Cloning of a PCR Amplified Gene

EXPERIMENT OBJECTIVE:

The objective of this experiment is to amplify a DNA fragment by Polymerase Chain Reaction (PCR) and to clone the amplified DNA by using the blue/white cloning system.

This experiment is designed for DNA staining with InstaStain® Ethidium Bromide.
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Material Safety Data Sheets can be found on our website:
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Components & Requirements

This experiment contains reagents to perform five PCR reactions and five cloning experiments.

Sample volumes are very small. For liquid samples, it is important to quick spin the tube contents in a microcentrifuge to obtain sufficient volume for pipeting. Spin samples for 10-20 seconds at maximum speed.

This experiment includes either BactoBeads™ or LyphoCells™. If you have received LyphoCells™, please refer to the addendum posted on the last page of this literature. If you have received the BactoBeads™, refer to the Pre-Lab Preparations on page 31.

All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

THIS EXPERIMENT DOES NOT CONTAIN HUMAN DNA. None of the experiment components are derived from human sources.

**ATTENTION!**

**Components & Requirements**

<table>
<thead>
<tr>
<th>Reagents for PCR</th>
<th>Storage</th>
<th>P1 DNA Template for Amplification</th>
<th>-20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2 Primer Set (two primers)</td>
<td></td>
<td>P3 Tubes with PCR Reaction Pellets™</td>
<td>-20°C</td>
</tr>
<tr>
<td>(Each PCR reaction pellet™ contains dNTP Mixture, Taq DNA Polymerase Buffer, Taq DNA Polymerase and MgCl₂)</td>
<td>Room temp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4 Enzyme Grade UltraPure Water</td>
<td></td>
<td>P5 200 bp DNA ladder</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Wax Beads (for thermal cyclers without a heated lid)</td>
<td>Room temp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• 10x Gel Loading Solution</td>
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</tr>
</tbody>
</table>

**Reagents to Clean and Prepare DNA for Ligation**

| C1 Purification Buffer            | Room temp. |
| C2 Wash Buffer                    | Room temp. |
| C3 Restriction Enzyme Reaction Buffer | -20°C  |
| C4 Enzyme Grade UltraPure Water  | -20°C  |
| C5 Eco RI                         | -20°C  |
| C6 Hind III                       | -20°C  |
| C7 Restriction Enzyme Dilution Buffer | -20°C  |
| • Spin Columns and Reservoirs     | Room temp. |

**Reagents for Ligation**

| L1 pUC19 Plasmid Vector cut with Eco RI and Hind III | -20°C  |
| L2 DNA Ligase                                     | -20°C  |
| L3 Ligation buffer                               | -20°C  |

**Reagents for Transformation**

| T1 Ampicillin                                   | -20°C  |
| T2 IPTG                                         | -20°C  |
| T3 X-Gal in Solvent                             | -20°C  |
| T4 CaCl₂                                        | -20°C  |
| • Recovery Broth                                 | Room temp. |
| • BactoBeads™ or LyphoCells™                    | Refrigerator |
| • Ready Pour Agar                                | Room temp. |

**Other Components:**

- UltraSpec-Agarose™
- Electrophoresis Buffer (50x)
- InstaStain® Ethidium Bromide
- Microcentrifuge Tubes
- PCR tubes (0.2 ml - for thermal cyclers with 0.2 ml template)
- Petri plates
- Sterile loops
Cloning of a PCR Amplified Gene

Requirements

- Thermal cycler (EDVOTEK Cat. # 541 highly recommended) or three waterbaths*
- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Balance
- Microcentrifuge
- Water bath (37°C & 65°C)
- Incubation oven (37°C)
- UV Transilluminator or UV Photodocumentation system
- UV safety goggles
- Automatic micropipets (5-50 µl & 0.5-10 µl) with tips
- Microwave, hot plate or burner
- Pipet pump
- 250 ml flasks or beakers
- Hot gloves
- Disposable vinyl or latex laboratory gloves
- Ice buckets and ice
- Distilled or deionized water
- Isopropanol

*If you do not have a thermal cycler, PCR experiments can be conducted, with proper care, using three waterbaths. However, a thermal cycler assures a significantly higher rate of success.

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- Kit lot number on box or tube
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Polymerase Chain Reaction, Ligation and Cloning

The Polymerase Chain Reaction (PCR) has made an extraordinary impact on various aspects of biotechnology. The success of utilizing PCR is due to the specificity endowed in the hybridization of nucleic acid and simplicity of the reaction. PCR has also made amplification of genes an alternate approach to traditional cloning experiments. It is currently being used in genome projects, in DNA mapping and sequencing. PCR is also applied in DNA based forensics, paternity and determination of evolutionary relationships.

In a typical PCR reaction, the first step is the preparation of the DNA sample that is extracted from various biological sources. Segments of DNA or genes to be amplified are referred to as the targets and the two synthetic oligonucleotides required for the PCR reaction are referred to as primers. Because PCR is very sensitive, only a few copies of the gene is required. Freshly isolated DNA will give the best amplification results compared to DNA extracted from older specimens that may be degraded. The set of two primers, usually in the range between 15 and 30 nucleotides, are chemically synthesized to correspond to the two ends of the gene or DNA to be amplified. The primer concentrations are always in excess of the DNA target. The nucleotide primer sequences for the DNA amplification reaction are determined to yield the best hybridization.

ABOUT DNA POLYMERASES

DNA Polymerase I is the best studied DNA polymerase. With some exceptions, the mechanism of DNA polymerization is essentially identical for all polymerases. This DNA polymerase is a single polypeptide with a molecular weight of 109,000 (approximately 1000 amino acids). Like other DNA polymerases it requires a primed template, the four deoxynucleotide triphosphates and magnesium for DNA synthesis. Polymerization of the nucleotides occurs in the 5' to 3' direction by the addition of a 5' phosphorylated nucleotide to the free 3' hydroxyl group of the growing DNA chain with the concomitant production of pyrophosphate. The primer is antiparallel and is base paired to the template strand. In vivo, RNA fragments containing about 10 nucleotides serve to prime DNA synthesis. RNA primers are synthesized by the enzyme primase which is a specialized DNA dependent RNA polymerase.

DNA polymerase I also has a 3'-5' exonucleolytic activity that cleaves DNA starting with a free terminal 3' hydroxyl group on the growing chain to yields 5'-deoxynucleotide monophosphates. This activity serves as a proof-reading function during DNA synthesis. It recognizes distortions in the 3' ends of the growing DNA chain caused by mismatched bases between the template and the growing chain. The enzyme also possesses a 5' to 3' nuclease activity. This activity is part of a repair enzyme system that removes damaged DNA bases.
Cloning of a PCR Amplified Gene

Limited proteolysis of DNA polymerase I by subtilisin or trypsin produces two polypeptide fragments having molecular weights of 76,000 and 36,000. The larger polypeptide, known as the Klenow fragment contains the polymerization and 3’ to 5’ exonuclease activities while the smaller fragment contains the 5’ to 3’ nuclease activity. The Klenow fragment was used in the initial PCR experiments and was subsequently replaced by Taq DNA polymerase that is thermally stable. The source of Taq DNA polymerase is Thermus aquaticus. Several other thermo-stable polymerases have also been purified from other thermophilic bacteria. Because of its thermal stability, Taq DNA polymerase will not be denatured during the high temperature PCR steps described below. In the initial experiments, the Klenow fragment had to be replenished after each DNA denaturation step (94°C). The thermostability of Taq DNA polymerase eliminates replenishment, thereby simplifying PCR.

