EXPERIMENT 8 – AMPLIFYING & CLONING A GENE UPSTREAM REGION (GENE TWO)

**Purpose:** To determine the activity of the promoter of the gene of interest at the cellular and tissue levels in *Arabidopsis* plants via the expression of the β-glucuronidase (GUS) or green fluorescent protein (GFP) gene fused to the promoter of the gene of interest.

**References:**
- iProof High-Fidelity DNA Polymerase Kit (Bio-Rad; See Appendix 1J)
- pENTR/D-TOPO Cloning Instruction Manual (Invitrogen; See Appendix 1K)
- QIAprep Miniprep Handbook (Qiagen; see Appendix 1L)

**STRATEGY**

I. AMPLIFICATION OF THE UPSTREAM REGION OF THE GENE OF INTEREST USING HIGH FIDELITY DNA POLYMERASE

II. CLONING THE AMPLIFIED PROMOTER REGION INTO A PLASMID VECTOR

   A. LIGATING THE PCR PRODUCT AND A PENTR/D-TOPO VECTOR
   B. TRANSFORMATION OF *E. COLI* CELLS WITH THE LIGATION MIXTURE
   C. SCREENING FOR *E. COLI* CELLS HARBORING THE RECOMBINANT PLASMID AND ISOLATING RECOMBINANT PLASMID DNA
   D. CONFIRMING THE AUTHENTICITY OF RECOMBINANT PLASMID DNA VIA RESTRICTION ENZYME DIGESTION
   E. VERIFYING THE SEQUENCE OF THE PROMOTER REGION BY SEQUENCING ANALYSIS
STRAIGHT FOR DETERMINING THE ACTIVITY OF A GENE UPSTREAM REGION

**Arabidopsis Genomic DNA**
- Amplify the promoter by PCR using High Fidelity DNA Polymerase and a modified FW primer beginning with CACC

**Amplified product beginning with CACC**
- Linearized pENTR vector bound by Topoisomerase, also containing GTGG overhang
  - CACC in PCR product anneals to complementary GTGG in pENTR vector
  - Topoisomerase seals the phosphodiester bond and is released from the plasmid

**Population of recombinant and non-recombinant plasmid**
- Transform E. coli cells using CaCl$_2$
- Screen for colonies containing recombinant plasmid by isolating plasmid DNA and performing a restriction digest and gel electrophoresis
- Sequence the promoter to make sure there are no mutations

**Promoter cloned into pENTR vector**
- pBGWFS7 T-DNA vector (T-DNA contains toxic gene upstream of GFP and GUS reporter genes, Basta$^R$ gene, and left and right border sequences)
  - Homologous recombination between the att sequences flanking the promoter in pENTR and flanking the toxic gene in pBGWFS7

**T-DNA plasmid (T-DNA contains promoter upstream of GFP and GUS reporter genes, Basta$^R$ gene, and left and right border sequences)**
- Transform E. coli cells using CaCl$_2$
- Screen for colonies containing recombinant plasmid by isolating plasmid DNA and performing a restriction digest and gel electrophoresis

**Promoter cloned into T-DNA plasmid**
- Transform Agrobacterium tumefaciens cells containing the Ti Helper plasmid, which encodes genes with T-DNA transfer functions
- Transform Arabidopsis plant with Agrobacterium cells containing Ti Helper plasmid and the T-DNA plasmid
- Select for transformed Arabidopsis plants by applying the herbicide Basta

**Transformed Arabidopsis plants containing the promoter upstream of GFP and GUS genes**

---

Amplifying & Cloning a Gene Upstream Region (Gene TWO) 8.2
I. AMPLIFICATION OF THE UPSTREAM REGION OF THE GENE OF INTEREST

Materials Needed:

- iProof High Fidelity DNA polymerase kit (Cat.# 172-5301, Bio-Rad)
- iProof High Fidelity DNA polymerase (included in iProof High Fidelity DNA polymerase kit)
- 5x iProof HF Buffer (included in iProof High Fidelity DNA polymerase kit)
- dNTP mix (included with the Ex Taq DNA polymerase, Takara)
- Sterile water
- 12 µM Gene-specific Promoter Forward primer
- 12 µM Gene-specific Promoter Reverse primer
- High quality Arabidopsis genomic DNA (12 ng/µL)
- PCR product of the promoter region amplified by TAs with Ex Taq DNA Polymerase (for use as positive control)
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain
- 6x Loading buffer containing ONLY bromophenol blue
- 100 bp DNA ladder (Invitrogen)

Materials Needed:

- Ice bucket
- Pipettes
- Filter pipet tips
- 0.2 mL PCR tubes
- PCR tube rack
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Microcentrifuge
- Vortex
- PCR machine (Bio-Rad MyCycler)
- Gel electrophoresis materials (Appendix 1A)

PROCEDURE

*Note: ALWAYS wear gloves and use PCR filter tips when performing PCR preparation to prevent contamination.*

1. Get ice from the icemaker.
2. Thaw out tubes of 5x iProof HF Buffer and dNTP mix (2.5 mM each dNTP) on a microcentrifuge tube rack for 1.5 mL microcentrifuge tubes at room temperature for a few minutes. Once the solutions are thawed, vortex for 5 seconds to mix the contents. Spin tubes for 10 seconds. Put the tubes on ice until needed.

