BIOTECHNOLOGY EXPERIMENTS

by

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# TABLE OF CONTENTS

Micropipetting ........................................................................................................................................... 2

Preparation of competent cells .................................................................................................................. 3

Transformation of cells with DNA ........................................................................................................ 5

DNA fingerprinting ................................................................................................................................. 7

Digestion of DNA with restriction endonucleases .................................................................................. 8

DNA fingerprinting and digestion of DNA .............................................................................................. 9

Electrophoresis ........................................................................................................................................ 10

Plasmid map ............................................................................................................................................ 13
PROCEDURE I – MICROPIPETTING

Pipetting microliter amounts requires maximum precision and consistency. Since we will be doing a considerable amount of micropipetting, it is important to make certain that we can do it proficiently. Colored water will be used for this practice session.

1. Set the desired volume by holding the pipet body in one hand and turning the volume adjustment knob until the correct volume shows on the digital indicator. Lock into place.

2. Attach a new disposable tip to the shaft of the pipettor.

3. The plunger of the pipettor is depressed to the first positive stop. The pipet tip is placed 1-2 mm into the sample liquid and the push button is allowed to return to the up position. Never allow the push button to snap up. Wait, with the tip still in the liquid, to insure that a full volume of sample is drawn into the tip.

4. The pipet tip is placed beneath the surface of the liquid in the receiving vessel. The sample is gently expelled from the tip and the solution is stirred with the tip to disperse the sample.

5. The solution is drawn back up into the pipet tip to rinse the tip with the liquid in the receiving vessel. The liquid is again gently expelled into the receiving vessel.

6. The plunger is returned to the top position and the tip is discarded.

7. When pipetting small volumes of fluid, e.g. 10 μl, into a dry microfuge tube, touch the tip of the pipettor to the inside wall of the microfuge tube while discharging the fluid.

Some general hints for a good experiment – Keep your hands away from all solutions. Your fingers may have endonucleases that will degrade the DNA. Always change pipet tips for each addition of a reagent. This will help make sure that you do not accidentally contaminate any of your solutions. Pipet in the exact order that the reagents are listed. Always try to keep your 1.5 ml microfuge tube and all reagent tubes on ice.

Dispose of all tubes and pipet tips in the biohazard bag.
PROCEDURE II – PREPARATION OF COMPETENT CELLS

Many bacteria are capable of taking up foreign DNA. These bacteria are said to be “competent”. A temporary state of “competence” can be induced by treating the bacteria with ice-cold solutions of calcium chloride followed by heat shock at 42°C. This treatment induces the bacteria to produce enzymes that degrade their bacterial cell walls at specific places. The open pores then allow the foreign DNA (plasmids) to enter the cell. The plasmids pUC18 and pBR322 are closed, circular rings of double stranded DNA that have a gene for ampicillin (antibiotic) resistance. Not all of the bacteria will take in the plasmids. The bacteria that take in the plasmids will be able to grow on the agar containing ampicillin because they will express the ampicillin resistance gene. They will produce an enzyme that inactivates ampicillin by breaking the antibiotic apart. The bacteria that did not take in the plasmid will die because they are sensitive to ampicillin. The ampicillin prevents the cell wall from staying together or repairing itself. The bacteria which have taken in the naked DNA plasmid are said to be transformed. The process is called transformation.

Bacterial transformation is a key technique in biotechnology. By transforming bacteria, scientists are able to produce natural occurring proteins, such as insulin, in commercial quantities. Transformation is the inserting of a gene or a few genes from one organism into another. Scientists put the gene of interest and an antibiotic resistance gene in the same plasmid and transform the bacteria. The scientists can tell which bacteria have received the plasmid containing the added gene because the bacteria are then able to grow on media containing the antibiotic for which the plasmid carries resistance.