ABOUT THE POLYMERASE CHAIN REACTION

A typical PCR reaction mixture contains DNA, the four deoxynucleotide triphosphates, Mg+2 Taq DNA polymerase and the reaction buffer. The total incubation reaction is usually small (10 to 25 µl) in volume. If water baths or a thermal cycler without a heated lid is used, the incubation reaction mixture is layered with mineral oil to minimize evaporation. With thermal cyclers that have heated lids layering with mineral oil is not required.

The PCR reaction mixture is then exposed to sets of three step temperature cycles. The first temperature 94°C melts the hydrogen bonds between the two DNA strands. The temperature is then reduced between 45°C to 60°C to hybridize the two primers to each of target DNA strands. The temperature is then increased to 72°C, the optimum for Taq DNA polymerase. During this step DNA is synthesized. The three temperature steps of a cycle are usually repeated 20 to 30 times (Figure 1). This process is made efficient by placing the reaction tubes in thermal cyclers that are programmed to alternate and maintain temperatures. Amplified DNA products are detected by gel electrophoresis analysis.

It should be noted that PCR amplification can introduce a small number of mutations in the amplified DNA product. Mutations introduced can be controlled by the use of low nucleotide concentrations. Various undesired amplification products such as short DNA fragments are also synthesized. For best results in subsequent steps such as ligation in plasmids, the amplified DNA is purified from unused dNTPs, primers and Taq DNA polymerase prior to further use.

The correct PCR product will be in three forms. These products are due to an intrinsic property of the Taq DNA polymerase that appends an extra nucleotide usually a dA, at the 3’ ends of blunt double-stranded DNA. This will yield three forms where the first form will be double-stranded with no dA appended at either 3’ end. The second form will have a dA on one of the
**Polymerase Chain Reaction, Ligation and Cloning**

**Figure I: The Polymerase Chain Reaction**
two DNA strands and the third form will have a dA residues on both DNA strands. This single nucleotide addition is referred to as “template independent polymerization”. The presence of this nucleotide will have a negative impact on a subsequent cloning step. When overhanging end cloning (sticky ends) is desired, the best strategy would be to use primers that flank restriction enzyme sites present at the two ends of the target DNA that can be amplified. The amplified product is then digested by a restriction enzyme.

ABOUT PLASMIDS

The plasmid pUC19 used for this experiment is derived from the pUC series. It has a single recognition site for Eco RI and Hind III (restriction enzymes), that are located in a polylinker. The polylinker region known as MCR (multiple cloning region) contains several single restriction enzyme sites that facilitates the insertion of DNA. The pUC19 plasmid (Figure 2) is present in multiple copies in a host E. coli cell, and has been cleverly modified by genetic engineering. These modifications include the addition of the lac Z gene that codes for beta-galactosidase, an enzyme involved in lactose metabolism. DNA inserted into the MCR interrupts the lac Z' gene and prevents the formation of a functional beta-galactosidase protein. As a result clones of interest will appear as white colonies instead of blue on selection agar plates that contain ampicillin.
CONSTRUCTION OF A RECOMBINANT PLASMID

Ligation of the PCR amplified DNA to the linearized plasmid is catalyzed by T4 DNA ligase. The enzyme catalyzes the formation of phosphodiester bonds by the condensation of a 5’ phosphate and 3’ hydroxyl group of adjacent nucleotides. Each phosphodiester bond formation results in the hydrolysis of ATP to AMP and pyrophosphate. Ligation of DNA fragments having cohesive termini is usually achieved at temperatures between 4°C to 22°C. These temperatures will allow for annealing between complementary DNA ends that serves as a prerequisite for ligation.

When the plasmid and insert have the same cohesive termini, the orientation of the sub-cloned DNA (ligated in the plasmid) will vary due to the symmetrical nature of the termini. Statistically one would expect to find a 50:50 occurrence for the DNA orientation in bacterial colonies obtained from the same transformation reaction. Therefore the insert in the recombinant plasmid can be in either one of two directions relative to a fixed point in the vector (Figure 3A).

Figure 3A: Use of a single restriction enzyme (Eco RI) for the ligation of DNA in an Eco RI pre-digested plasmid. As noted by the two arrows the DNA insert is ligated bi-directionally.
Background Information

In the simplest reaction, ligation of a plasmid and PCR amplified DNA (cleaved by the same restriction enzyme) will form a circular recombinant plasmid (Figure 3A). The stoichiometry of this reaction is complex and is based on the length and relative concentrations of the two DNAs, the amount enzyme and the ionic strength of the reaction. In this reaction the plasmid (without the amplified DNA) will also circularize (Figure 3A).

To obtain a recombinant with a DNA insert in the desired direction the plasmid is co-digested within the multiple cloning region by two restriction enzymes that will produce cohesive ends that do not match. A similarly co-digested DNA fragment will be ligated in the desired orientation (Figure 3B). In this reaction the plasmid will not circularize because the ends are derived from the co-digestion by two different restriction enzymes. White colonies that may be present would be due to undigested plasmid.

ABOUT TRANSFORMATION:

For this experiment competent cells are prepared from cultures of E. coli, strain JM109. This strain does not have any natural antibiotic resistance or plasmids and lacks restriction enzymes. Transformation with the recombinant DNA allows for its expression, propagation, and purification. Linear plasmids and large concatamers do not transform competent cells, while supercoiled DNA has the highest transformation efficiencies. Only small amounts of DNA, typically less than 10 nanograms, are required for transformation. In fact, transformation is inhibited by DNA exceeding 100 nanograms. Even with this amount of DNA, only 1 in 10,000 cells successfully incorporate the recombinant DNA.

Transformation efficiency is based on the number of transformants obtained per microgram of DNA. As an example to determine transformation efficiency, 10 nanograms of DNA were used for a reaction and cells were allowed to recover in a final volume of 1 ml but only one tenth of this volume
Cloning of a PCR Amplified Gene

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was plated and produced 100 colonies on a selective agar medium. Therefore, 1000 transformants are present per ml. Keeping in mind that each colony grew from one transformed cell, the efficiency would be 1000/0.01ug = 1 x 10^5. Transformation efficiencies of 10^5 to 10^6 are sufficient for most classroom cloning experiments. When cloning of single copy genes from genomic DNA is done, required efficiencies are 10^7 to 10^8.

This experiment has four modules with the following objectives:

1. To amplify a DNA fragment using the PCR reaction.
2. To prepare PCR amplified DNA for ligation.
3. To ligate the PCR amplified DNA in pUC19
4. To transform *E. coli* host cells and determine the number of white colonies (recombinant plasmid).
Experiment Overview and General Instructions

BEFORE YOU START THE EXPERIMENT

1. Read all instructions before starting the experiment.

2. If you will be conducting PCR using a thermal cycler without a heated lid, also read the Appendix entitled "Preparation and Handling PCR Samples with Wax ".

3. If you will be using three waterbaths to conduct PCR, read the two appendices entitled "Polymerase Chain Reaction Using Three Waterbaths" and "Handling samples with wax overlays".

4. Write a hypothesis that reflects the experiment and predict experimental outcomes.

EXPERIMENT OBJECTIVE:

The objective of this experiment is to amplify a DNA fragment by Polymerase Chain Reaction (PCR) and to clone the amplified DNA by using the blue/white cloning system.