3. Thaw gene-specific promoter forward and reverse primers corresponding to the gene of interest as in step 2.

4. Obtain THREE 0.2 mL sterile PCR tubes and set them on a PCR tube rack.

5. Label the lids and sides of the tubes with sample name and date.
   - Tube #1: **Name of a gene**
   - Tube #2: **Pos.** (Positive control for the gene of interest = PCR product of the promoter region amplified by TAs with Ex Taq DNA Polymerase)
   - Tube #3: **Neg.** (Negative control for the gene of interest containing the same components as in tube #1, but NO genomic DNA)

6. Label ONE 1.5 mL microcentrifuge tube “Mmix” (for Master mix). Keep the tube on ice.

7. Prepare a **Master mix** for 4 reactions (3 samples + 1 extra) (see table below)  
   **Caution:** Keep tube on ice at all times.
   **Note:** Amplification of targets greater than 4 kb may require more DNA polymerase, but do NOT exceed 2 units of enzyme per 50 µL reaction.
### Master Mix

<table>
<thead>
<tr>
<th></th>
<th>Mmix for 1 Reaction</th>
<th>Mmix for 4 Reactions</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>25.5 µL</td>
<td>102 µL</td>
<td></td>
</tr>
<tr>
<td>5x iProof HF Buffer</td>
<td>10 µL</td>
<td>40 µL</td>
<td>1x</td>
</tr>
<tr>
<td>dNTP mix (2.5 mM each dNTP)</td>
<td>4 µL</td>
<td>16 µL</td>
<td>0.20 mM</td>
</tr>
<tr>
<td>12 µM Gene-specific Promoter Forward primer</td>
<td>1 µL</td>
<td>4 µL</td>
<td>0.24 µM</td>
</tr>
<tr>
<td>12 µM Gene-specific Promoter Reverse primer</td>
<td>1 µL</td>
<td>4.0 µL</td>
<td>0.24 µM</td>
</tr>
<tr>
<td>iProof DNA polymerase (2.0 Units/µL)</td>
<td>0.5 µL</td>
<td>2 µL</td>
<td></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>42 µL</strong></td>
<td><strong>168 µL</strong></td>
<td></td>
</tr>
</tbody>
</table>

a. Pipet the reagents into the Mmix tube in order from the top down.

b. After pipetting all reagents into the master mix tube, close its lid. Mix the contents by vortexing at a slow setting (speed 2-3) for 2 seconds. **Caution:** Do NOT vortex a mixture with enzyme, such as DNA polymerase, vigorously, or for > 5 seconds because these two factors will break down enzymes, resulting in LOW or NO yield of PCR product.

c. Spin the tube in a microcentrifuge at full speed (13,200 rpm) for 10 seconds. Put the tube back on ice.

8. Prepare the PCR reactions according to the table below.

a. Pipet 42 µL of the Mmix solution into the labeled PCR tubes.

b. Pipet 8 µL of genomic DNA or water into tubes #1-3.

c. Mix the contents by pipetting up and down 5 times.
PCR reactions:

<table>
<thead>
<tr>
<th></th>
<th>Tube #1</th>
<th>Tube #2</th>
<th>Tube #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mmix</td>
<td>42 µL</td>
<td>42 µL</td>
<td>42 µL</td>
</tr>
<tr>
<td>~100 ng Arabidopsis genomic DNA (12 ng/µL)</td>
<td>8 µL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PCR product of the promoter amplified by TAs (Positive control)</td>
<td>-</td>
<td>1 µL</td>
<td>-</td>
</tr>
<tr>
<td>Sterile water (Negative Control)</td>
<td>-</td>
<td>7 µL</td>
<td>8 µL</td>
</tr>
<tr>
<td>Total volume</td>
<td>50 µL</td>
<td>50 µL</td>
<td>50 µL</td>
</tr>
</tbody>
</table>

9. Perform PCR amplification as follows:
   a. Turn ON the PCR machine (MyCycler). Wait for one minute for the machine to initialize.
   b. Put the PCR tubes in the wells of the 96-well hot plate of the Bio-Rad MyCycler.
   c. Select the “Protocol Library” by pressing “F1.”
   d. Select “HC70AL” by pressing the yellow arrowheads surrounding the “ENTER” button. Select the “HC70AL IProof” protocol. Press “ENTER.”
   e. The “CHOOSE OPERATION” menu will appear. Select “VIEW PROTOCOL.” The PCR profile of the iProof protocol is as follows:

**PCR profile for genomic DNA templates**

<table>
<thead>
<tr>
<th>Cycling parameters</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation Enzyme step</td>
<td>98°C for 30 seconds</td>
</tr>
<tr>
<td>Denaturation step</td>
<td>98°C for 10 seconds</td>
</tr>
<tr>
<td>Annealing step</td>
<td>63°C (or Tm+3°C) for 20 seconds</td>
</tr>
<tr>
<td>Extension step</td>
<td>72°C for 2 minutes (or 15-30 seconds/kb)</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C for 5 minutes</td>
</tr>
</tbody>
</table>
f. Press “F5” for “DONE.” The “CHOOSE OPERATION” menu will appear. Press “ENTER” to **run the protocol**.

g. Enter the **volume** of the PCR reaction. Press “F5” to “Begin Run.” **Note:** It will take about 3 hours for the PCR amplification to be completed.

10. Once the PCR amplification is complete, remove the PCR tubes from the PCR machine and store them in the **refrigerator** until gel electrophoresis or leave them in the PCR machine at 4°C until you have a chance to put them away later.

