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.
Table of Contents

Experiment Components ............................................. 3
Experiment Requirements ........................................ 4
Background Information ........................................... 5

Experiment Procedures
  Experiment Overview and General Instructions ............. 12
  Laboratory Safety ........................................... 13
  Module I: Amplification of a 270 bp DNA Fragment by
  PCR and Separation by Electrophoresis ..................... 14
  Module II: Preparation of Insert for Ligation .............. 17
  Module III: Ligation of the PCR Amplified 270 bp DNA
  into pUC19 .................................................. 19
  Module IV: Transformation .................................. 20
  Study Questions ............................................. 22

Instructor’s Guidelines
  Notes to the Instructor ..................................... 24
  Pre-Lab Preparations ....................................... 27
  Experiment Results and Analysis ............................ 32
  Study Questions and Answers ............................... 33

Appendices
  A PCR Experimental Success Guidelines ................. 36
  B Polymerase Chain Reaction Using Three Waterbaths .... 37
  C Preparation and Handling of PCR Samples With Wax .... 38
  D 1.0% Agarose Gel Preparation ........................... 39
  E 1.0% Agarose Gels - Quantity Preparations ............ 40
  F Staining and Visualization of DNA with
     InstaStain® Ethidium Bromide Cards .................. 41
  • Material Safety Data Sheets ............................ 42
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 pipetting. Spin samples for 10-20 seconds at maximum speed.

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

<table>
<thead>
<tr>
<th><strong>Reagents to Clean and Prepare DNA for Ligation</strong></th>
<th><strong>Storage</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>C1 Purification Buffer</td>
<td>Room temp.</td>
</tr>
<tr>
<td>C2 Wash Buffer</td>
<td>Room temp.</td>
</tr>
<tr>
<td>C3 Restriction Enzyme Reaction Buffer</td>
<td>-20°C</td>
</tr>
<tr>
<td>C4 Enzyme Grade UltraPure Water</td>
<td>-20°C</td>
</tr>
<tr>
<td>C5 Eco RI</td>
<td>-20°C</td>
</tr>
<tr>
<td>C6 Hind III</td>
<td>-20°C</td>
</tr>
<tr>
<td>C7 Restriction Enzyme Dilution Buffer</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Spin Columns and Reservoirs</td>
<td>Room temp.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Reagents for Ligation</strong></th>
<th><strong>Storage</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>L1 pUC19 Plasmid Vector cut with Eco RI and Hind III</td>
<td>-20°C</td>
</tr>
<tr>
<td>L2 DNA Ligase</td>
<td>-20°C</td>
</tr>
<tr>
<td>L3 Ligation buffer</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Reagents for Transformation</strong></th>
<th><strong>Storage</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 Ampicillin</td>
<td>-20°C</td>
</tr>
<tr>
<td>T2 IPTG</td>
<td>-20°C</td>
</tr>
<tr>
<td>T3 X-Gal in Solvent</td>
<td>-20°C</td>
</tr>
<tr>
<td>T4 CaCl₂</td>
<td>-20°C</td>
</tr>
<tr>
<td>1 Bottle of Recovery Broth</td>
<td>Room temp.</td>
</tr>
<tr>
<td>1 Vial of Bacterial LyphoCells™</td>
<td>Room temp.</td>
</tr>
<tr>
<td>1 Bottle of Ready Pour Agar</td>
<td>Room temp.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Other Components:</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• UltraSpec-Agarose™</td>
</tr>
<tr>
<td>• Electrophoresis Buffer (50x)</td>
</tr>
<tr>
<td>• InstaStain® Ethidium Bromide</td>
</tr>
<tr>
<td>• Microcentrifuge Tubes</td>
</tr>
<tr>
<td>• PCR tubes (0.2 ml - for thermal cyclers with 0.2 ml template)</td>
</tr>
<tr>
<td>• Petri plates</td>
</tr>
</tbody>
</table>

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.
Cloning of a PCR Amplified Gene

Requirements

- Thermal cycler (EDVOTEK Cat. # 532 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

Online Ordering now available

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

Technical Service Department

Mon - Fri
9:00 am to 6:00 pm ET

FAX: (301) 340-0582
Web: www.edvotek.com
email: edvotek@aol.com

Please have the following information ready:

- Experiment number and title
- Kit lot number on box or tube
- Literature version number (in lower right corner)
- Approximate purchase date
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' exonuclease 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.
Background Information

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
Background Information

Clone PCR Amplified Gene

Polymerase Chain Reaction, Ligation and Cloning

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.