BRIEF DESCRIPTION OF EXPERIMENT:

This experiment has four modules with the following objectives:

1. To amplify a DNA fragment using the PCR reaction.
2. To prepare PCR amplified DNA for ligation.
3. To ligate the PCR amplified DNA in pUC19.
4. To transform E. coli host cells and to quantitate the number of white colonies.

GEL SPECIFICATIONS

This experiment requires a gel with the following specifications:

- Recommended gel size: 7 x 7 cm or 7 x 14 cm
- Number of sample wells required: 6
- Placement of well-former template: first set of notches
- Agarose gel concentration: 1.0%
Laboratory Safety

1. Gloves and goggles should be worn routinely as good laboratory practice.

2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.

3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.

4. Exercise caution when using any electrical equipment in the laboratory.
   - Although electrical current from the power source is automatically disrupted when the cover is removed from the apparatus, first turn off the power, then unplug the power source before disconnecting the leads and removing the cover.
   - Turn off power and unplug the equipment when not in use.

5. EDVOTEK injection-molded electrophoresis units do not have glued junctions that can develop potential leaks. However, in the unlikely event that a leak develops in any electrophoresis apparatus you are using, IMMEDIATELY SHUT OFF POWER. Do not use the apparatus.

6. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.
Module I: Amplification by PCR and Separation by Electrophoresis

AMPLIFICATION OF A 270 BP DNA FRAGMENT BY PCR

1. Program the thermal cycler with the following schedule:

<table>
<thead>
<tr>
<th>Initial Denaturation</th>
<th>30 cycles @</th>
<th>Final Extension</th>
</tr>
</thead>
</table>
   | 94°C for 5 min.      | 94°C for 30 sec. | 72°C for 5 min.
   |                      | 50°C for 30 sec. |                |
   |                      | 72°C for 30 sec. |                |

2. Obtain one of the tubes (P3) containing the PCR Reaction pellet™. Transfer the pellet to the appropriate sized tube (e.g. 0.5 ml or 0.2 ml) for your thermal cycler.

3. Label the side of the tube and top of the cap with your lab group number or initials.

4. Add the following to tube P3:

   | 5 µl | DNA Template for Amplification (P1) |
   | 10 µl | Primer Set (P2) |
   | 15 µl | Enzyme Grade Ultrapure Water (P4) |

5. Gently mix the reaction tube and pulse spin it in a microcentrifuge to collect the entire sample at the bottom of the tube.

6. Check to see if your thermal cycler is equipped with a heated lid. If it is, proceed directly to placing the tube in the thermal cycler.

   If your thermal cycler does not have a heated lid, add one wax bead to the tube before placing the tube in the thermal cycler.

7. After the tubes are placed in the thermal cycler, process the samples for 30 cycles according to the schedule outlined in step 1.

8. To a clean tube add:

   | 3 µl | 10x Gel Loading Solution |
   | 12 µl | Distilled water |

9. After the final PCR cycle, remove 5 µl of the amplified DNA and transfer it to the tube containing the 10x gel loading solution and water (from step 8). This sample can be analyzed on a 1.0% agarose gel. The remaining amplified DNA (insert) is ready to be cleaned and purified for ligation in Module II.

OPTIONAL STOPPING POINT

Freeze at -20°C the amplified DNA and sample to be analyzed on a 1% gel, or continue with electrophoresis and/or Preparation of Insert for Ligation (Module II).
Module I: Amplification by PCR and Separation by Electrophoresis

AGAROSE GEL REQUIREMENTS
- Recommended gel size: 7 x 7 cm or 7 x 14 cm
- Placement of well-former template: first set of notches
- Agarose gel concentration: 1.0%

PREPARING THE AGAROSE GEL
1. Close off the open ends of a clean and dry gel bed (casting tray) by using rubber dams or tape.
2. Place a well-former template (comb) in the first set of notches at the end of the bed. Make sure the comb sits firmly and evenly across the bed.
3. To a 250 ml flask or beaker, add agarose powder and buffer as indicated in the Reference Tables (Appendix A) provided by your instructor. Swirl the mixture to disperse clumps of agarose powder.
4. With a marking pen, indicate the level of the solution volume on the outside of the flask.
5. Heat the mixture using a microwave oven or burner to dissolve the agarose powder.
6. Cool the agarose solution to 60°C with careful swirling to promote even dissipation of heat. If detectable evaporation has occurred, add distilled water to bring the solution up to the original volume marked in step 4.

After the gel is cooled to 60°C:
7. Place the bed on a level surface and pour the cooled agarose solution into the bed.
8. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes.
9. After the gel is solidified, be careful not to damage or tear the wells while removing the rubber dams or tape and comb(s) from the gel bed.
10. Place the gel (on its bed) into the electrophoresis chamber, properly oriented, centered and level on the platform.
11. Fill the electrophoresis apparatus chamber with the appropriate amount of diluted (1x) electrophoresis buffer (refer to Table B on the instruction Appendix provided by your instructor).
Module I: Amplification by PCR and Separation by Electrophoresis

BEFORE LOADING THE SAMPLES

This experiment requires a 1.0% agarose gel and is designed for staining with InstaStain® Ethidium Bromide.

LOADING DNA SAMPLES

1. Make sure the gel is completely submerged under buffer before loading the samples. Load 20 µl of the PCR sample.

   Lane
   1  200 bp DNA Ladder (P5)
   2  Reaction sample after 30 cycles

2. Record the position of your sample in the gel for easy identification after staining.

RUNNING THE GEL

3. After the DNA samples are loaded, properly orient the cover and carefully snap it onto the electrode terminals.

4. Insert the plugs of the black and red leads into the corresponding inputs of the power source.

5. Set the power source at the required voltage and conduct electrophoresis for the length of time determined by your instructor.

6. Check to see that current is flowing properly - you should see bubbles forming on the two platinum electrodes.

7. After the electrophoresis is completed, disconnect the power and remove the gel from the bed for staining.

STAINING AND VISUALIZATION OF DNA

After electrophoresis, agarose gels require staining to visualize the separated DNA samples. Your instructor will provide instructions for DNA staining with InstaStain® Ethidium Bromide.
Module II: Preparation of Insert for Ligation

The amplified DNA (insert) must be cleaned (the Taq DNA polymerase must be removed), then cut with Eco RI and Hind III before proceeding to the ligation step.

1. Transfer the amplified DNA (~25 µl) to a clean 1.5 ml microcentrifuge tube and label it with your group number or initials.

2. Add 300 µl of the Purification Buffer (C1) to the tube containing the amplified DNA insert. Mix by inversion several times.

3. Place a spin column into a reservoir tube and transfer all of the mixture from step 2 to the column. Close the cap on the column.

4. Balance the tubes in a microcentrifuge and spin at maximum speed for 1 minute. At this point, the DNA product is bound to the membrane in the column.

5. Empty the reservoir tube and replace the spin column to the empty reservoir tube.

6. Wash the column:
   - Add 750 µl wash buffer to the spin column.
   - Close the cap on the column.
   - Spin balanced tubes in a microcentrifuge at maximum speed for 1 minute.

7. Empty the contents of the reservoir tube and replace the spin column to the empty reservoir tube. Close the cap on the column and spin balanced tubes in a microcentrifuge at maximum speed for 1 minute.

8. Recover the DNA insert from the column:
   - Place the column into a clean 1.5 ml snap-top tube.
   - Add 12 µl of Enzyme Grade Ultrapure Water to the center of the column (directly onto the white membrane).
   - Close the cap on the column and let the tube stand for 1 minute.

