EXPERIMENT 4 – CLONING THE PROMOTER REGION OF THE GENE OF INTEREST

Purposes:
1. (Long term) 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 Green Fluorescent Protein (GFP) gene fused to the promoter.
2. (Short term) To clone the promoter region of the gene of interest into a plasmid vector.

Reference:
1. iProof High-Fidelity DNA Polymerase Technical Note (Bio-Rad) (see Appendix 1D)
2. pCR-Blunt II-TOPO Cloning Instruction Manual (Invitrogen) (see Appendix 1E)
3. QIAprep Miniprep Handbook (see Appendix 1F)

STRATEGY

I. AMPLIFICATION OF THE PROMOTER REGION OF THE GENE-OF-INTEREST USING HIGH FIDELITY (HIFI) DNA POLYMERASE
II. CLONING THE AMPLIFIED PROMOTER REGION INTO A PLASMID VECTOR
   A. LIGATING THE PCR PRODUCT AND A pCR-BLUNT II-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
STRATEGY OF PROMOTER ACTIVITY ANALYSIS

Cloning of the Promoter Region of the Gene of interest (Gene One)
Materials Needed:

High quality *Arabidopsis* genomic DNA (12 ng/µL)
PCR product of the promoter region tested with Ex-Taq DNA Polymerase
Filter tips for PCR
iProof High Fidelity DNA polymerase kit (Cat.# 172-5301, Bio-Rad)
Sterile water
dNTP mix (2.5 mM each dNTP, from Ex-Taq DNA polymerase package, Takara)
12 µM Promoter Gene-specific Forward primer
12 µM Promoter Gene-specific Reverse primer
Pipetman sets of P-10, P-20, P-200
PCR tubes or strip of tubes
1.5-mL Microcentrifuge tubes
Microcentrifuge-tube rack
Ice bucket
pCR-Blunt-II TOPO Cloning kit (Cat.# K2800-20, Invitrogen)
One Shot Competent cells (Included in the TOPO Cloning kit)
S.O.C. medium
Sterile toothpicks
Terrific Broth (TB) broth medium
50 mg/mL Kanamycin
LB + Kanamycin plates containing x-gal and IPTG
37°C Air incubator
Orbital shaker in the 37°C air incubator
42°C Water bath
Bacterial cell spreader
A glass jar containing 95% ethanol solution
Regular Pipet tips for P-10, P-20, P-200, P-1000
QIAGen Plasmid Miniprep kit
Nanodrop spectrophotometer
TE buffer
PROCEDURE

I. AMPLIFICATION OF THE PROMOTER REGION OF THE GENE OF INTEREST

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

1. Get ice from an icemaker in room 2911 or 3906.
2. Thaw out tubes of 5x iProof HF Buffer with 7.5 mM MgCl₂ and dNTP mix (2.5 mM each dNTP) on a microcentrifuge rack for 1.5-mL microcentrifuge tubes at room temperature for few minutes. Once the solutions are thawed out → vortex for 5 seconds to mix the contents → spin tubes for 10 seconds → put the tubes on ice until needed.
3. Thaw out Promoter Forward and Reverse primer solutions corresponding to a gene of interest as in step 2.
4. Obtain THREE 0.2-mL sterile PCR tubes and set them on a PCR rack.
5. Label Name and Date on the lids and sides of the tubes as follows: (your TA will show you how to write on the tubes)
   Tube #1: "Name of a gene" (same name as primer's gene)
   Tube #2: Pos. (Positive control for the gene of interest = PCR product using Ex-Taq DNA polymerase)
   Tube #3: Neg. (Negative control for the gene of interest containing same components as in tube #1, but NO genomic DNA)
6. Obtain ONE 1.5-mL microcentrifuge tube and set them on a microcentrifuge-tube rack.
7. Write on the lid of the tube with black ultra-fine sharpie as "Mmix" (for Master mix). Keep the tube on ice.
8. Prepare the Master Mix (Mmix) solution for 4 reactions (3 samples + 1 extra) (see table below)

*Caution:* Keep tube on ice at all the time.

