Lab 4.

Molecular Cloning of Viral DNA Fragments in a Bacterial Plasmid Vector

Reading:
● Appendix VIII. Restriction Enzymes

This lab exercise generally follows the sequence of numbered steps shown in Campbell’s Fig. 20.3, with the following exceptions:

1. Although we use a bacterial plasmid DNA vector (pUC19) similar to the one described in the text, our “gene-source” DNA will be the genome of a bacterial virus called Lambda (λ) rather than human DNA.

2. You begin the exercise with plasmid vector DNA and Lambda “gene-source” DNA that have already been isolated and purified.

3. Rather than identifying a “gene of interest” in our recombinant clones, we will use restriction digestion analysis to determine the size (identity) of the cloned Lambda DNA fragment.

Figure 1

The λ Bacteriophage Particle (Virion)

DNA is packaged in the icosahedral “head”
Several features of this exercise are frequently overlooked or misunderstood. To minimize confusion, try to keep the following points in mind:

The plasmid vector is only a tool; it is really the Lambda DNA that we are interested in as an object of study.

The plasmid vector contains four essential features:

I. Origin of replication

  - allows the plasmid DNA to replicate in an E. coli host cell

II. Ampicillin resistance gene

  - gene for an enzyme that degrades the antibiotic ampicillin
  - E. coli carrying plasmids that express this gene are ampicillin-resistant
  - E. coli cells that have acquired such a plasmid can be selected by plating on media containing the antibiotic

III. Unique restriction site/s

  - one or more restriction sites that occur only once in the plasmid
  - cutting such sites opens the circular plasmid to full-length linear form
  - occur in lacZ gene

IV. lacZ gene

  - sequence required for expression of the enzyme beta (β)-galactosidase
  - β-galactosidase converts the artificial substrate X-Gal to a blue-colored product
  - if foreign DNA is inserted in the plasmid at a unique restriction site in lacZ, the gene will be disrupted by “insertional inactivation”
  - this allows screening for recombinant plasmids carrying foreign DNA inserts
  - The restriction endonuclease merely creates the DNA fragments that serve as a substrate for cloning. The enzyme DNA ligase actually reconnects the fragments to create recombinant DNA molecules.
Although the recombinant DNA molecules are created by ligation of restriction fragments \textit{in vitro} (outside a living cell), they must be reintroduced to living cells \textit{(in vivo)} for replication (amplification).

The class will isolate a collection of clones carrying different fragments of the Lambda genome. This will constitute a Lambda “genomic library.” The lab exercise is broken down into 8 procedures (I-VIII) that are done sequentially over five lab periods. The instructor will give you the dates scheduled for each procedure this quarter. Write them in the appropriate boxes of Table 1.

\textbf{Table 1. Schedule}

<table>
<thead>
<tr>
<th></th>
<th>DATE</th>
</tr>
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<tbody>
<tr>
<td>I.</td>
<td>Restriction Digest #1</td>
</tr>
<tr>
<td>II.</td>
<td>Agarose Gel #1</td>
</tr>
<tr>
<td>III.</td>
<td>Ligation</td>
</tr>
<tr>
<td>IV.</td>
<td>Transformation</td>
</tr>
<tr>
<td>V.</td>
<td>Inoculation and Growth</td>
</tr>
<tr>
<td>VI.</td>
<td>Plasmid DNA Isolation</td>
</tr>
<tr>
<td>VII.</td>
<td>Restriction Digest #2</td>
</tr>
<tr>
<td>VIII.</td>
<td>Agarose Gel #2</td>
</tr>
<tr>
<td>IX.</td>
<td>Analysis of Results</td>
</tr>
<tr>
<td></td>
<td>Lab Report Due</td>
</tr>
</tbody>
</table>
I. **Restriction Digest (#1) of Lambda DNA and pUC19 DNA**

The purified Lambda and pUC19 DNAs have been purchased. You digest the pUC19 and Lambda DNAs separately with the same restriction enzyme (*Hind* III) and run an agarose gel to demonstrate that the digestion has been successful (Procedure II). While the gel is running, you set up the ligation (Procedure III).

