Materials Provided

Solution I -Tris Buffer
Solution II -Sodium Dodecyl Sulphate and Sodium Hydroxide
Solution III- Guanidinium Chloride and Acetic Acid
Equilibration Buffer- Sodium Hydroxide
Buffer HB -Guanidinium Chloride and Isopropanol
*Wash Buffer -Tris Buffer, Sodium Chloride and Ethanol Note: The ethanol must be added to this buffer prior to use.
Elution Buffer -Tris Buffer
HiBind DNA Columns
Collection Tubes
Transfer pipets
Satellite DNA (from Laboratory Session 2)
*EcoR1 - Buffer
DNA markers (from Laboratory Session 1)
*Electrophoresis buffer, agarose and gel stain
Electrophoresis sample buffer - This sample buffer contains RNAse

* Prepared as described in the Instructor Manual

Materials Not Provided

A microcentrifuge, 1.5 -2 ml tubes and a 37 C water bath, distilled water

I. PLASMID ISOLATION

Background

Methods for plasmid isolation have the following steps in common:

1. Growth and Harvesting of Plasmid-Containing Bacterial Cells - The cells from an antibiotic-resistant colony are usually grown overnight in a liquid culture to stationary phase and then harvested as a pellet by centrifugation.

2. Preparation of the Cleared Lysate. These steps involve the lysis (breaking open) of the bacteria and the separation of plasmid DNA from most other components in the lysate including chromosomal DNA, protein

and RNA.

3. Final Purification of the Plasmid DNA – These step involve the final purification of the plasmid DNA using a silica matrix.

Procedure

A. Harvesting the Bacteria

1. Transfer about 1.5 ml of your three overnight cultures to three labeled 1.5 ml microcentrifuge tubes and centrifuge at maximal speed for 1-2 minutes to pellet the bacteria. Discard the supernatant, fill the tubes again with the appropriate cultures and centrifuge for 1-2 minutes. Repeat this step one additional time.

2. Carefully remove and discard the supernatant fluid. Invert the tubes and allow the cell pellets to dry for a minute.

B. Preparing the Plasmid DNA

1. Add 250 ul of Solution I to each tube and suspend the pellets in the solution by vigorous shaking or by using a vortex mixer. This solution contains RNAse, which destroys RNA. Complete re-suspension (no visible clumps) is important in order to have high plasmid yield.
2. Incubate the tubes at room temperature for 2 minutes.

3. Add 250ul of Solution II, cap the tubes and mix by inversion several times until the contents are mixed thoroughly. The solution should become clear and viscous within 1-2 minutes. Solution II contains the detergent sodium dodecyl sulfate (SDS) and sodium hydroxide, which dissolve the bacterial membranes and causes cell lysis. The sodium hydroxide also denatures the DNA. Upon neutralization in the step below, the plasmid DNA strands renature because of their proximity while the bacterial DNA does not.

4. Add 350 ul of Solution III to each tube, cap the tubes and shake until the contents are mixed thoroughly. A white flocculent precipitate should form The acetic acid in Solution III will neutralize the solution. The

Guanidinium Chloride in Solution III and the SDS from the previous step produce a tangled network of long strands of bacterial chromosomal DNA and cell debris, and this matrix can be separated from the smaller plasmid DNA by centrifugation.

5. Centrifuge the tube for 10 minutes at maximal speed. After centrifugation, plasmid DNA and small amounts of cellular proteins are in the supernatant, and bacterial chromosomal DNA and most of the proteins are in the pellet. This supernatant is known as a "cleared lysate". A common procedure that is used to remove the proteins, SDS and salts that are present in the cleared lysate involves the use silica, which binds to the DNA but not to the contaminants. The silica is packed into the bottom of a column that you will use, and the lysate is added to the column over the silica. The column is then washed by centrifugation to remove the contaminants and the plasmid DNA is eluted by using a low salt buffer.