11. Prepare a **50 mL 1% agarose gel** in 1x TAE buffer with a 10-tooth comb.

12. Spin PCR tubes for **5 seconds**.

13. Label new 1.5 mL microcentrifuge tubes with **sample name**.

14. Pipet **20 µL** of PCR product into each labeled tube.

15. Add **2 µL** of **6x lower loading dye** to each tube.

16. Load **10 µL** of **1 Kb Plus DNA ladder** in the first well of the 1% agarose gel.

17. Load ~**22 µL** of each sample-dye mixture on the agarose gel.

18. Record the identity of the sample in each lane.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Tube #</th>
<th>Sample</th>
<th>Expected Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>1 Kb Plus DNA Ladder</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>Genomic DNA</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>PCR product amplified by TAs</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>Sterile water</td>
<td>-</td>
</tr>
</tbody>
</table>

19. Add **5 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.

20. Run the gel at ~**105 volts** for **1-2 hours**.

   Starting time:

   Ending time:

21. Take a **picture of the gel**.

22. Analyze the results.
II. CLONING THE AMPLIFIED PROMOTER REGION INTO A PLASMID VECTOR

Materials Needed:
- Gene-specific promoter PCR product
- pENTR/D-TOPO Cloning kit (Cat.# K2400-20, Invitrogen)
- Sterile water

Materials Needed:
- Ice bucket
- Pipettes
- Filter pipet tips
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Microcentrifuge
- Vortex

A. Ligating the PCR Product into the pENTR/D-TOPO Vector

1. Thaw the pENTR/D-TOPO vector solution on ice.
2. Label THREE 1.5 mL microcentrifuge tubes pENTR + Promoter, pENTR Only and Mmix and the date. Place the labeled tubes on ice.
3. Set up the Mmix as follows:
   a. Pipet the reagents into the Mmix tube.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>pENTR + Promoter</th>
<th>pENTR Only (Negative Control)</th>
<th>Mmix for 2.3 rxns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>2.5 µL</td>
<td>2.5 µL</td>
<td>5.75 µL</td>
</tr>
<tr>
<td>Salt Solution</td>
<td>1.0 µL</td>
<td>1.0 µL</td>
<td>2.3 µL</td>
</tr>
<tr>
<td>pENTR/D-TOPO vector</td>
<td>0.5 µL</td>
<td>0.5 µL</td>
<td>1.15 µL</td>
</tr>
<tr>
<td>Freshly prepared PCR product</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterile water</td>
<td>-</td>
<td>2.0 µL</td>
<td>-</td>
</tr>
<tr>
<td>Total Volume</td>
<td>6.0 µL</td>
<td>6.0 µL</td>
<td></td>
</tr>
</tbody>
</table>

   b. Mix GENTLY by flicking the tube. Do NOT vortex the tube!
4. Pipet 4 µL of Mmix into the pENTR + Promoter and pENTR Only tubes.
5. Pipet 2 μL of freshly prepared PCR product into the pENTR + Promoter tube.
6. Pipet 2 μL of sterile water into the pENTR Only tube.
7. Mix GENTLY by flicking the tubes. Do NOT vortex the tubes!
8. Incubate the reaction for 30 minutes at room temperature (22-24°C).

*Note:* For most applications, 5 minutes will yield many colonies for analysis. The length of the TOPO Cloning reaction can be varied from 30 seconds to 30 minutes.

For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (>1 kb), increasing the reaction time will yield more colonies *(Taken from TOPO Cloning Manual, Invitrogen).*

9. After the ligation reaction is done, place the ligation mixture tube on ice. *Note: If you don’t have time, you can store the ligation mixture at −20°C overnight.*

**B. Transformation of E. coli Competent Cells with the Ligation Mixture**

**Solutions Needed:**
- Ligation reactions
- pENTR/D-TOPO Cloning kit with One Shot TOP10 Competent cells (Cat.# K2400-20, Invitrogen)
- pUC19 plasmid (10 pg/μL) (Included with the TOP10 Competent cells)
- S.O.C. medium (Included with the TOP10 Competent cells)
- LB Kanamycin 50 μg/mL plates
- LB Ampicillin 100 μg/mL plates for pUC19 plasmid (control for transformation efficiency)

**Materials Needed:**
- Ice bucket
- Pipettes
- Pipet tips (non-filter)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Microcentrifuge
- Vortex
- 42°C water bath
- 37°C incubator
- Orbital shaker in the 37°C incubator
- Tape
Cell spreader
- A glass jar containing 95% ethanol solution
- Inoculating turntable
- Biological fume hood
- Parafilm

Note: Make sure to set a water bath 42°C before starting.

1. Thaw the pUC19 control plasmid on ice.

2. Thaw THREE vials of One Shot E. coli competent cells for transformation on ice for a few minutes. Note: Remove tubes of competent cells from the -70°C freezer and place them on ice just a few minutes before you need them.

3. Label the vials number 1-3 and your initials.

<table>
<thead>
<tr>
<th>Vial #1</th>
<th>Vial #2</th>
<th>Vial #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pENTR + Promoter</td>
<td>pENTR Only</td>
<td>pUC19</td>
</tr>
</tbody>
</table>

4. Pipet the TOPO ligation mixtures or control plasmid DNA (pUC19) into the vials of One Shot E. coli competent cells as shown below. Mix the contents by flicking each tube gently. Attention: Do NOT pipet the mixture up and down.