*Note:* Amplification of targets greater than 3 kbp may require more DNA polymerase, but NOT to 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>28.5 µL</td>
<td>114.0 µL</td>
<td></td>
</tr>
<tr>
<td>5x iProof HF Buffer, 7.5 mM MgCl₂</td>
<td>10.0 µL</td>
<td>40.0 µL</td>
<td>1x</td>
</tr>
<tr>
<td>dNTP mix (2.5 mM each dNTP)</td>
<td>1.0 µL</td>
<td>4.0 µL</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>12 µM Gene-specific Forward primer</td>
<td>1.0 µL</td>
<td>4.0 µL</td>
<td>0.24 µM</td>
</tr>
<tr>
<td>12 µM Gene-specific Reverse primer</td>
<td>1.0 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>2.0 µL</td>
<td>0.2 Unit</td>
</tr>
<tr>
<td>Total volume</td>
<td>42.0 µL</td>
<td>168.0 µL</td>
<td></td>
</tr>
</tbody>
</table>

a. Pipet into the tube the reagents with order from top down (*example:* water, 5x Buffer, dNTP mix, etc.).

b. After pipetting all reagents into the master mix tube, close its lid. Mix the contents by vortexing at **setting of slow speed** for **2 seconds. Caution:** Do NOT vortex the mixture with the enzyme, such as DNA polymerase, **vigorously** as well as for **> 5 seconds** because these two factors will **break down** enzyme, resulting **LOW** or **NO yield** of PCR product. Spin the tube in a microcentrifuge at full speed (13,200 rpm) for **10 seconds**. Put the tube back on ice.
9. Prepare PCR-reaction tubes by
   a. pipetting 42 µL of the Mmix solution into PCR tubes (see table below)
   b. pipetting 8 µL of genomic DNA or water to tubes #1-3.
   c. Immediately, mix the contents by pipetting up and down five 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 <em>Arabidopsis</em> genomic DNA (12 ng/µL)</td>
<td>8 µL</td>
<td>0 µL</td>
<td>0 µL</td>
</tr>
<tr>
<td>Positive control DNA template (PCR product using Ex-Taq DNA polymerase)</td>
<td>0 µL</td>
<td>1 µL</td>
<td>0 µL</td>
</tr>
<tr>
<td>Sterile water</td>
<td>0 µL</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>

10. Perform PCR amplification as follows:
   a. Turn on the PCR machine (MyCycler) by pressing the "Standby" button once.
   b. Put the PCR tubes in the wells of the 96-well hot plate of the MyCycler.
   c. Select the "Protocol Library" by pressing "F1" button.
   d. Select "iProof" protocol by pressing yellow arrowheads surrounding the "ENTER" button. Once it is selected, the "iProof" protocol is highlighted. Press the "ENTER" button.

   The **PCR profile for Genomic DNA templates.**

<table>
<thead>
<tr>
<th>Cycling parameters</th>
<th>Up to 3 kbp of PCR product</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation Enzyme step</td>
<td>98°C for 30 seconds</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation step</td>
<td>98°C for 10 seconds</td>
<td></td>
</tr>
<tr>
<td>Annealling step</td>
<td>63°C (or $T_m$+3°C) for 30 seconds</td>
<td>30</td>
</tr>
<tr>
<td>Extension step</td>
<td>72°C for 75 seconds (or 15-30 seconds/kb)</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C for 5 minutes</td>
<td>1</td>
</tr>
</tbody>
</table>
e. Under the "Choose Operation" window, "Run Protocol" is highlighted. Press the "ENTER" button to run the protocol.

f. Press "F5" button to "Begin Run" the protocol.

11. Analyze 10 µL of the reaction products on a 1% agarose gel containing 0.5 µg/mL ethidium bromide and visualize the DNA bands under UV illumination.
II. CLONING THE AMPLIFIED PROMOTER REGION INTO A PLASMID VECTOR

A. LIGATING THE PCR PRODUCT AND A pCR-BLUNT II-TOPO VECTOR

1. Thaw on ice a tube of TOPO vector.

2. Label a 1.5-mL microcentrifuge tube “pCR-Blunt + Gene Name” and Date.
   Place the labeled tube on ice.

3. a. Pipet the following reagents into the labeled tube as listed in the Table below:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly prepared PCR product</td>
<td>2.0 µL</td>
</tr>
<tr>
<td>Salt Solution</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>Sterile water</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>pCR-Blunt II – TOPO</td>
<td>0.5 µL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>6.0 µL</strong></td>
</tr>
</tbody>
</table>

b. Mix reaction GENTLY by flicking the tube. Do NOT vortex the tube!
c. Incubate the reaction for **5 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), increase the reaction time will yield more colonies (Taken from TOPO Cloning Manual, Invitrogen).