6. Obtain three blue columns, and three collections tubes, label each, and insert the columns into the collection tubes. Add 100 ul of Equilibration Buffer to the columns and centrifuge for 1 minute at maximal speed.

7. Carefully pour the three cleared lysates from step 5 into the columns making sure that the pellets are not dislodged. Centrifuge the columns with collecting tubes for 1 minute at maximal speed.

8. Discard the liquid in the collection tubes, place the columns back into the collection tubes and add 350 ul of HB Buffer to the columns. Centrifuge the columns/collecting tubes for 1 minute.
9. Discard the liquid in the collection tubes, place the columns back into the collection tubes and add 700 ul of Wash Buffer to the columns. Centrifuge the columns/collecting tubes for 1 minute.

10. Discard the liquid in the collection tubes, place the columns back into the collection tubes and centrifuge for 2 minutes to dry the columns.

11. Discard the collection tubes and insert your columns into three clean-labeled 1.5-2 ml centrifuge tubes. Place 25 ul of Elution Buffer onto the center of the silica in the columns and incubate for 2 minutes. Centrifuge for 2 minutes, discard the columns and cap the tubes that contain the eluted purified plasmid DNA. The plasmid DNA can be stored in the freezer until the next laboratory or processed as described below.

II. NUCLEASE DIGESTION AND ELECTROPHORESIS

Background

The plasmids that you have isolated should consist of circular molecules. In order to characterize the recombinants, you will digest each with EcoR1. The EcoR1 digestion of the plasmid from blue colonies should yield a single vector band containing 2686 base-pairs while plasmids from the white colonies should yield the vector fragment plus a fragment from the satellite when digested with this enzyme.

Procedure

1. Obtain 3 microtubes

2. Number the tubes 1 to 3 with a waterproof marking pen.

3. Place 10µl of the EcoR1-Buffer solution into each tube.

4. Add 10µl of the indicated DNAs to the tubes.

Tube Number	
1	Plasmid DNA from tube B
2	Plasmid DNA from tube W1
3	Plasmid DNA from tube W2

5. Tap the tubes to mix the solutions and then incubate the tubes for 60 minutes at 37°C. During this incubation, prepare 1.2% agarose gels. You should also view your sectored plate at this time in order to confirm that the color of your three colonies was retained upon re-plating. The bacteria on these plates could be used as a source of the plasmids that you have isolated today.

6. At the end of the incubation period, add 5µl of DNA sample buffer to each tube and incubate the tubes for 5 minutes at room temperature This sample buffer contains RNAse which will destroy RNA in your plasmid DNA sample.

7. Remove 20µl of the satellite DNA that you isolated from total sheep DNA during laboratory session 1 and add it to a tube containing 5µl of DNA sample buffer. Label this tube #4.

8. Load 20µl of the samples in tubes 1-3 on the 1.2% agarose gel as shown below. Load 10ul of DNA Markers into tube #8.

Sample Well Number	Sample Tube Number	
1	1	Group One
2	2	
3	3	
4	4	
5	1	Group two
6	2	
7	3	
8	4	

9. After electrophoresis, place each gel in a tray containing about 100ml of methylene blue gel stain and place the tray in the refrigerator.

10. After staining for 12-24 hours, decant and discard the stain, rinse the gels with water, and add 200-300ml of distilled water to the tray. The gels should be stored in water in the refrigerator until the next lab session.

LABORATORY SESSION V
(DATA ANALYSIS)

1. Place your gel over a light source and attempt to identify the following bands.

   A. A single vector band containing 2686 base-pairs in lanes 1-3.

   B. A cloned satellite DNA band (370,430 or 800 base-pairs) in lanes 2 and 3.

   C. A satellite DNA band of the same size in lane 4 that you isolated from sheep DNA.

2. Hopefully, all went as planned during these 5 laboratory sessions and you were able to clone one of the sheep satellite DNA fragments. Offer some possible explanations if you failed to recover recombinants.