Figure 2: Plasmid pUC19 and MCR Map

MCR: SacI SmaI XbaI SbfI
EcoRI KpnI BamHI SalI PstI SphI HindIII
agtgAAATTCAAGCTTCGGTACCCGGGGATCCTTAGAGTCGACCTGCAGGCATGCAAGCTTGGcgtaaatcatggtcat

lacZα translational start
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.
Polymerase Chain Reaction, Ligation and Cloning

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
Polymerase Chain Reaction, Ligation and Cloning

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 $1000/0.01\mu g = 1 \times 10^5$. 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.
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 thermal cycler for 30 cycles 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. Obtain one of the tubes (P3) containing the PCR Reaction pellet™ and label the side of the tube and top of the cap with your lab group number or initials.

3. 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).

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

5. If your thermal cycler has a 0.5 ml template, proceed to step 6.
   If your thermal cycler has a 0.2 ml template, transfer the entire contents of your PCR reaction tube into a clean 0.2 ml PCR tube before proceeding to step 6.

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

If you are unfamiliar with agarose gel preparation and electrophoresis, detailed instructions and helpful resources are available at www.edvotek.com

**AGAROSE GEL REQUIREMENTS**

- Recommended gel size: 7 x 7 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.

**Important Note**
Continue heating until the final solution appears clear (like water) without any undissolved particles. Check the solution carefully. If you see “crystal” particles, the agarose is not completely dissolved.

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. Make sure the gel is completely submerged under buffer before loading the samples and conducting electrophoresis.

LOADING DNA SAMPLES
1. 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 wires 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.

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  Eco RI enzyme
   - 10 µl  Hind III 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:
   • 9 µl “Cut PCR Insert” DNA
   • 3 µl pUC19 Plasmid Vector cut with Eco RI and Hind III
   • 3 µl Ligation buffer
   • 12 µl Enzyme Grade Ultrapure Water
   • 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 CaCl$_2$ 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 CaCl$_2$.  
   • Between your fingers, twist the toothpick vigorously in the CaCl$_2$ 
     solution to dislodge the cells.

5. Suspend the cells in the tube by tapping or vortexing (preferred).  
   At this point, the CaCl$_2$ cell suspension should look cloudy and slightly tur- 
   bid. 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.
Module IV: Transformation

PLATING CELLS

16. Balance and centrifuge both tubes at maximum speed for 1 – 2 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.

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

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?
Material Safety Data Sheet

Material Safety Data Sheet

EDVOTEK, Inc.

14676 Rothgeb Drive
Rockville, MD 20850

Section I - Personal Protective Equipment

OSHA PEL Yes
NTP? No

Section II - Hazardous Ingredients/Information

Material: EDVOTEK Multifunctional Solution

Section III - Physical/Chemical Characteristics

Flash Point: 146°F (63°C)
Evaporation Rate: 0.25 g/ml
Specific Gravity: 1.050
Vapor Pressure: 0.1 mm Hg

Section IV - Physical/Chemical Characteristics

Flammable Limits: Flash Point: 146°F (63°C)
Composition: 98.0% Water, 2.0% Solution

Section V - Reactivity Data

Stability: Stable
Compatibility: Stable

Section VI - Health Hazard Data

Health Hazard: Acute and Chronic

Section VII - Precautions for Safe Handling and Use

Precautions to Be Taken in Case Material Is Released for Spillage: None

Section VIII - Control Measures

Other Protective Clothing or Equipment: None

Other Precautions: None

Section IX - Large-Scale Accidents

Protein Precipitation: Yes
Eye Protection: Yes

Section X - Disposal

Disposal Method: None

Section XI - Transportation

DOT Classification: None

Section XII - Regulatory Information

Regulatory Information: None

Section XIII - Other Information

Other Information: None
### Material Safety Data Sheet

**IDENTITY (As Used on Label and List)**

**EDVOTek**

**Section I - Producer and Address**

**Manufacturer's Name:** EDVOTek, Inc.

**Address:** 14769 Rockledge Drive

**Rockville, MD 20850**

**Emergency Telephone Numbers:**

- **For Sales:** 1-800-255-5990
- **For Information:** 1-301-251-5990

**Section II - Hazardous Ingredients/Identity Information**

**Chemical Identity:** InstaStain® Ethidium Bromide

**Physical State:** Solid

**Appearance and Odor:** Odorless, white crystalline powder

**Material:** Water-soluble

**Solubility in Water:** Soluble

**Specific Gravity:** 1.07 to 1.10

**Boiling Point:** Data not available

**Melting Point:** Data not available

**Vapor Density:** (AIR = 1)

**Appearance and Odor:** Odorless, white crystalline powder

**Odor:** Odorless

**Miscellaneous:** Odorless

**Directive:** OSHA 29 CFR 1910.1200

**Standard:** OSHA Regulation

**ACGIH TL:** Not applicable

**OSHA PEL:** Data not available

**IPT:** No data available

**NTP:** Not studied

**IARC Monographs:** Not studied

**Section III - Physical/Chemical Characteristics**

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solubility in Water</strong></td>
<td>Soluble</td>
</tr>
<tr>
<td><strong>Specific Gravity</strong></td>
<td>1.07 to 1.10</td>
</tr>
<tr>
<td><strong>Boiling Point</strong></td>
<td>Data not available</td>
</tr>
<tr>
<td><strong>Melting Point</strong></td>
<td>Data not available</td>
</tr>
<tr>
<td><strong>Vapor Density</strong></td>
<td>(AIR = 1)</td>
</tr>
</tbody>
</table>