4. 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 CELLS WITH THE LIGATION MIXTURE

1. Thaw on ice ONE vial of One Shot E. coli competent cells for transformation for a few minutes.
2. Pipet 2 µL of the TOPO ligation mixture into the vial of One Shot E. coli competent cells. Attention: Do NOT pipet the mixture up and down.
3. Incubate the cell mixture on ice for 10-20 minutes.
4. Heat-shock the cells for 30 seconds in the 42°C waterbath without shaking.
5. Immediately, transfer the tube back on ice. Leave it on ice for 2 minutes.
6. Transfer the tube to a rack for microcentrifuge tubes at room temperature.
7. In the bacterial hood, pipet 250 µL of room temperature S.O.C medium to the cell mixture. Cap the tube tightly.
8. Incubate the tube at 100-150 rpm shaking on an orbital shaker in a 37°C incubator for 45-60 minutes.
9. Meanwhile, label on the bottom of TWO prewarmed (37°C) selective (Kanamycin) plates supplemented with x-gal and IPTG with the following information:
   pCR II + “gene promoter”,
   Date
   10 µL or 50 µL
   Your Initial
10. Spread 10-50 µL of transformation mixture on TWO prewarmed (37°C) selective (Kanamycin) plates.
    Plate #1: 10 µL of transformation mixture + 20 µL of S.O.C medium (for even spreading of a small volume)
    Plate #2: 50 µL of transformation mixture
11. Incubate the plates in the 37°C incubator overnight (14-16 hours).
12. **Next day**, count the number of **WHITE** and **BLUE** colonies. Seal the plates with pieces of parafilm and then store them at 4°C (cold room or fridge) until inoculation step.
C. SCREENING FOR E. COLI CELLS HARBORING THE RECOMBINANT PLASMID AND ISOLATING PLASMID DNA

INOCULATION OF MEDIUM WITH BACTERIAL COLONIES

1. Put SIX sterile glass tubes on a test tube rack.
2. Label on the side of each tube with your Initial and number (1-6).
3. Pipet ~1.5 mL of Terrific Broth (TB) medium containing 50 μg/mL Kanamycin into each of 6 tubes.
4. Inoculate the TB medium + Kanamycin with individual WHITE colonies by using a sterile toothpick to pick a single WHITE colony on the plate and drop that toothpick into the tube #1.
5. Repeat step 4 for FIVE other tubes.
6. Incubate the tubes at 37°C overnight by
   a. transferring all 6 tubes to a wired rack on an orbital shaker in the 37°C incubator.
   b. turning the SPEED dial (LEFT dial) to number 2 for 200 rpm shaking.
   c. turning the TIME dial (RIGHT dial) clockwise to a CONSTANT position.
7. Close the incubator door.

Next day, Inspect the growth of cells (appearing very cloudy) in culture tubes. If plasmid DNA is not isolated immediately, place culture tubes in the cold room.
**ISOLATING PLASMID DNA**

1. Label on the lids of **1.5-mL microcentrifuge tubes** with your **Initial** and the number (1-6). Set labeled tubes on the microcentrifuge rack.

2. Arrange the culture tubes and labeled microcentrifuge tubes in their corresponding order. For example, 1 to 1, 2 to 2, ..., 6 to 6.

3. **Carefully**, pour the liquid culture into the microcentrifuge tube. Close the lids of the tubes. *Note:* if the culture tubes sit in the fridge or coldroom for more than **ONE** hour, vortex the tubes for 5-10 seconds to mix the content before transfer it to the microcentrifuge tube.

4. Spin tubes in a microcentrifuge at **FULL** speed for **2 minutes**.

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

6. Place the tubes back on the microcentrifuge rack.

7. Pipet **250 µL** of **Buffer P1 (Resuspension buffer + RNase A)** to each tube. Close the lid **tightly**.

8. Resuspend pelleted bacterial cells by either raking the tube on a microcentrifuge rack for 10 times or vortexing the tube for a few minutes until NO cell lumps are observed.

9. Place the tube back on the microcentrifuge rack.

10. Add **250 µL** of **Buffer P2 (Lysis buffer)** to each tube. Close the lid.

11. Invert tubes for **10 times** or **until** the mixture is clear. This step is for breaking open bacterial cells to release their contents (chromosomal DNA, plasmid DNA, proteins, carbohydrates) into the solution. *Note:* Do **NOT** vortex the contents to prevent shearing bacterial chromosome DNA into many tiny pieces that have the same size as the plasmid DNA.