9. Balance and carefully position the tubes in the microcentrifuge so that the open caps of the snap-top tubes do not interfere with operation of the centrifuge.

10. Spin the tubes for 1 minute at maximum speed. The DNA insert will be recovered in a volume slightly less than 12 µl.

11. Label the tube “PCR insert” and discard the used spin column.

**IMPORTANT!**

Make sure that the Enzyme Grade Ultrapure Water (C4) is added directly onto the white membrane of the column (avoid touching the membrane with the pipet tip) for complete elution of the DNA.

**OPTIONAL STOPPING POINT**

Freeze the samples at -20°C or continue with restriction enzyme digestion.
Module II: Preparation of Insert for Ligation

**RESTRICTION ENZYME DIGESTION**

1. To the tube labeled “PCR insert” containing 10-12 µl the purified PCR mixture, add the following:
   - 5 µl Restriction Enzyme Reaction Buffer
   - 10 µl Enzyme Grade Ultrapure Water
   - 10 µl EcoRI enzyme
   - 10 µl HindIII enzyme

2. Mix the solution by gently pipeting up and down several times.

3. Incubate at 37°C for 60 minutes. Near the end of the incubation, prepare a separate 65°C waterbath.

After the 60 minute restriction enzyme digestion, the DNA digestion requires an incubation at 65°C to heat-inactivate the enzymes prior to being ligated into the vector.

4. Heat-inactivate the restriction enzymes by incubating the completely digested DNA at 65°C for 20 minutes.

5. Label the tube “Cut PCR Insert”. After this step, the DNA insert is now ready to be ligated into the vector.

**OPTIONAL STOPPING POINT**

Freeze the samples at -20°C or continue with the ligation.
Module III: Ligation of the PCR Amplified 270 bp DNA into pUC19

1. Equilibrate an ice water bath at 16°C for Step 4.

2. In a clean microcentrifuge tube, carefully mix together:
   - 21 µl “Cut PCR Insert” DNA
   - 3 µl pUC19 Plasmid Vector cut with Eco RI and Hind III
   - 3 µl Ligation buffer
   - 3 µl DNA Ligase

   30 µl Total

   Label this tube “PCR Insert+Vector/Lig”.

3. In another clean microcentrifuge tube, carefully mix together:
   - 3 µl pUC19 Plasmid Vector cut with Eco RI and Hind III
   - 3 µl Ligation buffer
   - 21 µl Enzyme Grade Ultrapure Water
   - 3 µl DNA Ligase

   30 µl Total

   Label this tube “Vector Control/Lig”.

4. Incubate the reaction tubes in a 16°C ice-water bath for 30 minutes.
   Alternatively, the reaction tubes can be incubated for 2 hours at room temperature.

OPTIONAL STOPPING POINT

Freeze the samples at -20°C or continue with the transformation.
Module IV: Transformation

1. Label one microcentrifuge tube "PCR+Vector/Transf". (This will be the transformation tube with the PCR amplified Gene.)

2. Label a second microcentrifuge tube "Vector Control/Transf". (This will be the transformation tube with the vector control.)

3. Using a sterile pipet, add 500 µl (0.5 ml) of ice cold CaCl₂ solution to one of the tubes from step 1 or 2.

4. Pick colonies from the source plate of E. coli cells.
   - Use a sterile toothpick to transfer 5 colonies (2-4 mm) from the source plate to the tube containing the CaCl₂.
   - Between your fingers, twist the toothpick vigorously in the CaCl₂ solution to dislodge the cells.

5. Suspend the cells in the tube by tapping or vortexing (preferred).
   - At this point, the CaCl₂ cell suspension should look cloudy and slightly turbid. If it does not, add a few additional colonies and/or make sure the cells are completely suspended by mixing and vortexing.

6. After the cells are completely resuspended, transfer half (0.25 ml) of the cell suspension to the other labeled tube.

7. Add 10 µl "PCR+Vector/Lig" DNA to the tube labeled "PCR+Vector/Transf" and vortex.

8. Add 10 µl "Vector Control/Lig" DNA to the tube labeled "Vector Control/Transf".

9. Incubate the two tubes on ice for 20 minutes.

10. Briefly vortex the tubes, then float both tubes at 42°C for 90 seconds for the heat shock step. This facilitates the entry of DNA in bacterial cells.

11. Return both tubes immediately to ice and incubate for 2 minutes.

12. With a sterile pipet, add 250 µl of Recovery Broth to each tube & vortex.

13. Incubate cells for 30 minutes in a 37°C waterbath for a recovery period.

14. While the tubes are incubating, label 2 agar plates as follows:
   - "PCR insert + Vector"
   - "Vector Control"
   - Put your initials or group number on both plates.

15. After the recovery period, remove the tubes from the waterbath and place them on the lab bench.

Note:
Sufficient colonies that have been completely suspended in CaCl₂ are critical to the success of the transformation portion of the experiment.
Module IV: Transformation

PLATING CELLS

16. Balance and centrifuge both tubes at maximum speed for 5 minutes to pellet the cells.

17. After the centrifuge has stopped, use a different pipet to carefully remove 0.4 ml of supernate from each tube. Vortex the tubes to completely resuspend the cells in the remaining liquid (0.1 ml).

18. Use a sterile pipet to transfer all of the suspended cells from the tube labeled “PCR Insert + Vector/Transf” to the middle of the plate “PCR Insert + Vector”.

19. Use a sterile pipet to transfer the suspended cells from the tube labeled “Vector Control/Transf” to the middle of the plate “Vector Control”.

20. Spread the cells with a sterile inoculating loop.

21. Cover both plates and allow the liquid to be absorbed.

PREPARING PLATES FOR INCUBATION

22. Stack your group's set of plates on top of one another and tape them together. Put your initials or group number on the taped set of plates.

23. Leave the plates in an upright position to allow the cell suspension to be absorbed by the agar. Place the set of plates in a safe place designated by your instructor.

24. After the cell suspension is absorbed by the agar for approximately 15-30 minutes, you or your instructor will place the plates in the inverted position (agar side on top) into a 37°C bacterial incubation oven for overnight incubation (15-20 hours).

   Note: The plates are inverted to prevent condensation on the lid, which could drip onto the culture and interfere with experimental results.

AFTER OVERNIGHT INCUBATION

25. Observe the plates and estimate the number of transformants (both white and blue colonies) on each plate. Keep track of the counted colonies by putting a dot over them on the outside of the plate with a lab marker.
Module IV: Transformation

26. Calculate the transformation efficiencies for total transformants and for colonies that contain vectors with inserts (white colonies). The final volume of cells was 50 µl and the volume plated was 50 µl.

\[
\text{Number of transformants} \times \frac{\text{Final volume of cells (ml)}}{\mu g \text{ of DNA}} = \frac{\text{Number of transformsants}}{\mu g \text{ volume plated (ml)}}
\]

The quantity of DNA plasmid used for the vector control was approximately 0.012 µg. Estimate the PCR+Vector DNA to be approximately 0.05 µg.

Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. Why does this cloning experiment yield both blue and white colonies?
2. Do all the white and blue colonies contain a plasmid?
3. Why is there a purification step prior to the restriction enzyme digestion step?
4. Why are there two different sticky ends on the PCR amplified DNA?
5. What is a diagnostic step to analyze the success of the various steps in this experiment?
Instructor’s Guide

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students. These guidelines can be adapted to fit your specific set of circumstances. If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK web site. In addition, Technical Service is available from 9:00 am to 6:00 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).

