Biotechnology &
Recombinant DNA Techniques
For Middle and High School Students and Teachers

by

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Additional reading on Recombinant DNA techniques:


INTRODUCTION

Genetic engineering has become one of the most powerful tools in modern biochemistry and biology. Recombinant DNA technology is widely used in both research and industry. Many new companies are being created for research and development of techniques that are now classified as biotechnology.

In the following experiments, you will isolate and analyze bacterial DNA using several recombinant DNA techniques. We hope that these experiments will stimulate both you and your students to answer and raise questions concerning modern biology as well as the applications and benefits of gene cloning.

Recombinant DNA technology historically has used the microorganism *Escherichia coli*, which is a naturally occurring gut microbe. Some *E. coli* strains not only contain the genomic DNA that encodes the information required for growth and replication, but also carry small autonomously replicating circular DNA molecules called plasmids.

Although plasmids do not encode genes that are required for normal cell growth, they do contain genes which can give the cell a selective advantage under certain growth conditions. For instance, several plasmids encode antibiotic resistance genes, while others encode for the genes that confer sexual recombination ability. By taking advantage of the observation that certain plasmids can confer antibiotic resistance, plasmids have been used as vehicles (vectors) to transfer the genes of one organism to another. The presence of the plasmid containing antibiotic resistance genes has been used to detect microorganisms that contain the recombinant plasmid. Thus, various plasmids have been used as the basis for most gene cloning experiments and have become powerful tools in recombinant DNA technology.

To understand how the acquisition of a new gene changes the phenotype of a cell, we will investigate several recombinant DNA techniques. This will require that you gain an understanding of how restriction enzymes work and how they are used to achieve site-specific cleavage of DNA; what technique is used to induce uptake of plasmid DNA by *E. coli* cells, resulting in a changing cellular phenotype; and how to isolate plasmid DNA from the *E. coli* cells that harbor it.
Two commonly used plasmid vectors are shown above. These were artificially constructed for the purpose of isolating and moving pieces of foreign DNA into microbial systems. pBR322 contains the genes for ampicillin resistance and tetracycline resistance. This means that a microorganism containing this plasmid is capable of growth on media containing both of these antibiotics, while microorganisms that do not contain this plasmid could not grow on the antibiotic-containing media. This antibiotic resistance can be used as a means of selecting and enriching for only those organisms that contain the plasmid.
Overall Scheme for Recombinant DNA Experiments

1. **Restriction Enzyme Digestion**
   - Enzyme digestion of pUC-18 polylinker
   - Resulting fragment(s) marked as Amp

2. **Transformation**
   - Transformation of Amp
   - Insertion of foreign DNA into polylinker

3. **Selection Plates**
   - TVE Plates with Amp
   - TVE Plates with Tet
   - TVE Plates without Amp or Tet

Diagram depicts the process of cloning and selection in recombinant DNA experiments.
Experiment One:  
Restriction Digestion and Agarose Gel Electrophoresis

This experiment involves the use of enzymes called restriction endonucleases that recognize and cleave specific sequences of DNA. For example, EcoR1 cleaves the sequence shown to produce specific ends and specific pieces of DNA that are unique and of reproducible sizes. For example, upon incubation with EcoR1, a plasmid containing five EcoR1 restriction sites yields five fragments of 100, 400, 600, 1000, and 1200 base pairs in length. Since the DNA contains negatively charged phosphates in its sugar-phosphate backbone, DNA fragments can be separated on the basis of size by agarose gel electrophoresis.

In this case, the smallest piece of DNA moves the fastest generating a separation of the fragments by size.

In this experiment, you will be using the restriction endonucleases EcoR1, and Sau3A (RsaI). Due to the fact that Sau3A (RsaI) contains a recognition sequence of only four bases instead of the six recognized by EcoR1, Sau3A (RsaI) is less specific and a given plasmid will contain more Sau3A restriction sites than EcoR1.
Restriction Endonuclease Recognition Sequences:

<table>
<thead>
<tr>
<th></th>
<th>EcoR1</th>
<th>Sau3A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><code>--GAATTC--</code></td>
<td><code>--GATC--</code></td>
</tr>
<tr>
<td></td>
<td><code>--CTTAAG--</code></td>
<td><code>--CTAG--</code></td>
</tr>
</tbody>
</table>

EcoR1 cleaves both plasmids pBR322 and pUC only once, causing the circular DNA molecule to open up and linearize, while Sau3A or RsaI cleave multiple times yielding many linear bands.