12. Add **350 µL** of **Buffer N3 (Neutralization buffer)** to each tube. Close the lid.

   Immediately, invert the tube to mix the solution as in **step 11**. The solution appears cloudy. *Note:* Do **NOT** vortex the mixture!

13. Repeat step 12 for other tubes (one by one).

14. Spin tubes in the microcentrifuge at **FULL** speed for **10 minutes**.
15. Meanwhile, label on the **SIDE** of the QIApred columns (**Light blue**) with your 
   **Initial** and the **number**. Set these columns in their collection tubes on the 
   microcentrifuge rack.

16. Also, label on the lids and side of a new set of tubes with the following information: 
   pCR-“gene name”; **Number**, your initial, date. (Tubes will be used at step 27).

17. After 10 minutes of spinning, transfer the **supernatant** from step 14 to the QIApred 
   column by pipetting. **Caution:** Make sure that the **numbers** on the **lids** of tubes and 
   the **SIDE** of QIApred columns are corresponding.

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

19. Lift the column off the collection tube and discard the flow-through liquid into a glass 
   BEAKER.

20. Put the column back in its collection tube.

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

22. Lift the column off the collection tube and discard the flow-through liquid into a glass 
   BEAKER.

23. 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 off residual salt and proteins 
   from the membrane on the column. **Note:** Make sure that ethanol is added to the **PE 
   buffer** before use.

24. Lift the column off the collection tube and discard the flow-through liquid into a glass 
   BEAKER.

25. Spin the columns at **FULL** speed for **1 minute** to remove residual wash buffer. **Note:** 
   if the residual wash buffer is NOT completely removed, DNA solution will float up 
   when the sample is loaded into the well of the agarose gel due to the presence of 
   ethanol in the DNA solution. Also, ethanol will inhibit enzyme activity in later steps.

26. Transfer the QIApred columns in **NEWLY labeled tubes (prepared in step 16)**. 
   Discard the **collection tubes** and ethanol residue. **Note:** make sure the numbers on 
   the columns and microcentrifuge tubes matched.
27. Pipet 50 µL of **Buffer EB (10 mM Tris-HCl, pH 8.5)** to the center of each QIAprep column.

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

29. Spin the tubes with columns at **FULL** speed for **1 minute**. Steps 28 and 29 are for eluting plasmid DNA off the column.

30. After spinning, discard the columns. **Save the eluted plasmid DNA** in the microcentrifuge tubes.

31. Determine **DNA concentration** and its **purity** using the Nanodrop UV spectrophotometer. Record DNA concentration,
D. CONFIRMING THE AUTHENTICITY OF RECOMBINANT PLASMID DNA VIA RESTRICTION ENZYME DIGESTION

**Purpose:** To ensure that plasmid DNA isolated from WHITE colonies is recombinant plasmid DNA, i.e. containing the cloned promoter region, not NON-recombinant DNA (i.e. the vector alone).

**Reagents and Materials Needed:**
1.5-mL Microcentrifuge tubes  
Microcentrifuge-tube rack  
Sterile water  
EcoRI restriction enzyme (Invitrogen, 10 units/µL)  
React buffer #3 (came with EcoRI)  
37°C water bath  
Agarose  
Gel Apparatus  
Gel Document system
PROCEDURE

1. Digest 300 - 500 ng of plasmid DNA with restriction enzyme EcoRI at 37°C for ONE hour.

(Why EcoRI? Check the presence of EcoRI sites in the Multiple Cloning Site of the pCRII-Blunt TOPO vector diagram in the previous page). 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.

a. Standard set up of a 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 React buffer #2 (Invitrogen)</td>
<td>2.0 µL</td>
<td>1x</td>
<td>2.0 µL</td>
</tr>
<tr>
<td>EcoRI (10 U/µL, Invitrogen)</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 µL** = 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 React buffer** is 1/10th the total volume of the reaction so that the final concentration of the React 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 the DNA completely in ONE hour under the conditions specified for that enzyme (most enzymes have optimal temperature at 37°C). To ensure that DNA is completely
Cloning of the Promoter Region of the Gene of interest (Gene One)

4.18

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 for bringing up the total volume.

**Note**: *: **Different restriction enzymes** require **Different 10x React buffers**. Therefore, **Check the enzyme to be used** and find the **appropriate buffer** for that enzyme.

b. Digestion set up for **at least TWO DNA samples**

**Tip**: When set up reactions for more than 2 DNA samples that are digested with the same restriction enzyme(s), it is highly recommended to work with a **Master mix**. Usually, the volume of DNA varies from sample to sample. To minimize errors, it is recommended to work with **2x Enzyme mix** as exemplified below. **Note**: the volume of DNA + sterile water = the volume of 2x Enzyme mix!