NATIONAL CONTENT AND SKILL STANDARDS

By performing this experiment, students will learn to load samples and run agarose gel electrophoresis. Analysis of the experiments will provide students the means to transform an abstract concept into a concrete explanation. Please visit our website for specific content and skill standards for various experiments.

EDUCATIONAL RESOURCES

Electrophoresis Hints, Help and Frequently Asked Questions

EDVOTEK experiments are easy to perform and designed for maximum success in the classroom setting. However, even the most experienced students and teachers occasionally encounter experimental problems or difficulties. The EDVOTEK web site provides several suggestions and reminders for conducting electrophoresis, as well as answers to frequently asked electrophoresis questions.

Visit our web site for information about EDVOTEK’s complete line of “hands-on” experiments for biotechnology and biology education.
Notes to the Instructor:

PCR EXPERIMENTAL SUCCESS GUIDELINES

Please refer to the Appendices section for a summary of important hints and reminders which will help maximize successful implementation of this experiment. This experiment has four modules:

I. Amplification of a 270 bp DNA Fragment (from pBR322 plasmid) by PCR and Separation by Electrophoresis
II. Preparation of PCR Amplified DNA for Ligation
III. Ligation of the PCR Amplified 270 bp DNA into pUC19
IV. Transformation

MICROPIPETTING BASICS AND PRACTICE GEL LOADING

Accurate pipeting is critical for maximizing successful experiment results. EDVOTEK Series 300 experiments are designed for students who have had previous experience with agarose gel electrophoresis and micropipeting techniques. If your students are unfamiliar with using micropipets, EDVOTEK highly recommends that students perform Experiment # S-44, Micropipetting Basics, or other Series 100 or 200 electrophoresis experiment prior to conducting this advanced level experiment.

APPROXIMATE TIME REQUIREMENTS

1. The PCR step (30 cycles) will take about 2 hours or can be processed overnight and held at 4°C.

2. The experiment can be temporarily stopped after the completion of Module I and/or Module II and later resumed. Experimental results will not be compromised if instructions are followed as noted under the heading “Optional Stopping Point” at the end of each module.

3. After setting up the ligation, Module III requires a 30 minute incubation.

4. JM109 colonies from the LyphoCells™ require an overnight incubation the night before the lab.

5. Module IV includes a 30 minute incubation in a 37°C waterbath. There is also an overnight incubation of plates in an incubation oven at 37°C before students can obtain the final results.

6. Whether you choose to prepare the gel(s) in advance or have the students prepare their own, allow approximately 30-40 minutes for this procedure. Generally, 20 minutes of this time is required for gel solidification. See section “Options for Preparing Agarose Gels” on the next page.
Notes to the Instructor:

7. The approximate time for electrophoresis will vary from 1 - 5 hours. Generally, the higher the voltage applied, the faster the samples migrate. However, depending upon the apparatus configuration and the distance between the two electrodes, individual electrophoresis units will separate DNA at different rates. Follow manufacturer’s recommendations. Time and Voltage recommendations for EDVOTEK equipment are outlined in Table C.

Table C  Time and Voltage
(1.0% - 7 x 14 cm gel)

<table>
<thead>
<tr>
<th>Volts</th>
<th>Recommended Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum</td>
</tr>
<tr>
<td>125</td>
<td>55 min</td>
</tr>
<tr>
<td>70</td>
<td>2 hrs 15 min</td>
</tr>
<tr>
<td>50</td>
<td>3 hrs 25 min</td>
</tr>
</tbody>
</table>

OPTIONS FOR PREPARING AGAROSE GELS

This experiment is designed for DNA staining after electrophoresis with InstaStain® Ethidium Bromide. There are several options for preparing agarose gels for the experiment.

1. Individual Gel Casting:
   Each student lab group can be responsible for casting their own individual gel prior to conducting the experiment.

2. Preparing Gels in Advance:
   Gels may be prepared ahead and stored for later use. Solidified gels should be stored under buffer in the refrigerator for up to 2 weeks.
   Do not store gels at -20°C. Freezing will destroy the gels.

   Gels that have been removed from their trays for storage, should be “anchored” back to the tray with a few drops of hot, molten agarose before placing the gels into the apparatus for electrophoresis. This will prevent the gels from sliding around in the trays and the chambers.

3. Batch Gel Preparation:
   A batch of agarose gel can be prepared for sharing by the class. To save time, a larger quantity of UltraSpec-Agarose can be prepared for sharing by the class. See instructions for "Batch Gel Preparation".

GEL CONCENTRATION AND VOLUME

The gel concentration required for this experiment is 1.0%. Prepare gels according to Table A.1 or A.2 in Appendix D.
Notes to the Instructor:

GEL STAINING AND DESTAINING AFTER ELECTROPHORESIS

After electrophoresis, the agarose gels require staining in order to visualize the separated DNA samples. This experiment features a proprietary stain called InstaStain®.

InstaStain® EtBr (Appendix F)

Optimal visualization of PCR products on gels of 1.0% or higher concentration is obtained by staining with InstaStain® Ethidium Bromide (InstaStain® EtBr) cards. Exercise caution when using Ethidium Bromide, which is a listed mutagen. Disposal of the InstaStain® EtBr cards, which contain only a few micrograms of ethidium bromide, is minimal compared to the large volume of liquid waste generated by traditional ethidium bromide staining procedures. Disposal of InstaStain® cards and gels should follow institutional guidelines for chemical waste.

PHOTODOCUMENTATION OF DNA (OPTIONAL)

There are many different photodocumentation systems available, including digital systems that are interfaced directly with computers. Specific instructions will vary depending upon the type of photodocumentation system you are using.
Pre-Lab Preparations

MODULE I: AMPLIFICATION OF A 270 BP FRAGMENT BY PCR

1. Program the thermal cycler with the following schedule:

<table>
<thead>
<tr>
<th>Initial Denaturation</th>
<th>30 cycles @</th>
<th>Final Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C for 5 min.</td>
<td>94°C for 30 sec.</td>
<td>72°C for 5 min.</td>
</tr>
<tr>
<td>50°C for 30 sec.</td>
<td>72°C for 30 sec.</td>
<td></td>
</tr>
</tbody>
</table>

2. Aliquot and assemble the following reagents and materials for each of the student groups.

   - P1 DNA Template for Amplification 6 µl
   - P2 Primer Set 12 µl
   - P3 PCR Reaction Pellet™ in tube that contains: 1 each
     - four dNTPs
     - MgCl2
     - Taq DNA Polymerase
     - Taq DNA Polymerase Buffer
   - P4 Enzyme Grade UltraPure Water 20 µl
   - P5 200 bp DNA Ladder 25 µl
   - 10x Gel Loading Solution 10 µl
   - Microcentrifuge tube (sterile)
   - Micropipet and sterile tips
   - Wax Bead (for thermal cyclers without a heated lid)

Notes and Reminders:

Accurate temperatures and cycle times are critical. A pre-run for one cycle (which will take approximately 3 to 5 min) is recommended to check that the thermal cycler is properly programmed.

For thermal cyclers which do not have a top heating plate, it is necessary to place a layer of wax above the PCR reactions in the microcentrifuge tubes to prevent evaporation.