When the above digest is analyzed on an agarose gel through electrophoresis, only one band is seen. This band can be distinguished from the uncut plasmid, which gives two bands, corresponding to two different conformations (supercoiled and relaxed circle) of the circular DNA molecule.
Restriction Digestion

1. Put 5 uL of sterile double distilled water into two separate small Eppendorf tubes. Label the tubes 1 and 2.
2. Add 1 uL of 10 X buffer to each tube (medium salt buffer is required by these two enzymes).
3. Add 4 uL of pUC18 DNA to each tube.
4. Add 1 uL of EcoR1 restriction enzyme to tube 1, and 1 uL of Sau3A restriction enzyme to tube 2.
5. Incubate tubes in a 37 degree C water bath for 1-2 hours.
6. One hour before your digest is completed, prepare a 0.7% agarose gel (for NSF program we will prepare one gel for the entire class):
   - 1.05 g agarose
   - 142 mL double distilled water
   - 7.5 mL 20 X agarose gel buffer
     - Mix all ingredients and heat in a microwave, or on a hot plate until agarose is dissolved. Add 25 uL of ethidium bromide (use caution) and swirl to mix. Place well-forming comb on glass plate and pour carefully. Allow 20-30 minutes for polymerization.
7. Remove 4 uL of your pUC restriction digestions to new small Eppendorf tubes and add 1 ul of agarose gel dye to each tube. In a separate tube, add 2 ul of uncut pUC18 DNA and 2 ul of dye, to run as a control (NSF program will run one control for the entire class).
8. Load the contents of the three tubes in three adjacent wells of the agarose gel.
9. Attach the electrodes and electrophorese at 200 mAmps for 0.5 to 1 hour.
10. Visualize the gel under a long wave ultraviolet light source and either photograph or draw a picture of what you see. Use UV eye protection.
Experiment Two:
Bacterial Transformation

Experiment Two demonstrates the process by which bacteria uptake foreign DNA. In nature, bacteria take in and incorporate pieces of DNA into their genome that are found in the environment. The bacteria then replicate the foreign DNA along with their own DNA. This is one form of natural genetic recombination. When this method is coupled with the use of the previously described antibiotic selection techniques, bacteria can be induced to incorporate certain DNA fragments and then can be selected for that incorporation.

Bacterial growth occurs in an exponential fashion. Bacteria can be made competent to uptake DNA in the early log phase of growth through treatment with calcium chloride. Calcium chloride essentially pokes holes in the cell wall and thereby allows the DNA to enter the cell. The presence of a plasmid such as pBR322 or pUC allows for the expression its of antibiotic resistance and/or sugar metabolism genes within the cell. Thus, you can use the selection conditions to isolate cells that contain the plasmid of interest.

Bacterial Transformation Procedure:

1. Prepare 50 mL of sterile 2XTY media in a 250 mL Erlenmeyer flask. This may be prepared first and sterilized in the flask.
2. Inoculate the 2XTY media using 1-2 mL of an overnight *E. coli* culture that has been grown at 37 degrees C.
3. Grow for 2-2.5 hours in a 37 degrees C shaker or water bath (NSF program: we will start this for the entire class in the morning).
4. Transfer 1 mL of culture to each of 4 large Eppendorf tubes and pellet the cells by spinning for 5 minutes in a microcentrifuge.
5. Pour off the supernatant by pouring away from the pellet and gently tapping the tubes upside down on a tissue to remove any remaining drops of media. Add 0.5 mL of ice

***Save one mL of culture for later plating as an untreated control culture (NSF program: we will save the flask of culture and will pass it around again later before plating)

***Note--from this point on, all solutions and cells used MUST BE KEPT ON ICE !!!!!!
cold 50 mM CaCl$_2$ solution to each tube and resuspend cells through gentle vortexing. Incubate on ice for 5 minutes. This treatment makes the cell wall permeable to DNA.

6. During the incubation of cells in CaCl$_2$, label 6 Falcon tubes and place on ice to chill. These should be labeled as, 1-"control-Amp", 2-"control-TYE", 3-"no DNA-Amp", 4-"no DNA-TYE", 5-"pUC 18-Amp", and 6-"pUC 18-TYE".

7. Spin the cells for 5 minutes in microcentrifuge to pellet the cells. Pour off the supernatant.

8. Resuspend the cells in 100 uL of ice cold 50 mM CaCl$_2$ as before. This yields competent cells.

9. Dispense 100 uL aliquots of the resuspended, competent cells into falcon tubes 3, 4, 5 and 6 (See the Table on the next page).