<table>
<thead>
<tr>
<th>Vial #1</th>
<th>Vial #2</th>
<th>Vial #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pENTR + Promoter</td>
<td>2 µL</td>
<td>-</td>
</tr>
<tr>
<td>pENTR Only</td>
<td>-</td>
<td>2 µL</td>
</tr>
<tr>
<td>pUC19</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

5. Incubate the cell mixture on ice for 10-30 minutes.

6. Heat-shock the cells for exactly 30 seconds in the 42°C water bath without shaking.

7. Immediately, set the tubes back on ice for 2 minutes.

8. Transfer the tubes to a rack for microcentrifuge tubes at room temperature.

9. In the bacterial hood, pipet 250 µL of room temperature S.O.C medium to the cell mixture. Cap the tube tightly.

10. Shake the tubes horizontally at 200 rpm on an orbital shaker in a 37°C incubator for 1 hour. Note: Attach tubes to shaker with tape.
11. Meanwhile, label the bottom of 6 prewarmed (37°C) plates (4 Kanamycin plates with no line on the side of the lids for pENTR samples and 2 Ampicillin plates with ONE RED LINE on the side of the lids for pUC19) the following:

<table>
<thead>
<tr>
<th>Plate #</th>
<th>Sample</th>
<th>Date</th>
<th>Initials</th>
<th>Volume of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin 1</td>
<td>pENTR + Promoter</td>
<td></td>
<td></td>
<td>10 µL</td>
</tr>
<tr>
<td>Kanamycin 2</td>
<td>pENTR + Promoter</td>
<td></td>
<td></td>
<td>50 µL</td>
</tr>
<tr>
<td>Kanamycin 3</td>
<td>pENTR Only</td>
<td></td>
<td></td>
<td>10 µL</td>
</tr>
<tr>
<td>Kanamycin 4</td>
<td>pENTR Only</td>
<td></td>
<td></td>
<td>50 µL</td>
</tr>
<tr>
<td>Ampicillin 1</td>
<td>pUC19</td>
<td></td>
<td></td>
<td>10 µL</td>
</tr>
<tr>
<td>Ampicillin 2</td>
<td>pUC19</td>
<td></td>
<td></td>
<td>50 µL</td>
</tr>
</tbody>
</table>

12. Spread 10 µL and 50 µL of each transformation mixture on the appropriately labeled plates in the hood (TAs will show you how). Note: Spread two volumes of cells per transformation mix in order to get at least one plate with enough colonies and well-separated colonies.

   Plate #1: 10 µL of transformation mixture + 40 µL of S.O.C medium (for even spreading of a small volume)

   Plate #2: 50 µL of transformation mixture

13. Incubate the plates in the 37°C incubator overnight (14-16 hours).

14. On the next day, count the number of colonies. Seal the plates with pieces of parafilm and then store them at 4°C (cold room or refrigerator) until the inoculation step.

   Do you expect to get the same number of colonies on the pENTR + Promoter plate as on the pENTR Only plate?

C. Screening for E. coli Cells Harboring the Recombinant Plasmid and Isolating Plasmid DNA

Reference: QIAprep Miniprep Handbook (Qiagen; see Appendix 1L)

Materials Needed:
- pENTR + Promoter plate with colonies
Materials Needed:

- 5 mL plastic pipette
- Bulb
- Black ultra-fine sharpie
- Vortex
- 37°C incubator
- Orbital shaker in the 37°C incubator
- Biological fume hood
- Sterile glass culture tubes
- Culture tube rack
- Sterile toothpicks
- Parafilm

Inoculation of a Liquid Medium with Bacterial Colonies

1. Put FOUR sterile glass tubes on a test tube rack.
2. Label the side of each tube with your initials and number 1-4.
3. Choose FOUR colonies and label them 1-4 on the agar plate.
4. Pipet 3 mL of Terrific Broth (TB) medium containing 50 µg/mL Kanamycin into each of the 4 tubes.
5. Inoculate the TB + Kanamycin solution in each tube with individual colonies by using a sterile toothpick or pipet tip to pick a single colony on the pENTR + Promoter plate and drop that toothpick/pipet tip into a tube.
6. Gently vortex to resuspend the colony.
7. Shake the tubes at 37°C overnight.
   a. Transfer all 4 tubes to a culture tube rack on an orbital shaker in the 37°C incubator.
   b. Turn the shaking SPEED dial (LEFT dial) to number 2 for 200 rpm.
   c. Turn the shaking TIME dial (RIGHT dial) clockwise to the “CONSTANT” position.
8. Close the incubator door.
9. Wrap your plates in parafilm and store at 4°C.
10. **On the next day** (after 12-16 hours), inspect the growth of cells (appearing very cloudy) in the culture tubes. If plasmid DNA is not isolated immediately, place the culture tubes in the cold room.