Once the bacteria containing the gene have been isolated, they can be grown up in fermentation tanks where they divide about every 20 minutes to create two identical bacteria, each containing the plasmid. The millions of bacteria grown express the gene that has been added to the plasmid. In this way the peptides that make human insulin can be produced by bacteria and can be purified. Other products such as human growth hormone, artificial sweetener, interferon (controls viral infections) are also being made this way.
PROCEDURE III – MAKING CELLS COMPETENT

1. Label 2 microcentrifuge tubes with your initials and 
   Label 1 tube + DNA  
   Label 1 tube – DNA control  
   Pipet 1 ml of cell culture (E. coli HB101 cells provided by instructor) into each of the 2 microcentrifuge tubes.

2. Pellet the cells by centrifuging the tubes for 10 seconds.  
   Balance the rotor.  
   The pellet should be 1-2 mm in diameter and white.

3. Pour out the liquid broth into the biohazard trash.  
   It is important to remove as much of the liquid as possible by tapping the tubes on a tissue.

4. Add 1 ml of sterile, cold 100 mM calcium chloride to each tube.  
   Resuspend the cells by mixing the tubes vigorously (can be vortexed).  Be sure that the cell pellets are thoroughly resuspended.  
   Keep these 3 tubes in an ice bath for 15 minutes.

5. Pellet the cells by centrifuging 10 seconds.  
   Pour out the liquid into the biohazard trash.

6. Add 100 μl of sterile, cold 100 mM calcium chloride to each tube.  
   GENTLY resuspend the cell pellet in each tube (no vortexing or stirring).  
   Keep tubes on ice.

7. Cells can be used right away or cells can be stored at 4°C.  
   If stored, the cell viability will decrease after 2 days.
PROCEDURE IV – TRANSFORMATION OF CELLS WITH DNA

Place all tubes on ice.

8. **To the + DNA control tube,** add 10 µl from the stock DNA solution. This DNA solution contains the commercial plasmid.

9. **To the – DNA control tube,** do not add any DNA.

10. Mix the tubes gently.  
    Keep on ice for **30 minutes.** (20 minute minimum)

11. Place tubes in a 42°C water bath for **2 minutes.**  
    Immediately return the tubes to the ice bath.

12. Add 1 ml of sterile LB broth to each tube. Do not shake tubes!!  
    Incubate at 37°C for **30 minutes.** (20 minute minimum)

13. Resuspend the cells by inverting each tube gently once or twice.

14. Label 1 LB agar plate without ampicillin with your initials and – **DNA.**  
    Pipet **100 µl** from the – DNA tube onto the plate and spread the cells with a sterile loop.

    Label 1 LB agar plate with ampicillin with your initials and – **DNA.**  
    Pipet **100 µl** from the – DNA tube onto the plate and spread the cells with a sterile loop.

    Label 1 LB agar plate with ampicillin with your initials and + **DNA.**  
    Pipet **100 µl** from the + DNA tube onto the plate and spread the cells with a sterile loop.
15. Incubate the plates upside down overnight at 37°C.

16. Examine the growth on each plate. Each colony was started by a single invisible bacterium. Since *E. coli* divides approximately every 20 minutes, each original bacterium has generated an entire colony of bacteria.
DNA fingerprinting was developed in 1985 by Alec Jeffreys. It is named DNA fingerprinting because the DNA sequence of an individual is as unique as are the fingerprints. Only one person out of 4 trillion might share the same DNA sequence (except identical twins). DNA fingerprinting is based on Restriction Fragment Length Polymorphisms (RFLP) analysis. Long stretches of DNA are the same in all persons, but certain areas have a variety of differences present in the DNA base sequences. These changes in the DNA sequences that have no effect on the protein product are called polymorphisms. (Frequently, a change in the base sequence does produce a different protein product.) These polymorphic, highly variable regions will never break in the same place in two individuals (except in identical twins) when cut with Restriction Enzymes and therefore create a pattern unique to each person.

Forensic scientists use DNA fingerprinting to examine the DNA in biological evidence and prove with almost certainty whether it came from a given individual. By doing so, the technology has helped to incriminate or exonerate accused rapists, identify murder suspects, and settle paternity questions. The DNA is very stable and remains intact in most environments. The RFLP analysis can be performed on less than 100 µl of blood, even if dried and stained for years; on small semen samples and on hair follicle cells that cling to hair roots which have fallen or been pulled out.