MODULE I: SEPARATION BY ELECTROPHORESIS

1. Students will share gels in this experiment. Each 1.0% gel should be loaded with the 200 bp DNA Ladder and samples from 4 or 5 students.

2. Aliquot 25 µl of the 200 bp Ladder (P5) into microcentrifuge tubes. Distribute one tube per gel.
**Pre-Lab Preparations**

**MODULE II: PREPARATION OF PCR AMPLIFIED DNA FOR LIGATION**

1. Thaw the Enzyme Grade UltraPure Water (C4).

2. Aliquot and assemble the following materials for each of the student groups for cleaning the amplified DNA:
   - C1 Purification Buffer 320 µl
   - C2 Wash Buffer 800 µl
   - C4 Enzyme Grade UltraPure Water 200 µl
   - Spin Columns and Reservoirs 1 each
   - Microcentrifuge tubes 3
   - Micropipet and sterile tips

3. Prepare the reagents for Restriction Enzyme Digestion of the amplified DNA 30 minutes before the procedures.
   - Thaw the Restriction Enzyme Reaction Buffer (C3) and the Restriction Enzyme Dilution Buffer (C7) and place on ice.
   - Dilute the *Eco* RI enzyme (C5) by adding 65 µl Restriction Enzyme Dilution Buffer (C7) to tube C5, *Eco* RI enzyme and place on ice.
   - Dilute the *Hind*III enzyme (C6) by adding 65 µl Restriction Enzyme Dilution Buffer (C7) to tube C6, *Hind* III enzyme and place on ice.

4. Aliquot the following for each of the student groups for restriction enzyme digestion of the amplified DNA and place on ice:
   - C3 Restriction Enzyme Reaction Buffer 10 µl
   - C4 Enzyme Grade UltraPure Water 20 µl
   - C5 Diluted *Eco* RI 12 µl
   - C6 Diluted *Hind* III 12 µl

Do not dilute the restriction enzymes more than 30 minutes before they are needed in Module II.
Pre-Lab Preparations

MODULE III: LIGATION OF PCR AMPLIFIED DNA INTO PUC19

1. Thaw the pUC19 Plasmid Vector cut with Eco RI and Hind III (L1), and place on ice.

2. Aliquot and assemble the following materials for each of the student groups for ligation:

   - L1 pUC19 Plasmid Vector cut with Eco RI and Hind III 7 µl
   - L2 DNA Ligase 7 µl
   - L3 Ligation Buffer 7 µl
   - C4 Enzyme Grade UltraPure Water 100 µl
   - Microcentrifuge tubes (sterile) 2
   - Micropipet and sterile tips

3. Set up a 16°C ice/waterbath by combining ice and water. Adjust temperature by adding more or less of one or the other. Alternatively, you may program your thermal cycler for 30 minutes at 16°C.

MODULE IV: TRANSFORMATION

Preparation of Antibiotic Agar Plates

Sterile agar plates can be prepared 1 week before the laboratory experiment. Store plates inverted for two days at room temperature. If poured more than one day before, store inverted in the refrigerator.

1. Thaw the X-Gal (T3).

2. Equilibrate a water bath at 60-65°C for later steps.

3. Loosen, but do not remove, the cap on the ReadyPour media bottle to allow for the venting of steam during heating.

4. Heat the ReadyPour™ bottle by microwave, hot plate or burner method outlined below. The amber-colored solution should appear free of small particles.

   A. Microwave method:
      • Heat the bottle on High for two 30 second intervals.
      • Swirl and heat on High for an additional 25 seconds, or until all the ReadyPour media is dissolved.

Wear Hot Gloves and Goggles during all steps involving heating.

Caution:
Failure to loosen the cap prior to heating or microwaving will cause the ReadyPour media bottle to break or explode.
Pre-Lab Preparations

When the ReadyPour™ media reaches approximately 60-65°C, the bottle will be warm to the touch but not burning hot.

Add ampicillin, IPTG, and X-Gal to media which has been cooled. Hot media will cause rapid decomposition of ampicillin.

B. Hot plate or burner method:
- Place the bottle in a beaker partially filled with water.
- Heat the beaker to boiling over a hot plate or burner.
- Using a hot glove, occasionally swirl to expedite melting.

5. Cool the melted ReadyPour media to approximately 60-65°C

6. Place the bottle in a 60-65°C water bath to prevent the agar from prematurely solidifying.

7. While the ReadyPour media is cooling, label petri plates on their bottom halves:

<table>
<thead>
<tr>
<th>Plates</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Amp, IPTG, X-Gal</td>
</tr>
<tr>
<td>14</td>
<td>Amp, IPTG, X-Gal</td>
</tr>
</tbody>
</table>

8. Pour the 6 source plates, 8 ml each.

Quick Reference: Pouring Agar Plates

1. Use a sterile 10 ml pipet and pump to transfer 8 ml of media to each petri plate. Pipet carefully to avoid forming bubbles.

2. Rock the petri plate back and forth to obtain full coverage.

3. If the molten media contains bubbles, they can be removed by passing a flame across the surface of the media.

4. Cover the petri plate and allow the media to solidify.

After the ReadyPour media has cooled:

9. Add the ampicillin (T1), IPTG (T2), and all of the X-Gal (T3) to the molten media. Swirl the media to mix.

10. Pour the 14 AMP, IPTG, XGal-labeled plates, 8 ml each. (See Quick Reference: Pouring Agar Plates.)

11. Let the agar cool and resolidify. If plates will be used within two days, store at room temperature, inverted without taping. If plates are prepared more than two days before use, rewrap in plastic sleeve and store inverted in refrigerator. Take out of refrigerator and leave inverted at 37°C for several hours prior to use.
PreLab Preparations

Preparation of *E. coli* Source Plates

For best results, the *E. coli* source plates should be streaked 16-20 hours before the experiment is performed. Preparing the source plates more than 24 hours before the laboratory may compromise the success of the transformation experiment. If you do not have an incubator, colonies will form at room temperature in approximately 24 - 48 hours.

1. **REMOVE** a single BactoBead™ from the vial using a sterile inoculating loop. Using aseptic technique, **TRANSFER** the bead to the edge of a large petri plate (LB source plate) and replace lid. **CAP** the vial immediately after using to limit exposure to moisture in the air.

2. Instantly **DISSOLVE** the bead by adding 10 µl of sterile liquid broth or sterile water.

3. **STREAK** the loop back and forth through the dissolved BactoBead™ to make a primary streak at the top of the plate. Try not to gouge the loop into the medium.

4. **STREAK** the loop through primary streak to a clean part of the agar several times to create a secondary streak.

5. **ROTATE** the plate. **STREAK** the loop through the secondary streak to a clean part of the agar several times.

6. **ROTATE** the plate once more. **STREAK** the loop through the third streak to a clean part of the agar. This should produce isolated colonies.

7. **COVER** the plate and **INCUBATE INVERTED** at 37°C for 16 to 20 hours. If you do not have an incubator, colonies will form at room temperature in approximately 24 - 48 hours.

8. **REPEAT** the above steps for each of the LB source plates.

**NOTE:** If growth on plates is heavy (i.e. lawn of colonies), instruct students to transfer a loopful of cells into the CaCl₂ solution.
Experiment Results and Analysis

MODULE I: AMPLIFICATION BY PCR AND SEPARATION BY ELECTROPHORESIS

Idealized Schematic

The idealized schematic at right shows the approximate relative position of the PCR amplified band. Actual results will yield broader bands of varying intensities. Smaller fragments will stain less efficiently and will appear as fainter bands. The idealized schematic shows the relative positions of the bands, but are not depicted to scale.

