Exercise 19
THE MINISCREEN - RAPID ISOLATION OF PLASMID DNA

Introduction

Bacterial cells often contain multiple segments of covalently closed circular deoxyribonucleic acid (ccc-DNA). The largest of these are the bacterial chromosomes while the smallest are loops of **extra-chromosomal** DNA called **plasmids**. Plasmids vary considerably in size (from 1 to over 200 kbp) and number within bacterial cells, but are generally much smaller than bacterial chromosomes (often over 2 million bp in length). The most thoroughly studied plasmids are F factor plasmids (fertility factors) and R factor plasmids (resistance factors).

The genes associated with plasmid DNA typically encode characteristics not essential to the life of bacteria under normal circumstances, therefore, plasmid DNA can be lost without inhibiting cell growth, development or reproduction. Because plasmids can be removed from cells, and reintroduced into other cells fairly easily, they are of great importance to procedures used in genetic manipulation known as **Recombinant DNA Techniques**.

There are currently numerous, artificially constructed plasmids (products of recombinant DNA technology) being used as **cloning vectors** in genetic manipulation procedures (genetic engineering). Each of these has an **origin of replication** and will reproduce independently of chromosomes when placed into a host cell. Genes from various sources can be inserted into these plasmids and will be replicated many times over within the host cell, i.e. they will be **cloned**. This allows for the **in vivo** amplification of genetic material necessary for its analysis and manipulation. Plasmids used as cloning vectors typically have three important features including:

1. One or more selectable **marker genes** that are typically nutritional genes or antibiotic resistance factors.
2. Multiple **cloning sites** or regions containing recognition sequences for restriction enzymes. These allow plasmids to be cut and segments of DNA to be inserted.
3. An **origin of replication** allowing the plasmid to be replicated independently of the chromosome.

The term **replicon** is sometimes applied to plasmids or the single origin of replication they carry. The number of any one type of plasmid within a cell is referred to as the **copy number** for that plasmid, and is quite variable. The plasmid pBR322 (developed by Boyer and Rodriguez) has a copy number of about 50, while the smaller pUC19 (developed by researchers at UC Davis) has a copy number ranging between 100-150.

In order to work with plasmid DNA, the cells containing the DNA must be lysed (broken open), and the plasmid DNA separated from other cell components. Differences in the physical properties of chromosomal and plasmid DNA facilitate this separation. The tendency of proteins to coagulate when exposed to heat can also be of significance.

The purification of large (milligram) amounts of plasmid DNA is generally time-consuming and involves the use of large batch cultures (typically 1 Liter) of bacteria. However, a variety of rapid isolation procedures have been developed that allow for the purification of small amounts of plasmid DNA (1-5 micrograms) from small cultures (1-10 mL). The DNA obtained by these small-volume, rapid-isolation methods, generally referred to as "mini-screens" or "mini-preps," is not as pure as that obtained by more elaborate methods, but is suitable for many characterization and cloning procedures.

In this exercise, *E. coli* cultures containing the cloning vectors (plasmids) **pUC19**, **pGLO** or **pGEM** will be provided and a mini-screen protocol used to purify the DNA from each culture. In later exercises, these DNA samples will be subjected to gel electrophoresis and students will be able to observe them. Finally plasmid DNA will be used to transform new *E. coli* cells.

Information about plasmids

The plasmids pUC18 and pUC19 are high copy number plasmids 2686bp in length. Both carry an origin of replication (pMB1 replicon), a gene for **ampicillin resistance** (*bla* – encoding **β-lactamase**) and multiple cloning sites arranged in opposite directions. These plasmids carry a portion of the *E. coli* lactose utilization operon including the CAP binding site, the promoter, the repressor binding site (operator site) and the 5' end of the *lacZ* gene (encoding β-galactosidase). The cloning sites occur within the *lacZ* gene.

The plasmid pBR322 is 4363bp in length and carries a replicon (*rep*) responsible for plasmid replication (from pMB1), a *rop* gene encoding Rop protein (which decreases copy number), the *bla* gene encoding β-lactamase (allowing for ampicillin resistance), and the *tet* gene encoding tetracycline resistance.

The pGLO plasmid is about 5400bp in length (5371bp), carries a replicon, a marker gene encoding ampicillin resistance (*bla* = β-lactamase gene), and portions of an inducible operon normally involved in the utilization of arabinose. Some structural genes of the operon have been replaced by a gene encoding green fluorescent protein (GFP) originally produced by the bioluminescent jellyfish, *Aequorea victoria*. The repressor protein (*ara*C protein) regulating the expression of the arabinose operon is encoded by the gene *ara*C. This gene and the *bla* gene have weak constitutive promoters. The promoter for the arabinose operon (P_{BAD}) is strongly regulated by the repressor protein (*ara*C protein). If arabinose is absent, the repressor is active and GFP is not produced. If arabinose is present, the repressor is inactivated and GFP is produced.