In RFLP analysis, the DNA sample must first be digested with a restriction enzyme which cuts the DNA into fragments by recognizing a specific base pair sequence. These fragments can then be separated by gel electrophoreses. Because DNA carries a negative charge, it will migrate from the negative pole to the positive pole in the chamber; the smallest fragments migrating the fastest. In order to identify specific fragments, a Southern Blot is further applied. The complementary dsDNA is first denatured to utilize only single-stranded DNA (ssDNA). The bands on the gel are transferred to a nylon sheet and a radioactive probe, short sequences of synthetic ssDNA, hybridizes those fragments to form dsDNA. Exposure of this nylon sheet to x-ray film produces black bands where the radioactive probes bind to the fragments. (Not all of the DNA fragments bind to the probe.) The resulting autoradiogram can be considered unique to each individual. The bands in the autoradiogram appear at different intensities and distances and the size of the fragment is disclosed by comparing them to known standards run in adjacent lanes on the same agarose gel.
DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASES

Restriction endonucleases (restriction enzymes) are isolated from bacteria. They are named by taking the first letter of the genus name and the first two letters of the species name of the bacterium they were isolated from. An example is EcoRI which was isolated from Escherichia coli Ry113 (RI).

The restriction enzymes used in molecular DNA experiments recognize, bind to, and cut double stranded (ds)DNA at specific nucleotide sequences, usually 4 - 6 nucleotides long.

The enzyme EcoRI recognizes the following 6 bp sequence

Strand 1 .......... GAATTC ........... Strand 1
Strand 2 .......... CTTAAG ........... Strand 2

Remember G&C pair and A&T pair with each other on the opposite strands.

When EcoRI cuts the dsDNA sequence it has recognized, it produces pieces which have ends that look like:

Strand 1 .......... G and AATTC ........... Strand 1
Strand 2 .......... CTTAA G ............... Strand 2

The enzyme used in these experiments is BglII which is isolated from the bacterium Bacillus globigii. This enzyme recognizes the sequence

Strand 1 .......... GCCNNNNNGGC ............. Strand 1
Strand 2 .......... CGGNNNNNCCG ............. Strand 2

(N can be either A or C)

When it cuts the dsDNA sequence it has recognized, it produces pieces which have the ends:

Strand 1 .......... GCCNNNN and NGGC ............. Strand 1
Strand 2 .......... CGGN NNNNCCG ........... Strand 2

Restriction enzymes require different conditions when they digest DNA. The major variable is the composition of the buffer and buffers differ chiefly in the concentration of salt (NaCl) that they contain. The enzyme BglII requires a high salt buffer. It is most active at 37°C.
PROCEDURE V – DNA FINGERPRINTING

We are going to generate a fingerprint pattern to identify an unknown DNA sample.

1. Label 3 sterile 1.5 ml microcentrifuge tubes with your initials.
   label 1 tube “unknown”,
   label the second tube “pBR 322”
   and label the third tube “pUC 18”

2. Pipet the following in the order indicated into the labeled tubes

<table>
<thead>
<tr>
<th></th>
<th>Unknown</th>
<th>pBR 322</th>
<th>pUC 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>17 µl</td>
<td>17 µl</td>
<td>17 µl</td>
</tr>
<tr>
<td>10X buffer</td>
<td>2 µl</td>
<td>2 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>Bgl I</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

3. Centrifuge these tubes 5 seconds in the microfuge.

4. Incubate all 3 tubes at 37°C for 45 minutes.

During this 45 min incubation period, prepare your agarose gels for your subsequent electrophoresis. (Page 12)

5. Centrifuge the tubes for 5 seconds.

6. Add 4 µl of tracking dye to each microfuge tube.
   Centrifuge the tubes for 5 seconds.

7. You may stop here and store in the refrigerator overnight.

8. Add 20 µl of each sample to the wells in the agarose gel.

9. The gel will run about 3 h at 48V.

10. Photograph the gel.
PROCEDURE VI – ELECTROPHORESIS

There are two types of electrophoresis that are used to separate nucleic acid molecules. Polyacrylamide gel electrophoresis is used to separate very small fragments of DNA and agarose gel electrophoresis is used to separate larger fragments of single and double-stranded DNA. Since we will be working with relatively large pieces of DNA, we will be using agarose gel electrophoresis.