**Isolating Plasmid DNA**

**Materials Needed:**
- Bacterial cultures
- QIAprep Spin Miniprep kit (Qiagen, cat. #27104)

**Materials Needed:**
- Ice bucket
- Pipettes
- Pipet tips (non-filter)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Microcentrifuge
- Vortex
- NanoDrop spectrophotometer
- Kimwipes

1. Label the lids of **FOUR 1.5 mL microcentrifuge tubes** with your initials and number 1-4. Set the labeled tubes on a microcentrifuge tube rack.
2. Arrange the culture tubes and labeled microcentrifuge tubes in their corresponding order. For example, 1 to 1, 2 to 2, …, 4 to 4.
3. Pipet **1 mL** of liquid culture into the appropriate microcentrifuge tube. Close the lids of the tubes. **Note: If the culture tubes sit in the refrigerator or cold room for more than ONE hour, vortex the tubes for 5-10 seconds to mix the contents before transferring it to the microcentrifuge tube.**
4. Spin tubes in a microcentrifuge at **FULL speed for 30 seconds**.
5. Pour off the supernatant into a glass Erlenmeyer flask labeled “CULTURE WASTE.” Dab off the extra liquid on a piece of paper towel or Kimwipes.
6. Place the tubes back on the microcentrifuge tube rack.
7. Pipet another 1 mL of liquid culture into the appropriate microcentrifuge tubes. Close the lids of the tubes.

8. Spin the tubes in a microcentrifuge at FULL speed for 30 seconds.

9. Pour off the supernatant into a glass Erlenmeyer flask labeled “CULTURE WASTE.” Dab off the extra liquid on a piece of paper towel.

10. Place the tubes back on the microcentrifuge tube rack.

11. Use a P-200 pipet to remove any remaining liquid from the tubes containing cell pellets. Discard the liquid into a glass Erlenmeyer flask labeled “CULTURE WASTE.”

12. Shake or vortex Buffer P1 (Resuspension buffer) to ensure that all particles are completely dissolved. Note: Make sure that RNase A and LyseBlue are added to Buffer P1 before use.

13. Pipet 250 µL of Buffer P1 (Resuspension buffer) to each tube. Close the lids tightly.

14. Resuspend pelleted bacterial cells by either vortexing or pipetting up and down until NO cell clumps are observed.

15. Place the tube back on the microcentrifuge tube rack.

16. Add 250 µL of Buffer P2 (Lysis buffer) to each tube. Close the lids. The solution will turn blue.

17. Gently mix by inverting the tubes 5 times or until a homogeneously colored suspension is achieved. This step is for breaking open the bacterial cells to release their contents (chromosomal DNA, plasmid DNA, proteins, carbohydrates) into the solution. Note: Do NOT vortex the contents. Vortexing can shear bacterial chromosome DNA into many tiny pieces that have the same size as the plasmid DNA. Note: Do NOT allow the lysis reaction to proceed for more than 5 min.

18. Add 350 µL of Buffer N3 (Neutralization buffer) to tube #1. Close the lid. Immediately, invert the tube 5 times to mix or until all trace of blue has gone and the suspension is colorless. The solution will appear cloudy. Note: Do NOT vortex the mixture!

19. Repeat step 17 for the other tubes (one by one).

20. Spin tubes in the microcentrifuge at FULL speed for 10 minutes.
21. Meanwhile, label the lids of the QIAprep columns and sides of the collection tubes (light blue) with your initials and number 1-4. Set the columns in their collection tubes on the microcentrifuge tube rack.

22. Also, label the lids and sides of a new set of 1.5 mL tubes with the following information: pENTR-gene name, number, your initials, and the date. (Tubes will be used in step 31).

23. After 10 minutes of spinning, pour the supernatant from step 19 into the QIAprep column. **Caution:** Make sure that the numbers on the tubes the QIAprep columns match.

24. Spin the columns in their collection tubes at **FULL** speed for **30 seconds**.

25. Lift the column off of the collection tube and discard the flow-through liquid into a glass beaker for waste.

26. Put the column back in its collection tube.

27. Pipet **500 µL** of Buffer PB to each column. Spin the columns at **FULL** speed in the microcentrifuge for **30 seconds**.

28. Lift the column off of the collection tube and discard the flow-through liquid into a glass beaker for waste.

29. Pipet **750 µL** of Buffer PE to each column. Spin the columns at **FULL** speed in the microcentrifuge for **30 seconds**. This step is to wash residual salt and proteins from the membrane of the column. **Note:** Make sure that ethanol is added to the PE buffer before use.

30. Lift the column off of the collection tube and discard the flow-through liquid into a glass beaker for waste.

31. Spin the columns at **FULL** speed for **1 minute** to remove residual wash buffer. **Caution:** If the residual wash buffer is NOT completely removed, the DNA solution will float up when the sample is loaded into the well of an agarose gel due to the presence of ethanol in the DNA solution. Also, ethanol may inhibit enzymatic activity in later steps. **Note:** If wash buffer remains on the column, spin for another 1-2 minutes.
32. Transfer the QIAprep columns to the labeled tubes (prepared in step 21). Discard the collection tubes. *Note: Make sure the numbers on the columns and microcentrifuge tubes match.*

33. Pipet 50 µL of Buffer EB (10 mM Tris-HCl, pH 8.5) to the center of each QIAprep column. *Note: If the plasmid is >10 kb, pre-heat Buffer EB to 70°C prior to eluting DNA from the QIAprep membrane.*

34. Let the columns stand for 1 minute. *Note: It is okay to incubate longer than 1 minute.*

35. Spin the tubes with columns at FULL speed for 1 minute. *Steps 32-34 are for eluting plasmid DNA off the column.*

36. After spinning, discard the columns. Save the eluted plasmid DNA in the 1.5 mL microcentrifuge tubes.

37. Determine the DNA concentration and its purity using the NanoDrop spectrophotometer. Record DNA concentration.

**D. Confirming the Authenticity of Recombinant Plasmid DNA Via Restriction Enzyme Digestion**
**Purpose:** To ensure that the plasmid DNA isolated from colonies is recombinant plasmid DNA (i.e. contains the cloned promoter region), not non-recombinant DNA (i.e. the vector alone).