<table>
<thead>
<tr>
<th>Components</th>
<th>Sample A (300 ng/µL)</th>
<th>Sample B (200 ng/µL)</th>
<th>Sample C (100 ng/µL)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 ng of DNA</td>
<td>1.7 µL</td>
<td>2.5 µL</td>
<td>5.0 µL</td>
<td>25 µL/µL</td>
</tr>
<tr>
<td>Sterile water</td>
<td>8.3 µL</td>
<td>7.5 µL</td>
<td>5.0 µL</td>
<td>---</td>
</tr>
<tr>
<td>2x Enzyme mix</td>
<td>10.0 µL</td>
<td>10.0 µL</td>
<td>10.0 µL</td>
<td>1x</td>
</tr>
<tr>
<td>Total volume</td>
<td>20.0 µL</td>
<td>20.0 µL</td>
<td>20.0 µL</td>
<td></td>
</tr>
</tbody>
</table>
c. Label on the lid of a microcentrifuge tube as “2x Enz Mix” and Prepare a 2x Enzyme Mix for the number of plasmid DNA + 1 Extra reaction to be analyzed. Keep the Enzyme Mix tube on ice. Mix the contents by flicking the tube gently.

*How many DNA samples will be digested?*

<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 React buffer #3</td>
<td>2.0 μL</td>
<td>______ μL</td>
</tr>
<tr>
<td>Sterile water</td>
<td>7.5 μL</td>
<td>______ μL</td>
</tr>
<tr>
<td>EcoRI (10 U/μL)</td>
<td>0.5 μL</td>
<td>______ μL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>10.0 μL</td>
<td>______ μL</td>
</tr>
</tbody>
</table>

d. Label on the lids of microcentrifuge tubes sample number, EcoRI, and your initial. Keep tubes on ice. Set up restriction digestion reactions for a number of plasmid DNA to be analyzed by pipetting the following components into the tubes.

<table>
<thead>
<tr>
<th>Components</th>
<th>Sample 1 (ng/μL)</th>
<th>Sample 2 (ng/μL)</th>
<th>Sample 3 (ng/μL)</th>
<th>Sample 4 (ng/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 ng of 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>2x Enzyme mix</td>
<td>10.0 μL</td>
<td>10.0 μL</td>
<td>10.0 μL</td>
<td>10.0 μL</td>
</tr>
<tr>
<td>Total volume</td>
<td>20.0 μL</td>
<td>20.0 μL</td>
<td>20.0 μL</td>
<td>20.0 μL</td>
</tr>
</tbody>
</table>

c. Mix the contents by flicking the tubes several times. Spin the tubes in the microfuge for 10 seconds to bring down liquid to the bottom of the tubes.

d. Incubate the reactions in the 37°C water bath for about 1 hour.
2. Meantime, prepare a 1% agarose gel in 1x TAE buffer with a 20-tooth comb as usual.
3. At the end of incubation, spin tubes briefly for 10 seconds.
4. Add 3 µL of 6x Loading dye to each restriction-digested DNA sample. Mix the contents by pipetting the mixture or flicking the tube.
5. Load 20 µL of restriction-digested DNA samples on the agarose gel. Also, load 10 µL of 1-kb DNA ladder solution next to the DNA samples. Record the loading pattern.

<p>| | | | | | | | | | | |</p>
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>11</td>
</tr>
</tbody>
</table>

6. Run the gel at ~105 volts for 1-2 hours.
   Starting time:
   Ending time:
7. Take a picture of the gel and paste it below.
8. 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 or clone will be used for sequencing analysis?
F. 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.

**SEQUENCING REACTION WITH BIG DYE V. 3**

**Reference:** Perkin Elmer/Applied Biosystems

**Solutions Needed:**

- Applied Biosystems Big Dye version. 3 (Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- Dye Dilution Mix (Sigma, Cat. # S3938; also, obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- 3 μM T7 primer
- 3 μM SP6 primer
- Sterile water

**Materials Needed:**

- Applied Biosystems GeneAmp 9700 or BioRad MyCycler
- 0.2 mL PCR tubes or Strips of 8 tubes/strip
- PCR Rack
- Aerosol-barrier (or PCR) Pipet Tips
- Sequencing Reaction Purification Columns (Edge Biosystem) (can be bought directly from Edge Biosystem or Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
Overview:
Generally, 20-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 number of pipettings and mistakes of not adding some components into the individual reaction tubes resulting in negative.