Agarose, which is made from seaweed, is a bit like jell-o in that it needs to be heated to go into solution and then solidifies as it cools. Agarose gels are made by melting agarose in the desired buffer until it becomes a clear solution. The melted solution is poured into a mold and allowed to harden.

The DNA samples can then be added to the wells in the gel that were formed by the well mold when the agarose solidified. When an electric field is applied across the gel, DNA (which is negatively charged at pH 8) migrates to the positive pole. Three important features affect the way DNA moves in the gel; 1. the size of the DNA pieces, 2. the shape of the DNA, and 3. the concentration of the agarose in the gel. Smaller DNA molecules migrate faster than larger DNA molecules. DNA fragments that have extra twisting patterns (coiled) migrate faster than DNA fragments that are linear (relatively straight). Usually a scientist will select the concentration of agarose for a gel depending upon the estimated size of the DNA fragments they wish to separate. Gels containing relatively low concentrations of agarose (0.3%) are used to separate very large DNA fragments (1-60 kp) and gels containing relatively high concentrations of agarose (2.0% are used to separate smaller DNA fragments (.1-2 kb). We will be using gels that are 0.8% agarose.

The loading buffer is added to the DNA sample and serves three purposes. It increases the density of the sample so that the DNA drops evenly into the well, it adds color to the sample which makes the loading of the DNA easier, and it contains dyes that move toward the anode. Bromphenol blue migrates through agarose gels at approximately the same rate as linear dsDNA 300 bp in length, whereas xylene cyanol FF (green) migrates at approximately the same rate as linear dsDNA 4 kb in length. The DNA we are separating will be between the two dyes.

DNA can be detected in agarose gels by using the fluorescent dye, ethidium bromide (ETBR). ETBR sticks between the two strands of a piece of DNA and can be seen when an ultraviolet light shines on it.
CAUTION!  

ETBR is a powerful mutagen. It has been shown to be able to cause cancer in tissue culture cells. Gloves should be worn when working with gels containing ETBR.

Ultraviolet light is dangerous to the eyes and skin. Always wear protective goggles or a full safety mask that efficiently blocks UV light.
PROCEDURE VI – ELECTROPHORESIS

1. Assemble the agarose gel casting mold.

2. Prepare 1000 ml of 1X TBE electrophoresis buffer by diluting 200 ml of 5X TBE with 800 ml of distilled water.

3. Weigh 0.4 g of agarose and place into a flask. Add 50 ml of 1X TBE buffer. Melt the agarose in the microwave (approx. 1 min), or on a hot plate (approx. 3-5 min).

4. Cool the agarose by running cool tap water over the surface of the glass bottle until it is very warm but not scalding hot.

5. Add 1 µl of ethidium bromide (ETBR) stock solution to the agarose solution, swirl gently to mix, and pour the solution into the gel casting tray.

6. Position the comb near one end of the gel and allow the casting chamber to stand undisturbed until the gel is firm (about 20-30 min).

7. After the gel has solidified, remove the comb and place the gel into the electrophoresis chamber. Fill the unit, covering the gel to a depth of about 2 mm with 1X TBE buffer. Load gel so that cells are on the negative (black) pole – buffer has a pH of 8 so DNA will pick up a negative charge and move from negative to positive pole.

8. The gel is now ready for adding the DNA samples.

CAUTION! CAUTION!

The gel contains ETBR which is a powerful mutagen. It has been shown to be able to cause cancer in tissue culture cells. Gloves should be worn when handling the gel. The gel should be disposed of in the biohazard bag.