The digestion setup is complicated, so be sure you understand how it is set up (See Table 2. and Figure 3.)

<table>
<thead>
<tr>
<th>Table 2. Restriction Digest #1 Setup</th>
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<tbody>
<tr>
<td><strong>Conc.</strong></td>
</tr>
<tr>
<td>2X Buffer</td>
</tr>
<tr>
<td>pUC 19 DNA</td>
</tr>
<tr>
<td>Lambda DNA</td>
</tr>
<tr>
<td><em>Hind</em> III</td>
</tr>
<tr>
<td>TOTAL</td>
</tr>
</tbody>
</table>

You may find this table easier to use if rows and columns were switched. If so, reformat the table in your notebook so that you can refer to it in lab.

There are 4 reaction tubes. Tubes #2 and #4 are real digests of pUC19 DNA and λ DNA, respectively. Tubes #1 and #3 are “mock” digests where no restriction enzyme is used. Notice that the amounts of DNA used in tubes #2 and #4 (real digests) are larger than the amounts used in tubes #1 and #3. This is because DNA from the real digests will be used for analysis by electrophoresis AND for ligation. DNA from the mock digests will be used only for analysis by electrophoresis.
Procedure

1. Add all the reagents except the restriction enzyme (*Hind* III) to your tubes in the order shown in the table.
   
   (Think about when it is necessary/not necessary to change pipette tips.)

2. Mix the reagents with your pipette tip. (It may be necessary to spin the tubes briefly to pool everything at the bottom.)

3. Ask the instructor to dispense the restriction enzyme.

4. Gently mix the tubes. (It may be necessary to spin the tubes briefly to pool everything at the bottom.)

5. Incubate the tubes at 37°C in a water bath for 45 minutes.

6. Prepare a 0.7% agarose gel (Appendix II) while the digestion is running.
II. Agarose Gel Electrophoresis #1

You should have your gel ready to load by the time the restriction digest is finished. See Fig. 4 for a diagram of the following steps.

1. Prepare digest tubes #1 and #3 only for electrophoresis by adding 1 µl of 6X tracking dye to each and mixing.

2. For tubes #2 and #4, remove 4 µl from each. Mix each 4 µl sample with 1 µl of 6X tracking dye, in separate microfuge tubes.

3. Keep tubes #2 and #4, with the remaining 6 µl in each, on ice. This DNA will be used for the ligation.

4. Load the entire volume (5 µl) of each of the 4 prepared samples to separate wells.

5. Also load 18 µl of Lambda BstE II standard DNA to the wells on opposite sides of your samples.

   You should be running DNA in 6/8 lanes in your gel.

6. Run the gel under the same conditions (100 volts approx.) as in Lab 7.

7. Stain and photograph the gel.

8. Analyze the gel photograph carefully.

   See Appendix IX. for a more detailed guide to analyzing the gel and interpreting the restriction digests.

Use the Lambda BstE II standard DNA fragments to construct a semi-log plot of DNA fragment size vs. migration distance.

Is the size of the pUC19 DNA fragment consistent with the restriction map (Fig. 2)?

Are the sizes of the Lambda-Hind III fragments consistent with the restriction map (Appendix VII)?

If either or both of your restriction digests failed, you should set up the ligation with DNA samples we provide.
Figure 4. Preparation of Samples for Electrophoresis #1
III. Ligation

To “ligate” means to “tie or connect”, and this is an apt description of this phase of the procedure. In procedure I, you prepared DNA fragments by digestion of the complete Lambda genome with *Hind* III. These are to be mixed with the linear pUC19 DNA you prepared by digestion of the intact circular plasmid with the same enzyme. When the mixture of DNA fragments is cooled, the fragments will associate endwise due to complementary base-pairing between the single-strand extensions created by the restriction enzyme. These potential recombinants are unstable because the individual fragments are held together largely by weak hydrogen bonds. At room temperature they would largely dissociate into separate fragments again. The enzyme DNA Ligase is added to form covalent recombinants.