10. Add 2 uL of the pUC18 DNA into tubes 5 and 6. Add no DNA to the other tubes!!!

11. Incubate on ice for 15 minutes. During this time, plates should be labeled as, 1-"control-Amp", 2-"control-TYE", 3-"no DNA-Amp", 4-"no DNA-TYE", 5-"pUC 18-Amp", and 6-"pUC 18-TYE".

12. Heat shock the cells in a 42 degrees C water bath for 5 minutes.

13. Add 1 ml of fresh 2XTY (cell food) to tubes 3, 4, 5, and 6, and place in a 37 degrees C water bath for 15-30 minutes. Note--this allows the competent cells to recover before they are plated on selective media.

14. Now the transformations and controls are ready to be plated.

   a. We will do the control first. Add 500 ul of the untreated cells (from the flask of culture that is passed around) to tube 1-"control-Amp", then add 2.5 ml of lambda top agar that has been heated to melt it and then cooled to about 50 degrees C. (It should be comfortable to the touch). Vortex the mixture, and pour onto the surface of the plate labeled 1-"control-Amp" (that is at room temperature). Be sure to pour the agar mixture as quickly as possible so that it does not solidify in the tube.

   b. Repeat Step a. adding 500 ul of untreated cells to tube 2-"control-TYE" and plate onto the labeled TYE plate.

   c. Add 2.5 ml of lambda top agar to tube 3-"no DNA-Amp", vortex, and plate onto an Amp plate.

   d. Repeat Step c. and plate tube 4-"no-DNA-TYE" on a TYE plate.
e. To tube 5 -"pUC 18-Amp" add 2.5 ml of top agar, vortex, and plate on an Amp plate.
f. Similarly, add 2.5 ml of top agar to tube 6-"pUC 18-TYE", vortex, and plate onto a TYE plate.

### Transformation and Controls

| Tubes   | plate | DNA | cells | untreated | 2XTY  | Top Agar | Growth?
|---------|-------|-----|-------|-----------|-------|----------|--------
| 1.controlAmp | no    | no  | 500 ul| no        | 2.5 ml|
| 2.controlTYE | no    | no  | 500 ul| no        | 2.5 ml|
| 3.no-DNA   | Amp   | 100 ul| no   | 1 ml      | 2.5 ml|
| 4.no-DNA   | TYE   | 100 ul| no   | 1 ml      | 2.5 ml|
| 5.pUC 18   | Amp   | 100 ul| no   | 1 ml      | 2.5 ml|
| 6.pUC 18   | TYE   | 100 ul| no   | 1 ml      | 2.5 ml|
Experiment Three:
Plasmid DNA Isolation

In this experiment you will pick colonies from selective media plates and grow the cells overnight. You will then isolate the plasmid DNA and visualize the resulting isolate after electrophoresis on an agarose gel. A flowchart and detailed instructions for the procedures are included with an explanation of the theory involved.

You will be given plates that have the bacteria *E. coli* containing a plasmid (pUC18). These are selective media plates, they contain the antibiotic ampicillin, so only cells that contain the appropriate plasmids will grow.

1. Pick one colony off the plate using a sterile toothpick and place in a sterile test tube containing 2 ml of growth media (2XTY) containing ampicillin to insure the growth of the appropriate plasmid. Incubate overnight at 37 degrees C in a shaker or water bath.
2. Transfer the culture to a 1.5 ml Eppendorf tube and spin 5 minutes in a microfuge to pellet the cells.
3. Remove and discard the supernatant by pouring. Allow the tubes to remain inverted on a clean tissue for a few minutes.
4. Add 200 ul of lysis solution to the pelleted cells and resuspend the cells by vortexing. Incubate at room temperature for 15 minutes.
5. Add 200 ul of alkaline SDS solution and incubate at room temperature for 5 minutes. This step completes cell lysis, because the detergent SDS (sodium dodecyl sulfate) solubilizes cell membranes and cell wall lipids, which thereby causes the cell to lose its encapsulating features.
6. Add 150 ul of 3M sodium acetate, pH 4.8 and incubate on ice for 10-30 minutes. This high salt solution causes the cellular debris to precipitate from the solution.
7. Spin the tube for 15 minutes to pellet cellular debris and decant the supernatant into a clean 1.5 ml Eppendorf tube by pouring carefully.
8. Add 1 ml of cold 95% ethanol and precipitate the DNA by placing it on ice for 15 minutes (can be left in the freezer overnight if necessary).
9. Spin the tube 15 minutes in a microfuge. Pour off the supernatant carefully.
10. Air dry the pellet by inverting the tube on a clean tissue for 5-10 minutes.
11. When the pellet is dry, resuspend in 20 ul of fresh TE (store in
freezer if desired).