Lane 1  200 base pair ladder
Lane 2  Reaction sample after 30 cycles

MODULE IV: TRANSFORMATION

Blue and white colonies from the PCR + Vector Ligation Reaction

Blue colonies from the Vector Ligation Reaction (a few white colonies may be visible)

Blue colonies are from uncut plasmid.
White colonies are from recombinant plasmid.
Please refer to the kit insert for the Answers to Study Questions.
Appendices

A  PCR Experimental Success Guidelines
B  Polymerase Chain Reaction Using Three Waterbaths
C  Preparation and Handling of PCR Samples With Wax
D  1.0% Agarose Gel Preparation
E  1.0% Agarose Gels - Quantity Preparations
F  Staining and Visualization of DNA with InstaStain® Ethidium Bromide Cards

Material Safety Data Sheets
PCR Experimental Success Guidelines

EDVOTEK experiments which involve the amplification of DNA are extremely relevant, exciting and stimulating classroom laboratory activities. These experiments have been performed successfully in many classrooms across the country, but do require careful execution because of the small volumes used. The following guidelines offer some important suggestions, reminders and hints for maximizing success.

THE PCR REACTION

1. **Add Primers and DNA to the PCR Reaction Bead:** Add the primer mixture (forward and reverse primers) and the cell DNA (supernatant) as specified in the experimental procedures to the microcentrifuge tube containing the PCR reaction bead. Make sure that the bead (which contains the Taq DNA polymerase, the 4XdTPs, Mg and the PCR reaction buffer) is completely dissolved. Do a quick spin in a microcentrifuge to bring the entire sample to the bottom of the tube.

2. **The Thermal cycler:** It is critical that the thermal cycler be accurately programmed for the correct cycle sequence, temperatures and the time for each of the cycles.

3. **Oil or Wax:** For thermal cyclers that do not have a top heating plate, the reaction in the tubes must be overlaid with wax to prevent evaporation.

4. **Manual Water Bath PCR:** Three water baths can be used as an alternative to a thermal cycler for PCR, but results are more variable. Samples require wax layers. This method requires extra care and patience.

GEL PREPARATION AND STAINING

5. **Concentrated agarose:** Gels of higher concentration (> 0.8%) require special attention when dissolving or re-melting. Make sure that the solution is completely clear of “clumps” or glassy granules. Distorted electrophoresis DNA band patterns will result if the gel is not properly prepared.

6. **Electrophoretic separation:** The tracking dye should travel at least 6 cm from the wells for adequate separation before staining.

7. **Staining:** Staining of higher concentration gels (> 0.8%) require additional care to obtain clear, visible results.
   - After staining (15 to 30 min.) with InstaStain® Ethidium Bromide or liquid ethidium bromide, examine the results using a UV (300 nm) transilluminator. Repeat the staining as required.

8. **DNA 200 bp ladder:** After staining the agarose gel, the DNA 200 bp ladder (markers) should be visible. If bands are visible in the markers and control lanes, but bands in the sample lanes are faint or absent, it is possible that DNA was not successfully extracted from the cells. If the ladder and DNA bands are all faint or absent, potential problems could include improper gel preparation, absence of buffer in the gel, improper gel staining or a dysfunctional electrophoresis unit or power source.
**Preparation of the PCR Reaction:**

1. The PCR reaction sample should be prepared as specified in the experiment instructions. Each PCR reaction sample contains three critical components:
   - PCR Reaction pellet™
   - Primer mix
   - DNA for amplification

2. After adding the components of the PCR reaction sample, use clean forceps to transfer one wax bead to the PCR tube. At the start of the PCR reaction, the wax will melt and overlay the samples to prevent evaporation during heating.

**Polymerase Chain Reaction Cycling**

3. In the three-waterbath PCR method, the PCR reaction sample is sequentially cycled between three separate waterbaths, each set at different temperatures, for a specified period of time. The sequential placement of the reaction sample in the waterbaths maintained at three different temperatures constitutes one PCR cycle. One example of a PCR cycle might be as follows:

   - 94°C for 1 minute
   - 50°C for 1 minute
   - 72°C for 1 minute

   See experiment instructions for specific program requirements.

4. The PCR tube must be handled carefully when sequentially cycled between the three waterbaths. For each cycle:
   - Carefully place the PCR tube in a waterbath float. Make sure that the sample volume is at the bottom of the tube and remains undisturbed. If necessary, pulse spin the tube in a balanced microcentrifuge, or shake the tube to get all of the sample to the bottom of the tube.
   - Use forceps to carefully lower the waterbath float (with tubes) sequentially into the waterbaths.

5. Process the PCR reaction sample for the total number of cycles specified in the experiment instructions. On the final cycle the 72°C incubation can be extended to 5 minutes.

6. After all the cycles are completed, the PCR sample is prepared for electrophoresis.

**Appendix B**

Polymerase Chain Reaction Using Three Waterbaths

Superior PCR results are obtained using an automated thermal cycler. However, if you do not have a thermal cycler, this experiment can be adapted to use three waterbaths (Cat. # 544). Much more care needs to be taken when using the three-waterbath PCR method. The PCR incubation sample is small and can easily be evaporated. Results using three waterbaths are often variable. Please refer to the Appendix entitled "PCR Samples with Wax Overlays" for sample handling and preparation tips.

**Important Note**

It is imperative that temperatures are accurately maintained throughout the experiment.
Preparation and Handling of PCR Samples With Wax

For Thermal Cyclers without Heated Lids, or
PCR Using Three Waterbaths

Automated thermal cyclers with heated lids are designed to surround the entire sample tube at the appropriate temperature during PCR cycles. Heating the top of the tubes during these cycles prevents the very small sample volumes from evaporating. For thermal cyclers without heated lids, or when conducting PCR by the three-waterbath method, it is necessary to add a wax bead to the reaction sample. During the PCR process, the wax will melt and overlay the samples to prevent evaporation during heating.

PREPARING THE PCR REACTION:

1. The PCR reaction sample should be prepared as specified in the experiment instructions. Each PCR reaction sample contains the following three critical components:
   - PCR Reaction pellet™
   - Primer mix
   - DNA for amplification

2. After adding the components of the PCR reaction sample, use clean forceps to transfer one wax bead to the PCR tube.

3. Process the PCR reaction sample for the total number of cycles specified in the experiment instructions.

PREPARING THE PCR REACTION FOR ELECTROPHORESIS:

4. After the cycles are completed, transfer the PCR tube to a rack and prepare the PCR sample for electrophoresis.
   - Place the PCR tube in a 94°C waterbath long enough to melt the wax overlay. Use a clean pipet to remove most of the melted wax overlay.
   - Allow a thin layer of the wax to solidify.
   - Use a clean pipet tip to gently poke a hole through the solidified wax. Remove the tip.
   - Use another clean pipet tip to enter the hole to remove the volume of mixture specified in the experiment instructions. Transfer this volume to a clean tube.
   - Add other reagents according to experiment instructions, if applicable.
   - Add 3 µl of 10x Gel Loading solution and 12 µl of distilled water to the sample and store on ice.

5. Proceed to delivery of the sample onto an agarose gel for electrophoresis as specified in the experiment instructions.
1.0% Agarose Gel Preparation

For DNA analysis, the recommended electrophoresis buffer is Tris-acetate-EDTA, pH 7.8. The formula for diluting EDVOTEK (50x) concentrated buffer is one volume of buffer concentrate to every 49 volumes of distilled or deionized water. Prepare buffer as required for your electrophoresis unit.