Materials:

E. coli JM83 cultures containing the plasmid pUC19
E. coli JM101 cultures containing the plasmid pBR322
E. coli DH5a cultures containing the plasmids pGLO or pGEM
SET buffer: 20% Sucrose, 50 mM Tris-HCl pH 7.6, 50 mM EDTA
Sodium (Na) acetate: 3.0 M, pH 4.8
Lytic mix: 1% SDS, 0.2 N NaOH
RNase stock: Pancreatic ribonuclease A, 10 mg/ml
Isopropanol: Ice cold
70% Ethanol
Water (dH_20): Sterile, deionized or distilled
Ice buckets and ice
Sterile 1.5 ml Eppendorf microcentrifuge tubes
Microcentrifuge
Micropipettes and sterile tips

SET buffer is osmotically balanced to keep cells whole, buffered for DNA stabilization, and contains EDTA to chelate Mg^{++} ions necessary for DNAse activity.
Lytic mix is highly alkaline and will lyse cells. SDS, a surfactant, will also lyse cells and coats proteins.
Isopropanol is hydrophobic, causes Na^{+} ions to bind with the phosphate groups of DNA, neutralizing their interaction with water. Ultimately this causes DNA to precipitate.
The 70% ethanol is used to rinse the salt out of the DNA pellet.

Procedure: Each student will prepare one tube of plasmid DNA.

1. Obtain **one** tube containing *E. coli* host cells carrying plasmid DNA **(JM83 with pUC19 = blue tubes, DH5α with pGLO = green tubes, or DH5α with pGEM = orange tubes)**. Spin these tubes for 1 minute in a microcentrifuge to pellet the cells. **Make certain the centrifuge rotor is balanced before you start the spin**.

2. Pour off the supernatant (liquid material) into the flasks provided.

3. Add 150μl of SET buffer to each tube and resuspend the cells by vortex mixing or stirring the tube contents with a sterile loop. Be sure that the cell pellet is broken up completely.

   Alternate method: As an alternate method to steps 1-3 you may obtain a plate culture of *E. coli* host cells (JM83**,** or DH5α) carrying a specified plasmid type, and scrape the cells from 1/4 of the plate surface using a sterile loop. Place this cell mass into 150μl of SET buffer in a sterile microfuge tube **(JM83 with pUC19 = blue, DH5α with pGLO = green, and DH5α with pGEM = orange)**. Vortex or mix the tube contents to thoroughly break up the cell mass.

4. Add 350μl of Lytic mix to each tube and invert several times to mix the contents. Under these conditions, the cells will lyse, causing the solution to clear slightly and the viscosity to increase.

5. Place the tubes in an ice water bath for at least 15 minutes. The solution will become cloudy as the SDS precipitates.

6. Add 250μl ice-cold sodium acetate buffer to each tube and invert several times to mix. Immediately return the tubes to the ice water bath for at least 20 minutes.

7. While the tubes incubate in the ice bath, each student should obtain and a new clean sterile microcentrifuge tube of the same color (i.e., if you were initially using a blue tube, get a new blue tube, and if you were using an orange tube, get a new orange tube, etc.).

8. Remove the tubes of cellular material from the ice bath, and centrifuge for 10 minutes (this spin should be run at 4 degrees centigrade if possible).

9. Carefully pour the supernatant from the original tubes into the clean labeled tubes obtained in step seven. Discard the tubes containing the cell pellets in the flask provided (leave these open).

10. Using a cooled, sterile loop, add one loopful (about 2μl) of RNase stock solution to each tube of supernatant and invert several times to mix. Incubate these tubes for 10 minutes at 37^{o} C.

11. Add enough isopropanol to equal the volume of supernatant present in each tube. (This will be approximately 700μl, so you may simply fill the remainder of the space in the tube being careful to allow space for closure of the tube cap.) Invert the tubes several times to mix.

12. Immediately centrifuge the tubes for 5 minutes at room temperature. When you place the tubes into the centrifuge rotor, position them so that the "hinged" portion of the snap-cap is up (toward the outside of the rotor). This will insure the position of the plasmid sample collected and will prevent the tubes from splitting when spun a second time. When the spin is complete, pour (shake out) the supernatant into the flask provided. Be careful not to dislodge the plasmid sample at the tube bottom.

13. Add 1ml (1000µl) of 70% ethanol to each tube and invert several times to rinse the plasmid sample. Centrifuge for 3 minutes at room temperature (positioning the tubes as indicated above). Pour the supernatant into the flasks provided.

14. Carefully label your tube cap with a glass marker (using your initials or other unique marking). Open the tube, invert it, and place it open-end-down on the paper towel provided at the front of the laboratory. When most of the excess ethanol has drained into the towel, place your tube in the rack provided (leave the cap open). The tubes will be placed in the freezer overnight or until the next laboratory period to remove the residual ethanol. These dehydrated DNA samples will be resuspended during a later lab by adding 20µl of sterile distilled water or Tris buffer to each and allowing them to stand in an ice bucket for about 10 minutes.

    **** STOP POINT ****

The DNA solutions prepared above should be kept frozen at -20^{o} C until you are ready to continue working with them. If they are not to be used within 48 hours, the caps to the microfuge tubes should be closed. The samples must be thawed before using.

The DNA samples prepared during this exercise will be saved for use in future experiments (restriction endonuclease digestion, electrophoresis and transformation of new bacterial cells).

Questions:

1. How does plasmid DNA differ from chromosomal DNA?

2. What are cloning vectors, and what are they used for in genetic manipulation experiments?

3. Why is it possible to separate plasmid DNA from chromosomal DNA using fairly simple centrifugation steps?

4. Why might investigators want to remove plasmid DNA from bacterial cells, i.e., what purpose does this procedure serve?