DNA ligases are ubiquitous enzymes. They are essential participants in DNA replication, DNA repair and recombination. The activity of DNA ligase basically reverses the action of the restriction endonuclease (Fig. 5).

![Fig. 5 Activity of DNA Ligases](image)

Notice that in order to completely rejoin two separate restriction fragments it is necessary to ligate 2 nicks, one in each DNA strand. That is, to form a circular recombinant plasmid (one with a foreign DNA insert) it is necessary to ligate 4 nicks.
Procedure

1. Label a new sterile microfuge tube and keep it on ice.

2. Consult Table 3 for the appropriate volumes. Add water, buffer and DNAs to the tube. Do not add ligase.

3. Place the tube in a 65°C water bath for 3 minutes, then return it to an ice bath for 5 minutes.

4. Ask your instructor to add the DNA ligase to your tube.

5. Place the tube at 15°C for 24-48 hours.

<table>
<thead>
<tr>
<th>Table 3. Ligation Setup</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Sterile di Water</td>
</tr>
<tr>
<td>10X Ligase Buffer</td>
</tr>
<tr>
<td>pUC19 <em>Hind</em> III digest (from step I)</td>
</tr>
<tr>
<td>λ <em>Hind</em> III digest (from step I)</td>
</tr>
<tr>
<td>DNA Ligase</td>
</tr>
<tr>
<td>TOTAL</td>
</tr>
</tbody>
</table>
IV. Transformation

Recombinant plasmids that have been created by the ligation step are unavailable for further analysis unless they are separated from other ligation products and amplified by replication within living host cells. As the bacteria replicate, so does the plasmid and the DNA insert it is carrying. This is the actual “cloning” aspect of the overall process.

The desired ligation product is a circular plasmid with a single λ DNA insert at the Hind III site in lacZ. However, an array of additional products will be formed. Under our conditions the most common product will probably be monomeric (non-recombinant) circles formed by the ligation of the two ends of the same linear fragment to each other. These are especially prevalent when the DNA fragments in the ligation mix are dilute. Multimeric ligation products become more prevalent at higher DNA concentrations. The ratio of vector fragments to λ DNA fragments also influence the yield of the desired recombinant molecules.

There are additional strategies to increase the yield of recombinant plasmids in a ligation mix that we are not using. Ultimately though, the yield of desired products will never be 100%; so it is always necessary to isolate clones of living cells containing the desired products by selection and screening.

Obtaining pure cultures of cells carrying a specific recombinant plasmid involves:

i. Transformation

E. coli cells are treated with CaCl_2 to render them “competent”, able to take up DNA molecules from solution. The competent cells are mixed with the DNA ligation products. In this exercise, you will be provided with competent cells.

ii. Selection

Cells that acquire a plasmid from the ligation mix are said to be “transformed” and are called “transformants”. Transformants are able to grow on agar plates containing ampicillin by virtue of the ampicillin-resistance gene in the plasmid. Cells without a plasmid are sensitive to ampicillin.

iii. Screening

Among the colonies of transformed cells on an LB + Ampicillin + X-Gal plate we should observe some blue colonies and some not-blue (“white”) colonies. The blue color is due to hydrolysis of the X-Gal substrate in the medium and is the result of expression of the lacZ gene fragment in the plasmid. A λ DNA insert at the Hind III site should inactivate lacZ, so colonies of transformed cells carrying a recombinant plasmid should not be blue (because they do not hydrolyze X-Gal).
Selection and screening are done simultaneously by plating on LB + Ampicillin + X-Gal plates, so it is easy to confuse the two concepts. Remember that selection involves growth vs. no growth in the presence of ampicillin, based on the presence vs. absence of a plasmid. Screening involves a difference in the appearance of a colony based on expression of a gene (lacZ) that is inactivated by a foreign insert. That is, if a cell is subject to negative selection (i.e. doesn’t grow in the presence of ampicillin) it cannot be screened. This is summarized in Table 4.