Represents covalently bound topoisomerase I
Reagents and Materials Needed:

- Plasmid DNA
- Sterile water
- Ascl restriction enzyme (New England Biolabs, 10 units/µL)
- 10x NEB Buffer 4 (supplied with Ascl)
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain
- 6x Loading buffer containing ONLY bromophenol blue
- 1 Kb Plus DNA ladder (Invitrogen)

Materials Needed:

- Ice bucket
- Pipettes
- Pipet tips (non-filter)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Microcentrifuge
- Vortex
- 37°C water bath
- Gel electrophoresis materials (Appendix 1A)

PROCEDURE

1. Digest 300 - 1000 ng of plasmid DNA with the restriction enzyme Ascl at 37°C for 60 minutes.

   Why Ascl? Check the presence of the Ascl site in the Multiple Cloning Site of the pENTR/D-TOPO vector diagram on the previous page.


Table 1. Setting up a standard restriction enzyme reaction

<table>
<thead>
<tr>
<th>Components</th>
<th>Standard Reaction</th>
<th>Final Concentration</th>
<th>Example 500 ng DNA (200 ng/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>x µL</td>
<td>----</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Sterile water</td>
<td>y µL</td>
<td>---</td>
<td>15.0 µL</td>
</tr>
<tr>
<td>10x NEB buffer 4</td>
<td>2.0 µL</td>
<td>1x</td>
<td>2.0 µL</td>
</tr>
<tr>
<td>AscI (10 U/µL, NEB)</td>
<td>0.5 µL</td>
<td>0.25 U/µL</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>20.0 µL</td>
<td>----</td>
<td>20.0 µL</td>
</tr>
</tbody>
</table>

Explanation of volumes

\( x \, \mu L \) = Volume of Plasmid DNA depends on the amount (in ng) of DNA to be digested and the concentration of plasmid DNA (in ng/µL)

- **Volume** of 10x NEB buffer is 1/10\(^{th}\) the total volume of the reaction so that the final concentration of the buffer in the reaction is 1x.

- **The volume of restriction enzyme** depends on the amount of DNA to be digested. Usually, **ONE Unit of Restriction endonuclease** (Enzyme) is defined as the amount of enzyme required to digest 1 µg (or 1,000 ng) of DNA completely in **ONE hour** under the conditions specified for that enzyme (most enzymes have an **optimal temperature** of 37°C). To ensure that DNA is completely digested after 1 hour, we use **2-10 units** of enzyme per microgram of DNA.

- **y µL** = The volume of sterile water is the remaining volume added to the reaction to bring up the total volume.

**Note:** It is best to set up reactions with a master mix to minimize the chance of leaving out one or several components in the reactions. **The volume of NEB buffer + AscI for each reaction is fixed as 2.5 µL**, while the volume of the plasmid DNA + water is 17.5 µL. Therefore, it is best to make an **Enzyme Mix containing the buffer and AscI enzyme** (see Table 2 below) for five reactions (4 samples + 1 extra reaction).
a. Label the lid of a 1.5 mL microcentrifuge tube “Enz Mix.” Prepare the Enzyme Mix for the number of plasmid DNA samples + 1 Extra reaction (use Table 2 below). Keep the Enzyme Mix tube on ice. Mix the contents by flicking the tube gently. Spin the tube for 10 seconds (if necessary). Keep the tube on ice.

How many DNA samples will be digested?

Table 2. Preparation of the Enzyme Mix

<table>
<thead>
<tr>
<th>Components</th>
<th>Enz Mix for 1 Reaction</th>
<th>Enz Mix for ___Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x NEB Buffer 4</td>
<td>2.0 µL</td>
<td>___ µL</td>
</tr>
<tr>
<td>AscI (10 U/µL)</td>
<td>0.5 µL</td>
<td>___ µL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>2.5 µL</td>
<td>___ µL</td>
</tr>
</tbody>
</table>

b. Determine the volume of plasmid DNA and the volume of water so that you have a total volume of 17.5 µL and fill in Table 3 below.

Table 3.

<table>
<thead>
<tr>
<th></th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 - 500 ng plasmid DNA</td>
<td>_____ µL</td>
<td>_____ µL</td>
<td>_____ µL</td>
<td>_____ µL</td>
</tr>
<tr>
<td>Sterile water</td>
<td>_____ µL</td>
<td>_____ µL</td>
<td>_____ µL</td>
<td>_____ µL</td>
</tr>
<tr>
<td>Enzyme Mix</td>
<td>2.5 µL</td>
<td>2.5 µL</td>
<td>2.5 µL</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 µL</td>
<td>20 µL</td>
<td>20 µL</td>
<td>20 µL</td>
</tr>
</tbody>
</table>

c. Label the lids of 1.5 mL microcentrifuge tubes with the sample number, AscI and your initials. Keep tubes on ice. Set up restriction digestion reactions by pipetting the components from Table 3 into the tubes. Mix the contents by flicking the tubes several times. Spin the tubes in the microcentrifuge for 10 seconds to bring liquid to the bottom of the tubes.
d. Incubate the reactions in the 37°C water bath for about 1 hour.