12. To view the isolated DNA, add a 5 ul aliquot of your isolated DNA to a separate tube containing 2 ul of agarose gel dye. Load in a single well of an agarose gel. Electrophorese for 0.5-1 hour at 200 mA. (NSF program: we will make one gel for the entire class).
Experiment 4: Chromosomal DNA Isolation from Onions

1. Chop 1 onion in large pieces. Place in blender with 15 ml distilled water. Blend on low speed for 30 seconds, scraping down the onion material if necessary. Follow with 3 ten second bursts at high speed, scraping down the onion material for best mixing. The blended onion should be a wet paste.

2. Add 7 ml onion paste to a 15 ml tube. Add 7 ml lysis solution and mix gently by inversion. May need to mix paste and solution with spatula before inverting. Mix until the blue lysis solution is evenly dispersed.

3. Place tube in 55-65 degree C waterbath (or water heated to correct temperature in microwave) for 15 minutes. Mix by inversion every 3 minutes.

4. Place tube on ice for 5 minutes.

5. Filter mix through a coffee filter folded to fit in a 50 ml tube.

6. Transfer filtrate to a fresh 15 ml tube. Add an equal volume of cold isopropanol to filtrate. Leave at room temperature for 3 minutes. Note the interface, where a white precipitate will begin forming.

7. Invert tube gently 5 times. Swirl gently through the mixture with a glass spooling rod to collect the precipitated DNA strands.

8. Air dry for 30 minutes. Resuspend in 1 ml of 10:0.1 TE resuspension solution.

9. To visualize, run 20 ul of resuspended DNA on agarose gel.

NOTES:
- From one medium onion there should be sufficient material for at least 10 isolation reactions.
- The SDS lysis solution must be heated in waterbath for several minutes to allow SDS to go completely into solution.
- Blue dextran is optional and will result in a blue color in the precipitated DNA which is otherwise white or clear. Its presence allows for easier observation when mixing with onion paste and later when observing the isopropanol interface.
- Care should be used to avoid shearing of DNA by always gently inverting tube when mixing.
Experiment # 5
Butter Dish Electrophoresis

Apparatus Assembly

1. Gather materials to assemble gel apparatus.

   Materials Needed:

   - Covered butter dish with 4 holes drilled for electrode wires
     - one in each corner of dish base)
   - 3 pieces of 1/8 in. Lucite for tray - One large (top) and two small (legs)
   - 2 lengths stainless steel wire-approx. 8 in. each
   - 9-V transistor radio batteries (5) connected in series
   - 2 Jumper cables with insulated alligator clips -Super glue

2. Attach legs of tray to the underside of tray top using super glue.

3. Bend both pieces of wire into U shape. Insert ends of wire into holes in base so that the U will hang down into the buffer (in butter dish top) when upside down.
Gel Assembly

You will need:
- Agarose
- Buffer
- Tape
- Comb or Nails (to make wells in gel)
- Carolina Blu™ Safe dye Stain

1. Prepare Electrophoresis Buffer (see recipes-20X AGB) & dilute to 1X AGB.

2. Tape agarose gel plate.

3. Prepare Agarose Gel:
   - Weigh 0.3 g agarose
   - Add 30 mls 1X AGB
   - Heat to dissolve
   - When cool (55º-65ºC) add 1 drop of Carolina Blu™ Staining solution
   - Pour gel into taped plate

4. Place lid on butter dish and insert nails into pre-drilled holes (or insert comb into gel)

5. After gel solidifies (25-35 minutes), remove nails or comb. Take gel out of apparatus and remove tape.

6. Place gel into butter dish and add 1X AGB (add dye) until gel is covered. Add loading dye to DNA and pipet into wells. Cover with lid.

7. Attach batteries in series. Attach the alligator electrode clips.

8. Run gel 30-45 minutes.

10. Place the gel in a staining tray. Cover the gel with the Final Carolina Blue Stain™ and process for 5-10 minutes. Agitate gel and stain if possible. View bands to determine whether to develop longer or to destain.

11. Pour Stain™ back into bottle (can use about 8 times).

12. Cover gel with deionized water and destain. Repeat 3-4 times over 30-40 minutes. (Leave overnight to destain completely)

13. Wrap gel in plastic wrap and store in refrigerator. (1-2 days)