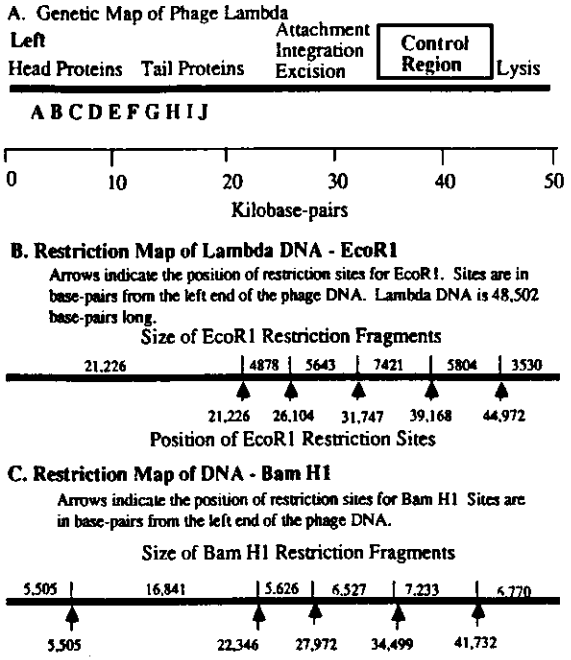


Laboratory Session 6:
Southern Blot Hybridization Analysis

All bands on lanes 1 (above gels) and the starred bands on lanes 3 and 4 were detected by Southern blot hybridization analysis.

APPENDIX 4 - ANALYSIS AND CONCLUSIONS

For discussion, the restriction map of phage λ is reproduced below.



A. Possible recombinant plasmids - Digestion of Phage λ with EcoR1 produces 6 fragments but the two terminal fragments (coordinates 0 - 21,226 and 44,972 - 48,502) are unlikely to be present in the population of recombinant plasmids since the sequences at the termini will not anneal to EcoR1 cut DNA (but see below). Thus, the amplified λ DNA is most likely derived from the internal 4 fragments. Some plasmids may contain more than one of these fragments since two or more λ fragments can be joined to each other during the ligation reaction. However, there is an upper limit to the length of DNA that can be amplified in pUC18 and recombinants containing more than 10-15 KB of λ DNA will probably not be recovered. Similarily, although the two terminal fragments could be joined to each other via the complementary single strands which are located on the ends of λ, it is unlikely that such a large molecule would be propagated in pUC18.

B. Restriction maps of λ DNA in recombinants W1 and W2
(See Appendix 3 for gels)