**Table 4. Selection vs. Screening**

<table>
<thead>
<tr>
<th>Selection</th>
<th>Screening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relevant feature of Plasmid Vector</td>
<td>Ampicillin-Resistance Gene</td>
</tr>
<tr>
<td>Relevant Component of Agar Medium</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>What you observe</td>
<td>Growth vs. No-growth of bacterial colony</td>
</tr>
<tr>
<td>What you learn</td>
<td>Bacteria contain a plasmid</td>
</tr>
<tr>
<td></td>
<td>X-Gal</td>
</tr>
<tr>
<td></td>
<td>X-Gal hydrolysis (blue) vs no hydrolysis (not blue)</td>
</tr>
<tr>
<td></td>
<td>Plasmid is recombinant</td>
</tr>
</tbody>
</table>
**Procedure**

In this procedure you work with *E. coli* bacteria. Review the hazard and safety information for working with bacteria that was included in the earlier exercise on serial dilutions and plating.

It is crucial to maintain sterile conditions during your manipulations.

You will set up 2 microfuge tubes in this procedure (Table 6). Tube #1 is the actual transformation where competent *E. coli* cells are mixed with the DNA from your ligation (Step III). Tube #2 is a control with no DNA. This demonstrates that there are no ampicillin-resistant cells present originally in the competent cells. (We omit a “no –cells” control that would confirm that there are no bacteria present in the DNA ligation mixture. We do this to conserve agar plates and DNA.)

**Table 6. Transformation Setup**

<table>
<thead>
<tr>
<th></th>
<th>#1</th>
<th>#2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile diH₂O</td>
<td>20 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>DNA from ligation</td>
<td>20 µl</td>
<td></td>
</tr>
<tr>
<td>Competent Cells</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>TOTAL</td>
<td>70 µl</td>
<td>70 µl</td>
</tr>
</tbody>
</table>

1. Retrieve your ligation mix from the 15°C incubator and keep it on ice.

2. Thaw a tube of competent cells on ice.

   Thawing takes about 10 minutes; use within 30 minutes after thawing. Do not let them sit around.

3. Add all reagents to tubes #1 and #2 according to Table 6.

4. Incubate the tubes on ice for 30 minutes.

   DNA binds to the cell surface.

5. Heat shock the transformation reaction for 90 seconds at 42°C.

   Use the 42°C WATER BATH.

   DNA enters the cells.

   Cells are fragile at this point—*handle gently*. Be careful not to jostle tubes.
6. Add 0.93 ml LB medium to each tube.

    Note that there is **NO ANTIBIOTIC** in this medium.

7. Incubate the tubes at 37°C for approximately 45 minutes.

    Although entry of the plasmid DNA into the cells alters their **genotype** immediately, an incubation period is required to allow for expression of the antibiotic resistance gene on the plasmid to alter the **phenotype**. The incubation is too short to allow significant cell division. Letting cells divide is undesirable because it leads to overestimation of transformation efficiency.

    The volume of this culture, 1 ml, will be used as the basis to calculate transformation efficiency (see Step V).

    **Prepare a flowsheet summarizing steps 8 and 9 before class and have it checked before proceeding.**

8. Dilute culture #1 for plating.

    Prepare 10^-1, 10^-2, and 10^-4 dilutions of the transformed cells in tube #1 using sterile saline (0.9% w/v NaCl) as diluent.

9. Plate cells

    Plate 50 µl samples from tube #1 on LB + Ampicillin + X-gal agar plates at dilutions of 10^0, 10^-1 and 10^-2.

    Plate a 50 µl sample from tube #2 on an LB + Ampicillin + X-gal plate at a dilution of 10^0.