2. In the meantime, prepare a 1% agarose gel in 1x TAE buffer with a 20-tooth comb.

3. At the end of the incubation, spin tubes for 10 seconds.

4. Add 2 µL of 6x Loading dye to each restriction-digested DNA sample. Mix the contents by pipetting the mixture or flicking the tube.

5. Load 10 µL of 100 bp DNA Ladder in the first well.

6. Load 20 µL of each sample-dye mixture on the agarose gel.

   *Note:* You may also load uncut plasmid DNA in other lanes for reference. Run 300-1000 ng of each uncut plasmid mixed with 2 µL of 6x Loading dye.

7. Load 10 µL of 1 Kb Plus DNA Ladder in the first well.

8. Record the identity of the sample in each lane.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>Expected Sizes (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 bp DNA Ladder</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Plasmid #1 x AscI</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Plasmid #2 x AscI</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Plasmid #3 x AscI</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Plasmid #4 x AscI</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>1 Kb Plus DNA Ladder</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Plasmid #1 Uncut</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Plasmid #1 Uncut</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Plasmid #1 Uncut</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Plasmid #1 Uncut</td>
<td>-</td>
</tr>
</tbody>
</table>

9. Add 10 µL of 10,000x SYBR Safe DNA gel stain to the running buffer at the anode.

10. Run the gel at ~105 volts for 1-2 hours.
    
    Starting time:
    
    Ending time:

11. Take a picture of the gel.

12. Analyze the results.

   *How many DNA fragments do you see from each plasmid DNA sample?*
Which fragment corresponds to the vector?
What is the size of the vector?
Which fragment corresponds to the PCR-amplified promoter region?
What is the size of the PCR-amplified promoter region?
How many samples have the PCR-amplified promoter region?
Which plasmid DNA prep (or clone) will be used for sequencing analysis?
E. Verifying the Sequence of the Promoter Region by Sequencing Analysis

**Purpose:** To verify that the cloned promoter region has the **exact** sequence as the one reported in the *Arabidopsis* database.

**References:** Applied Biosystems
UCLA WebSeq website [http://genoseq.ucla.edu/action/view/Sequencing](http://genoseq.ucla.edu/action/view/Sequencing)

**Solutions Needed:**
- Applied Biosystems Big Dye version. 3 (Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- 5x Sequencing Buffer (Sigma, Cat. # S3938; also, obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- Plasmid DNA
- 20 µM M13 Forward primer
- 20 µM M13 Reverse primer
- Sterile water

**Materials Needed:**
- Pipettes
- Filter pipet tips
- 0.2 mL PCR tubes or strips of 8 tubes/strip
- PCR tube rack
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Microcentrifuge
- Vortex
- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- Sequencing reaction purification columns (Qiagen DyeEx 2.0 Spin Kit; obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)

**Overview:**
Generally, **10-µL** reactions are set up with the following components in **0.2 mL PCR** tubes for a **single DNA template** (see table below). **Note:** If you set up several sequencing reactions that share all, except one component, such as DNA template or primer, you should use the format of Master Mix (Mmix) solution to minimize the...
number of pipettings and mistakes of not adding some components into the individual reaction tubes.

Important: This is NOT a regular PCR reaction. Do NOT add TWO primers into a single reaction tube. Each reaction contains only ONE primer.

General Components of One Reaction:

<table>
<thead>
<tr>
<th></th>
<th>ONE Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template *</td>
<td>x µL</td>
</tr>
<tr>
<td>Sterile water</td>
<td>y µL</td>
</tr>
<tr>
<td>20 µM Sequencing primer</td>
<td>1 µL</td>
</tr>
<tr>
<td>Big Dye v. 3 Solution</td>
<td>1 µL</td>
</tr>
<tr>
<td>5x Sequencing buffer</td>
<td>2 µL</td>
</tr>
<tr>
<td>Total volume</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

x µL = the volume depends on concentration and amount of DNA (see Table below)

y µL = the remaining volume to bring the total volume to 10 µL

* Amount of DNA template depends on type of DNA:

- For plasmid DNA, use 800 ng. We found that 250 ng of plasmid DNA will work, but more DNA gives the better reads.
- For PCR product, use the amount of DNA according to the table on the next page (Taken from UCLA WebSeq website. Also, see Perkin-Elmer Big Dye Protocol).

Note: If the DNA concentration is too low, you may not be able to add the recommended amount of DNA. In this case, just add 6 µL.

For this exercise, there is ONE DNA template, i.e. the plasmid containing the promoter of the gene of interest; but, there are TWO primers, M13 forward and M13 reverse primers. Therefore, it is best to prepare a master mix with all components, except the primers, which will be added to individual reaction tubes.

What is the concentration of the plasmid DNA? _______ ng/µL
What is the amount of DNA to be used? ______ ng
What is the volume of plasmid DNA to be used? ______ µL

PROCEDURE

1. Get ice from the icemaker.
2. Label the sides of TWO 0.2 mL PCR tubes with your initials and primer name. Set the tube on a PCR tube rack sitting on ice.
3. Label the lid and side of a 1.5 mL microcentrifuge tube as “Mmix” and your initials. Set the tube on ice.
4. Prepare a master mix (Mmix) for 3 reactions (2 reactions + 1 extra) by pipetting the following components into the Mmix tube as shown in the table below. Note: Use the information on the previous page to fill in the volume of DNA solution to be added and calculate the volume of water to be added to the Mmix tube for 3 reactions.