If preparing the gel with concentrated (50x) buffer, use Table A.1.

If preparing the gel with diluted (1x) buffer, use Table A.2.

Table A.1

<table>
<thead>
<tr>
<th>Size of Gel (cm)</th>
<th>Amt of Agarose (g)</th>
<th>Concentrated Buffer (50X) (ml)</th>
<th>Distilled Water (ml)</th>
<th>Total Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 x 7</td>
<td>0.25</td>
<td>0.5</td>
<td>24.5</td>
<td>25</td>
</tr>
<tr>
<td>7 x 14</td>
<td>0.5</td>
<td>1.0</td>
<td>49.0</td>
<td>50</td>
</tr>
</tbody>
</table>

Table A.2

<table>
<thead>
<tr>
<th>Size of Gel (cm)</th>
<th>Amt of Agarose (g)</th>
<th>Diluted Buffer (1x) (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 x 7</td>
<td>0.25</td>
<td>25</td>
</tr>
<tr>
<td>7 x 14</td>
<td>0.5</td>
<td>50</td>
</tr>
</tbody>
</table>

Table B

<table>
<thead>
<tr>
<th>EDVOTEK Model #</th>
<th>Total Volume Required (ml)</th>
<th>Dilution 50x Conc. Buffer (ml) + Distilled Water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6+</td>
<td>300</td>
<td>6 + 294</td>
</tr>
<tr>
<td>M12</td>
<td>400</td>
<td>8 + 392</td>
</tr>
<tr>
<td>M36 (blue)</td>
<td>500</td>
<td>10 + 490</td>
</tr>
<tr>
<td>M36 (clear)</td>
<td>1000</td>
<td>20 + 980</td>
</tr>
</tbody>
</table>

Table C

<table>
<thead>
<tr>
<th>Volts</th>
<th>Recommended Time (1.0% - 7 x 14 cm gel)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum</td>
</tr>
<tr>
<td>125</td>
<td>55 min</td>
</tr>
<tr>
<td>70</td>
<td>2 hrs 15 min</td>
</tr>
<tr>
<td>50</td>
<td>3 hrs 25 min</td>
</tr>
</tbody>
</table>

Time and Voltage recommendations for EDVOTEK equipment are outlined in Table C. The approximate time for electrophoresis will vary from approximately 1 - 5 hours depending upon various factors. Conduct electrophoresis until the tracking dye has migrated up to 7 cm, or for the length of time determined by your instructor.
1.0% Agarose Gels - Quantity Preparations

To save time, electrophoresis buffer and agarose gel solution can be prepared in larger quantities for sharing by the class. Unused diluted buffer can be used at a later time and solidified agarose gel can be remelted.

**BULK ELECTROPHORESIS BUFFER**

Quantity (bulk) preparation for 3 liters of 1x electrophoresis buffer is outlined in Table D.

**BATCH AGAROSE GELS (1.0%)**

For quantity (batch) preparation of 1.0% agarose gels, see Table E.

1. Use a 500 ml flask to prepare the diluted gel buffer.
2. Pour the appropriate amount of UltraSpec-Agarose™ into the prepared buffer (see Table E). Swirl to disperse clumps.
3. With a marking pen, indicate the level of solution volume on the outside of the flask.
4. Heat the agarose solution as outlined previously for individual gel preparation. The heating time will require adjustment due to the larger total volume of gel buffer solution.
5. Cool the agarose solution to 60°C with swirling to promote even dissipation of heat. If evaporation has occurred, add distilled water to bring the solution up to the original volume as marked on the flask in step 3.
6. Dispense the required volume of cooled agarose solution for casting each gel. The volume required is dependent upon the size of the gel bed.
7. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Then proceed with preparing the gel for electrophoresis.

---

**Table D**

<table>
<thead>
<tr>
<th>Concentrated Buffer (50x) (ml)</th>
<th>Distilled Water (ml)</th>
<th>Total Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>2,940</td>
<td>3000 (3 L)</td>
</tr>
</tbody>
</table>

**Table E**

<table>
<thead>
<tr>
<th>Amt of Agarose (g) +</th>
<th>Concentrated Buffer (50x) (ml) + Distilled Water (ml) = Total Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>6.0</td>
</tr>
<tr>
<td>4.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Note: The UltraSpec-Agarose™ kit component is often labeled with the amount it contains. Please read the label carefully. If the amount of agarose is not specified or if the bottle’s plastic seal has been broken, weigh the agarose to ensure you are using the correct amount.
Staining and Visualization of DNA

INSTASTAIN® ETHIDIUM BROMIDE CARDS

1. After electrophoresis, place the gel on a piece of plastic wrap on a flat surface. Moisten the gel with a few drops of electrophoresis buffer.

2. Wearing gloves, remove the clear plastic protective sheet, and place the unprinted side of the InstaStain® EtBr card on the gel.

3. Firmly run your fingers over the entire surface of the InstaStain® EtBr. Do this several times.

4. Place the gel casting tray and a small empty beaker on top to ensure that the InstaStain® card maintains direct contact with the gel surface. Allow the InstaStain® EtBr card to stain the gel for 10-15 minutes.

5. After 10-15 minutes, remove the InstaStain® EtBr card. Transfer the gel to an ultraviolet (300 nm) transilluminator for viewing. Be sure to wear UV protective goggles.

Visit our web site for an animated demonstration of InstaStain® EtBr.

Caution: Ethidium Bromide is a listed mutagen.

Disposal of InstaStain

Disposal of InstaStain® cards and gels should follow institutional guidelines for chemical waste.

Additional Notes About Staining

- If bands appear faint, or if you are not using EDVOTEK UltraSpec-Agarose™, gels may take longer to stain with InstaStain® EtBr. Repeat staining and increase the staining time an additional 10-15 minutes.

- DNA 200 bp markers should be visible after staining even if the amplified DNA samples are faint or absent. If markers are not visible, troubleshoot for problems with the electrophoretic separation.
LyphoCells™ for Transformation
Substitute for BactoBeads™

Day before the experiment

This experiment requires preparation of isolated *E.coli* host transformation colonies 16-20 hours before the laboratory experiment, so plan accordingly.

*Important: Do not prepare source plates more than 20 hours before the experiment. Older source plates will compromise the success of the transformation experiment.*

Preparation of *E. coli* Cells

1. Use a sterile pipet to aseptically add 2 ml of recovery broth to the vial of LyphoCells™.
2. Replace the rubber stopper of the LyphoCell™ vial and cap. Mix by gently inverting until the freeze dried plug is dissolved.
3. Incubate the vial of cells for 30 - 60 minutes in a 37°C incubation oven.
   
   Growth should be evident (Broth should be slightly turbid or cloudy). If growth is not evident, incubate for a longer period of time.
4. Transfer 50 - 75 µl of cells to each source plate and streak the cells on one quadrant of each plate with a sterile loop. (figure top right).
5. With the same loop, streak through the cells once or twice into another clean section of the plate (figure bottom right) to obtain isolated colonies.
6. Label the plates "*E. coli*", invert and incubate the plates overnight (16-20 hours) at 37°C in an incubation oven.
   
   If growth on plates is heavy (i.e. few or no isolated colonies), instruct students to touch the toothpick to a small amount of cells.