    Plate a 50 µl sample from tube #1 on an LB + X-gal plate (without Ampicillin) at a dilution of 10^-4.

    The plating protocol is summarized in Table 7.

<table>
<thead>
<tr>
<th>Sample #1 +DNA</th>
<th>Sample #2 -DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB X-gal</td>
<td>10^-4</td>
</tr>
<tr>
<td>LB Ampicillin X-gal</td>
<td>10^0; 10^-1; 10^-2</td>
</tr>
</tbody>
</table>

Use alcohol-rinsed, flamed bent metal rod to spread these suspensions. After allowing liquid to soak in (for these small volumes, around 10 minutes or less), invert plates and incubate 24-36 hours at 37°C.
Check: you are incubating 4 LB Amp X-gal plates and 1 LB X-gal plate, right? Your instructor will give you specific directions regarding removing the agar plates from the incubator.
V. Inoculation and Growth of Recombinant and Non-Rencombinant Transformants

1. Examine all the agar plates from Phase IV. Record your results and, before proceeding, be sure you understand the basis of the results you observed.

   What is the evidence that you have transformed cells? Which plate/s should have colonies of cells containing recombinant plasmids that can be isolated?

2. Prepare 2 sterile tubes with 3 ml LB + Ampicillin liquid medium in each tube.

   Be sure you use adequate sterile transfer technique.

   The ampicillin in the medium maintains positive selection for cells that retain the plasmid.

3. Transfer single colonies to the tubes of LB + Ampicillin liquid medium.

   Use sterile wooden sticks. Pick one “white” colony and one blue colony. Inoculate one colony per tube.

4. Incubate at 37°C.

   Put the tubes in a shaking water bath or forced air shaker/incubator at 37°C for 24-48 hours. Following incubation, the cultures should be stored at 4°C, if necessary, until the phase VI. Your instructor will give specific instructions regarding removal of the cultures from the shaker.

5. Count the colonies on the agar plates. Be sure that you record the number of blue and white colonies separately. If there are more than 500 colonies on a plate you should estimate.

   The plates can be kept in the refrigerator for analysis later.
Analysis of Transformation

The blue and white colonies that you selected on the LB + Ampicillin plates must contain plasmid DNA. (Why?) You now analyze the transformation to see how well it worked. The efficiency of a transformation depends on the quality of your competent cells and on the nature of the DNA.

If some of the plasmids you have transformed into the bacteria contained foreign DNA, then that DNA has been cloned, meaning that a single piece now resides throughout a single bacterial clone and can be amplified as much as you like simply by growing the bacteria in culture. The X-gal screening technique will signal the presence of foreign DNA in the vector, but typically one wants to actually “see” the insert to absolutely validate its presence and to determine its size. This means isolating it by cutting it out of the plasmid. You will do this in the subsequent steps of this laboratory exercise.

There are several ways to measure your results.

We will calculate:

i. the frequency of cells among the total population of competent cells that become transformants

ii. the number of transformed cells per µg of DNA (This is the standard measure of “transformation efficiency”.)

iii. the % of transformants that carry recombinant plasmids

**HINTS:**

It will be convenient to base the calculations on the 1 ml cultures in step IV.7. The calculations will be similar to those you performed in the earlier *E. coli* plating exercise (Lab 3).

Several preliminary calculations are necessary:

- the TOTAL cell concentration in tube #1.
- the concentration of transformants in tube #1.
- the total DNA concentration in tube #1.

Many variables will affect the efficiency of transformation, including the conformation of the DNA. For supercoiled plasmids, efficiencies between $10^6$ and $10^{10}$ transformants per µg of DNA are generally expected. Ligation products such as yours are “relaxed” (i.e. not supercoiled) and give much lower transformation efficiencies.
VI. Isolation of Plasmid DNA from *E. coli* using the Promega Wizard™ DNA Purification System

In order to confirm that you have cloned a recombinant plasmid containing a Lambda DNA insert, and to identify which Lambda DNA fragment/s are inserted, it is necessary to isolate the recombinant plasmid DNA from transformed cells and subject it to restriction analysis. As a comparison, the non-recombinant plasmid will also be analyzed. The liquid cultures you inoculated in Step IV will provide the plasmid DNA’s for analysis.