<table>
<thead>
<tr>
<th>Components</th>
<th>Mmix for ONE reaction</th>
<th>Mmix for 3 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>250-500 ng of plasmid DNA</td>
<td>x µL</td>
<td>x (x 3) µL</td>
</tr>
<tr>
<td>Sterile water</td>
<td>y µL</td>
<td>y (x 3) µL</td>
</tr>
<tr>
<td>Big Dye v. 3</td>
<td>1 µL</td>
<td>3 µL</td>
</tr>
<tr>
<td>5x Sequencing buffer</td>
<td>2 µL</td>
<td>6 µL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>9 µL</td>
<td>27 µL</td>
</tr>
</tbody>
</table>

a. Mix the contents by flicking the tube five times or vortexing at the mixer setting of 2-3 for 5 seconds.
b. Spin the tube for 10 seconds to bring all the contents to the bottom of the tube.
c. Set the tube back on ice.
5. Pipet Mmix and M13 primer into TWO labeled 0.2 mL PCR tubes.
<table>
<thead>
<tr>
<th>Components</th>
<th>M13 Forward primer</th>
<th>M13 Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mmix</td>
<td>9 µL</td>
<td>9 µL</td>
</tr>
<tr>
<td>20 µM M13 Forward primer</td>
<td>1 µL</td>
<td>-</td>
</tr>
<tr>
<td>20 µM M13 Reverse primer</td>
<td>-</td>
<td>1 µL</td>
</tr>
<tr>
<td>Total volume</td>
<td>10 µL</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

6. Carry out cycling reaction using either **Applied Biosystems GeneAmp 9700**

**PROGRAM:** HC70AL BIG DYE

The profile of the Big Dye program is:
- 25 cycles of 96°C 10 sec
- 55°C 5 sec
- 60°C 4 min

or **Bio-Rad MyCycler** with a **Big Dye** protocol with the same profile as above.

7. After the cycling reaction is finished, clean up sequencing reactions using DyeEx 2.0 Spin Columns (stored in the refrigerator drawer) as following:
   a. Resuspend the resin by inversion or gently vortexing.
   b. Loosen the cap of the column a quarter turn. *This is necessary to avoid a vacuum inside the spin column.*
   c. Snap off the bottom closure of the spin column, and place the spin column in a 2 mL collection tube.
   d. Centrifuge at 3,000 rpm for 3 minutes at room temperature.
   e. Meanwhile, label a new set of 1.5 mL microcentrifuge tubes according to your reactions.
   f. Carefully transfer the spin columns to the new tubes.
   g. Slowly apply the sequencing reactions to the gel beds of the appropriate columns.

   **Note:**
   - Pipet the sequencing reaction directly onto the center of the slanted gel-bed surface. Do not allow the reaction mixture or the pipet tip to touch the
sides of the column. The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel-bed surface with the pipet tip.

- For easier handling, more reproducible pipetting, and reduced error with small sample volumes, you may adjust the volume of your sequencing reaction to 20 µL using distilled water, before application to the gel bed.

h. Spin the columns as in step d.
i. Remove the spin columns from the microcentrifuge tubes. The eluate contains the purified DNA.

8. Keep samples on ice or in the refrigerator. Take the purified sequencing reactions to the UCLA Sequencing Facility located on the 5th floor in the Gonda Building. Note: Use the primer name as the name of your sequence. Make sure to copy down the assigned file number (example, #5678), which is automatically given by the Facility, after you enter the samples into the Facility computer.

9. After one to two days, retrieve your sequences from the Sequencing Facility webpage.

Retrieving and Analyzing DNA Sequences

Purpose: To verify that the sequence corresponds to that of the gene of interest.

1. Log into WebSeq at http://www.genetics.ucla.edu/webseq/
   a. Enter Username: goldberg_r
   b. Enter Password: embryo
   c. Click “LOGIN.”

2. Find your sequence files by looking up the assigned file number and the name of the gene you are working on.
   Example: The assigned file number is 106203, and the gene of interest is At5g09250. You would see the following files:
   106203GoldR At5g09250Fw A12.ab1
106203GoldR At5g09250Rv B12_ab1

What are the annotations?

106203 = assigned file number; GoldR = user name; At5g09250Fw = name of sequence obtained with the Forward sequencing primer, A12 = capillary position used in loading sequencing sample in the Sequencer (Biosystems 3730 Capillary DNA Analyzer), ab1 = ABI file format.

3. Check the boxes next to the sequences to be downloaded, and click “Download selected.” Alternatively, click on each filename that you want to download.

4. Open the ab1 files in the “Downloads” window using a sequence viewer program (CHROMAS on Windows, or 4PEAKS on Mac).

5. Copy DNA sequences to a Microsoft Word file. Note: Name the files according to the name of gene of interest (for example, Promoter At5g09250).

6. Process the DNA sequences by “BLASTN” search. See Appendix 2. Note: Blast search may take a few minutes or longer to complete depending on how busy the NCBI server in Washington D.C. is.

7. Determine if the DNA sequence corresponds to the upstream sequence of the gene of interest.

8. View the sequence alignment to the upstream sequence of the gene of interest.

Are there any mutations in the promoter that you cloned?

Do you think a mutation could affect the transcription of your reporter gene?

9. Print out the Blast result as a hard-copy record for your lab notebook.

10. Save the Blast result in the pdf format so that you can upload them to your webbook.