**Other Limits**

- **Flash Point:** Data not available
- **Lower Explosive Limit (LEL):** Data not available
- **Upper Explosive Limit (UEL):** Data not available
- **Flash Point Method Used:** Data not available
- **Flammable Limit (% Vol.):** Data not available
- **Flammable Limits:** Data not available
- **Less Reactive:** Data not available

**Special Fire Fighting Procedures**

- Do not use water spray, carbon dioxide, dry chemical, or dry chemical.
- Use carbon dioxide, dry chemical, or regular foam.
- Use fire resistant clothing and SCA to prevent contact with skin & eyes.
- Unusual Fire and Explosion Hazards: Emits toxic fumes.

**Other Precautions**

- Avoid dust or contact with skin.

**Waste Disposal Method**

- Dissolve in a combustible solvent and burn in a chemical incinerator with afterburner and scrubber, or sweep up and return in original container.

**Section IV - Reactivity Data**

<table>
<thead>
<tr>
<th>Property</th>
<th>Incompatibility</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stability</strong></td>
<td>Stable</td>
</tr>
<tr>
<td><strong>Incompatibilities</strong></td>
<td>Strong oxidizing agents</td>
</tr>
</tbody>
</table>

**Special Fire Fighting Procedures**

- Use fire resistant clothing and SCA to prevent contact with skin & eyes.
- Unusual Fire and Explosion Hazards: Emits toxic fumes.

**Other Precautions**

- Avoid dust or contact with skin.

**Waste Disposal Method**

- Dissolve in a combustible solvent and burn in a chemical incinerator with afterburner and scrubber, or sweep up and return in original container.

**Section V - health Hazard Data**

**Route(s) of Entry:**

- Inhalation
- Ingestion
- Skin

**Health Hazards:**

- Acute: Material irritating to mucous membranes, upper respiratory tract, eyes, skin
- Sensitizers may result in allergic reaction
- Repeated exposure may result in sensitization and possible sensitization

**Signs and Symptoms of Exposure:**

- May affect the respiratory system.
- Sensitizers may result in allergic reaction.
- Repeated exposure may result in sensitization and possible sensitization.

**Medical Conditions Generally Aggravated by Exposure:**

- Data not available

**Emergency First Aid Procedures**

- In case of inhalation, get fresh air.
- In case of ingestion, give nothing by mouth.
- In case of skin contact, wash with water.
- If on Eyes: Flush with water.

**Emergency Phone Numbers:**

- In case of fire, call 1-800-255-5990
- In case of spill, call 1-301-251-5990

**Protective Equipment:**

- No special protective equipment required.

**Other Precautions:**

- Avoid dust or contact with skin.

**Section VI - Control Measures**

**Respiratory Protection (Specify Type of Respirator):** Not required.

**Other Protective Clothing:** Not required.

**Other Protective Equipment:** Not required.

**Other Precautions:**

- Avoid dust or contact with skin.

**Section VII - Precautions for Safe Handling and Use**

**Steps to be Taken in Case Material is Released for Spillage:**

- If spilled material may emit toxic fumes.
- If inhaled, get fresh air.
- If swallowed, induce vomiting.
- If on skin, wash with water.

**Other Precautions:**

- Avoid dust or contact with skin.

**Section VIII - Exposure Limits**

**OSHA PEL:** Data not available

**ACGIH TL:** Data not available

**IPT:** Data not available

**NTP:** Not studied

**IARC Monographs:** Data not available

**Section IX - Other Information**

**Material and Process Compatibility:** Data not available

**Material and Substance Compatibility:** Data not available

**Section X - Other Information**

**Material and Process Compatibility:** Data not available

**Material and Substance Compatibility:** Data not available

**Section XI - Transportation Information**

**UN/NA:** Not applicable

**Class:** Not applicable

**DOT:** Not applicable

**IATA:** Not applicable

**ILO:** Not applicable

**ICAO:** Not applicable

**Section XII - Disposal Considerations**

**Material and Process Compatibility:** Data not available

**Material and Substance Compatibility:** Data not available

**Section XIII - Other Information**

**Material and Process Compatibility:** Data not available

**Material and Substance Compatibility:** Data not available

**Section XIV - Other Information**

**Material and Process Compatibility:** Data not available

**Material and Substance Compatibility:** Data not available

**Section XV - Other Information**

**Material and Process Compatibility:** Data not available

**Material and Substance Compatibility:** Data not available