The isolation of plasmid DNA from cells is a prerequisite for further manipulation and study, including sequence determination and restriction analysis of the cloned DNA fragment. Small-scale (<10 ml of bacterial culture) isolations of plasmid DNA, known as “minipreps”, yield sufficient DNA for restriction analysis.

Separating DNA from the protein, RNA, lipid, carbohydrate and other material released by cell lysis, while minimizing DNA degradation, is not a trivial biochemical task. Separating plasmid DNA from the bulk of *E.coli* chromosomal DNA is even more difficult. This plasmid DNA isolation technique makes use of the fact that the plasmid DNA is free in the bacterial cytoplasm, while the bacterial chromosome is attached to the cell membrane. In a cell lysate, membrane fragments with attached chromosomal DNA are precipitated by one means or another. The plasmid DNA remains in solution.

The Wizard Miniprep DNA Purification System (a product of the Promega Corp.), provides a simple and reliable method for rapidly isolating plasmid DNA. This system works most efficiently with plasmids <20 kb. The process can be completed in as little as 15 minutes. Plasmid DNA prepared by this system is of sufficient purity that it can be used directly for restriction digestion or transformation without further purification.

Promega Wizard minipreps cost about $0.75 per DNA isolation. The major cost element is the proprietary DNA binding resin. The Wizard kits for this laboratory exercise have been donated by the Promega Corporation.

The yield of plasmid DNA will vary depending on a number of factors, including the volume of bacterial culture, plasmid copy number (# of plasmid molecules/cell), type of culture medium and the bacterial strain. pUC19, the plasmid used in this exercise, has a copy number of 500 plasmids per cell and yields of approximately 5 µg DNA/ml culture are typical. Note that large inserts (>8 kb) in recombinant derivatives of pUC19 may lower the copy number substantially. This will usually be reflected in a lower DNA yield. The exact copy number and yield of a particular recombinant plasmid cannot be predicted with certainty.
General Summary of procedure:

- Lysis of cells by exposure to high pH and detergent. RNase enzyme is present to reduce RNA contamination.
- pH neutralization causes precipitation of cell wall debris
- Wall debris with attached chromosomal DNA is pelleted and discarded.
- Binding of plasmid DNA to an insoluble resin.
- Elution of plasmid DNA from resin after washing.

Further information on the Promega Wizard DNA Purification System is available at:  
Procedure

Prepare a flow diagram of this procedure before coming to class and have it checked before proceeding. Although the procedure is worded as if you were doing one miniprep, you will be doing 2; one of the culture grown from a white transformant and the other of the culture grown from a blue transformant. Thus, **you need to complete all steps below in duplicate.** Be careful not to mix up the two preparations. Note that sterile technique is not required in this procedure.

1. Transfer 1.2 ml of overnight culture to a microfuge tube.

   Agitate the culture first in case the cells have settled on the bottom of the tube.

2. Spin in a microfuge for one minute at maximum speed to pellet the cells.

   Balance your tubes in the rotor.
   Your cell pellet will be small but should be visible near the bottom of the tube.

3. Withdraw the clear supernatant carefully with your P-1000 and discard it by pipetting back into the culture tube it came from. Remove as much of the supernatant as possible without disturbing the pellet.

   The culture tubes are biohazard waste.

4. Re-suspend the cell pellet completely in 200 µl of Cell Resuspension Solution (RS) (Don’t use any other solution by mistake!)

   **Thoroughly** disperse the cell clumps. You should vortex and/or draw the solution repeatedly in and out of the micropipette tip to disperse the cell clumps.

   *PUT ON GOGGLES AND GLOVES THROUGH STEP 13!*

5. Add 200 µl of Cell Lysis Solution (LS) to your cell suspension and mix by inverting the microfuge tube 4 times.

   The turbidity (cloudiness) of the cell suspension should decrease and you may notice the solution has become viscous. Viscosity is good - it means you have liberated high molecular weight DNA from the cells.
   LS contains the detergent Sodium Dodecyl Sulfate (SDS) at pH 12.

6. Add 200 µl of Neutralization Solution (NS) and mix by inverting the tube several times.

   NS contains Potassium Acetate at pH 4.8. Cell wall debris should form an insoluble precipitate.
7. Centrifuge the lysate in a microcentrifuge for 5 minutes.

Balance your tubes.

The pellet will contain cell wall and membrane fragments, with the bulk of the chromosomal DNA attached (unless extensive shearing of the DNA has occurred through rough handling). This pellet will be less well defined than the cell pellet in step 3 and may be smeared along the side of the tube. If a pellet has not formed, centrifuge for an additional 15 minutes. The plasmid DNA remains in the supernatant.

During the spin you can begin to set up for steps 8 and 9.

8. **For each miniprep** prepare one Wizard Minicolumn as follows:

- Remove the plunger from a 3ml disposable syringe and set it aside.
- Attach the syringe barrel to the Luer-Lok® extension of the Minicolumn.

9. Obtain one microfuge tube per prep with 1 ml of resuspended DNA Purification Resin (PR) each.

Note: The resin itself is insoluble and should be visible as a cloudy suspension collected at the bottom of the tube. Thoroughly resuspend the DNA Purification Resin by vortexing before removing an aliquot.

Use the P-1000 to transfer the resin suspension to the barrel of the syringe/minicolumn assembly.

10. Carefully remove all (0.6 ml approx.) of the DNA sample from the microfuge tube (i.e. the supernatant from Step 7) and transfer it to the barrel of the syringe/minicolumn assembly.

Try not to suck up any of the gunk on the side of the tube.

11. Carefully insert the syringe plunger into the barrel and gently push the slurry into and through the Minicolumn.

The filtrate must be collected in the special waste vial provided and the empty microfuge tube must be placed in the special dry waste container provided. At this point, your plasmid DNA should be bound to the resin beads entrapped in the minicolumn.

The empty microfuge tubes must be placed in the special dry waste container provided in the hood.
12. Detach the syringe from the Minicolumn AND THEN remove the plunger from the syringe barrel. Reattach the syringe barrel to the Minicolumn.

*IMPORTANT! It is critical that you detach the syringe from the Minicolumn BEFORE you remove the plunger from the syringe. Otherwise, you will suck the resin out of the Minicolumn and back into the syringe barrel. This destroys your prep.*

13. Pipet 2.0 ml of Column Wash Solution (CW) into the barrel of the Minicolumn/syringe assembly. Insert the plunger into the syringe and gently push the Column Wash Solution through the Minicolumn. The filtrate must be collected in the special waste vial provided. Cap the waste vial and set it aside for proper disposal. You may now remove your goggles and gloves.

14. Remove the syringe and transfer the Minicolumn to a 1.5 ml microcentrifuge tube with the cap removed. Centrifuge the Minicolumn in a microcentrifuge for 2 minutes to dry the resin. The microfuge tubes and syringe must be placed in the special dry waste rack container provided in the hood.

15. Transfer the Minicolumn to a new 1.5 ml microcentrifuge tube (with cap removed but saved).

16. Add 50 µl of Tris-EDTA Buffer (TE) to the Minicolumn and centrifuge in a microcentrifuge for 20 seconds to elute the DNA.

17. Remove and discard the Minicolumn.

Proceed directly to the restriction digest (Step VII) or store the plasmid DNA in the microcentrifuge tube at 4°C or -20°C.

Typical DNA yield: 50 µl @ 0.1 µg/µl (approx.)
VII. Molecular Analysis of Recombinant Plasmid by Restriction Analysis

Using minipreps, you have isolated your recombinant plasmid DNA, and native pUC19 DNA from E. coli clones obtained by transformation of competent cells with DNA from the ligation. To analyze the recombinant, it is cut with Hind III and the fragments examined by gel electrophoresis. The goal is to identify the lambda DNA insert cloned into pUC19.

You performed restriction digests of plasmid DNA in two previous lab periods (Lab 8 and Step I of this exercise). In both cases you followed an explicit procedure set forth in detail in this manual. Here we expect you to create your own procedure, based on the guidance given below. It should be helpful to go back and review the procedural details of the 2 previous digests.

1. Develop a general strategy. How many different digest tubes will you set up? What DNA samples will they contain and which restriction enzyme/s will you use for each? Don’t forget to include controls that will verify the identity of all fragments from both minipreps.

2. The fundamental issue in working out an exact procedure is to determine how much DNA to use in each digest. This is governed by what you want to do with it after digestion. In phase I, “for example”, the digested DNAs were used in 2 ways (electrophoretic analysis and ligation).

   Here, the DNA will be used only for gel electrophoresis. You must use enough DNA so that each band on the gel is visible. An important rule of thumb applicable here is that a band in an agarose gel should ideally have between 10 ng and 100 ng of DNA. (It is not always possible to realize this ideal when fragment sizes within a given vary over more than a 10X range.) On the other hand, digesting excessive amounts of DNA is wasteful.

   By considering the relative sizes of pUC19 (2.686 kb) and the average size of the expected insert/s, we can make a rough estimate of the total amount of DNA needed for each digest. We recommend cutting 0.5 µg.

   Later when the size of your DNA insert has been determined, it will be very worthwhile for you to come back and calculate how much DNA is present in the insert band on your gel.

3. Next, determine the volume of DNA sample to use in each digest based on the expected yield figures for the miniprep given earlier. This can only be roughly estimated.

4. Determine the amount of restriction enzyme/s to use based on the amount of DNA in the digest, the definition of unit activity, and the concentration of the enzyme stock. Use a 5 – 10-fold excess of enzyme to insure complete digestion. Think ahead to the possibility that you may need to dilute the enzyme stock.
5. Next determine what the total volume of the digest will be. Remember that the maximum volume you can load into the wells of our gel system is 20 µl, and that you will add 6X tracking dye to each digest before loading.

6. Determine the nature and amount of restriction buffer to add. Restriction buffer is available at concentrations of 2X or 10X.

7. Determine the volume of water necessary for each digest tube to bring them all to the same total volume.

8. Summarize your procedure in a tabular format such as the one used to describe the previous digests.
VIII. Agarose Gel Electrophoresis #2

This gel identifies the Lambda\textit{Hind} III fragment/s you have cloned in your recombinant plasmid.

Run a 0.7\% w/v agarose gel under the same conditions as previous gels.

Run 2 DNA standards (provided) in addition to the samples from your digests (cut and uncut; white and blue). The 2 standards are:

1. \(\lambda\) \textit{BstE} II DNA
2. \(\lambda\) \textit{Hind} III DNA

IX. Analysis of Results

If the gel shows that you have cloned more than one Lambda\textit{Hind} III fragment, your report should reflect your consideration of how this might have happened. There are several possibilities:

- The original Lambda DNA digest did not go to completion and you cloned one of the partially digested fragments.

- Your plasmid is a ternary ligation product (i.e. results from ligation of two different Lambda\textit{Hind} III DNA fragments with the plasmid).

- Your clone acquired two different recombinant plasmids, each containing a single Lambda\textit{Hind} III DNA fragment.

How can you distinguish these possibilities?

You should also reflect on the class results. Were some fragments cloned more frequently awhile others were cloned less frequently or not at all? If so, why?