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:

| DNA template * | x µL |
| Sterile water | y µL |
| 3 µM Sequencing primer | 1 µL |
| Big Dye v. 3 Solution | 2 µL |
| Dye Dilution Mix (Sigma, S3938) | 2 µL |
| **Total volume** | **20 µL** |

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 20 µL

* Amount of DNA template depends on type of DNA:
  
  For plasmid DNA, use **250-500 ng**. We found that 500 ng of plasmid DNA gives the best read.

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

**PROCEDURE**

1. Get ice from the icemaker in room 2911 or 3906.
2. Label on the **side** of **TWO 0.2-mL PCR tubes** with your **initial** and **primer name**. Set the tube on a PCR rack sitting on ice.

3. Label on the **lid** and **side** of a **1.5-mL microcentrifuge tube** as “**Mmix**” and your **initial**. 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 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.*

### Master Mixes (Mmix) of Sequencing 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 DNA template</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>2.0 μL</td>
<td>6.0 μL</td>
</tr>
<tr>
<td>Dye Dilution Mix (Sigma, S3938)</td>
<td>2.0 μL</td>
<td>6.0 μL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>19.0 μL</td>
<td>57.0 μL</td>
</tr>
</tbody>
</table>

- Mix the content by flicking the tube five times or vortexing at the mixer setting of 2-3 for **5 seconds**.
- Spin the tube for **10 seconds** to bring all the contents to the bottom of the tube.
- Set the tube back on ice.
5. Pipet **Mmix** and **gene-specific primer** into TWO labeled 0.2-mL PCR tubes.

<table>
<thead>
<tr>
<th>Components</th>
<th>T7 primer</th>
<th>SP6 primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mmix</td>
<td>19 µL</td>
<td>19 µL</td>
</tr>
<tr>
<td>3 µM T7 primer</td>
<td>1 µL</td>
<td>1 µL</td>
</tr>
<tr>
<td>3 µM SP6 primer</td>
<td>1 µL</td>
<td>20 µL</td>
</tr>
</tbody>
</table>

6. Carry out cycling reaction using either **Applied Biosystems GeneAmp 9700 USER: <<pe>> PROGRAM: Big Dye**
   The profile of the Big Dye program as:
   25 cycles of 96°C, 10 sec. -> 50°C, 5 sec. -> 60°C, 4 min. Followed by 4°C, ∞

   or **BioRad MyCycler** with a **Big Dye** protocol with the same profile as above.

7. After the cycling reaction is finished, clean up sequencing reactions using Edge Biosystems spin columns (stored in the cold room) as following:
   a. Spin the pre-packed columns in a microcentrifuge at 3,000 rpm for 2 minutes at room temperature.
   b. Meanwhile, label a new set of 1.5 mL microcentrifuge tubes according to your reactions.
   c. Transfer the columns to new tubes.
   d. Pipet 20 µL of sequencing reaction to appropriate columns.
   e. Spin the columns as in step a.
   f. Discard the columns.

8. Take the purified sequencing reaction to UCLA Sequencing Facility located on the 5th floor in Gonda Building. **Note: Make sure to copy down the assigned file number** (example, # 5678); that 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 web page.

**RETRIEVING AND ANALYZING DNA SEQUENCES**
**Purpose:** To verify that the sequence corresponds to that of the promoter region of the gene of interest.

1. From any computers in the lab, Log in to the UCLA Sequencing Retrieval System via [http://www.genetics.ucla.edu/webseq/](http://www.genetics.ucla.edu/webseq/).
2. Enter in the USER NAME field: `goldberg_r`
3. Enter in the PASSWORD field: `embryo`
4. 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 **5677**, and the gene of interest is **At5g09250**. You would see the following files:
   
   ```
   5677 GOLDR_At5g09250Fw_080.ab1
   5677 GOLDR_At5g09250Rv_081.ab1
   ```
   
   What are the annotations?

   **5677** = assigned file number; **GOLDR** = user name; **At5g09250Fw** = sequence name obtained with the Forward sequencing primer, **080** = capillary position used in loading sequencing sample in the Sequencer ABI 7700 (Perkin-Elmer/Applied Biosystems); **abi** = ABI file format. Select "PROCESS INDIVIDUAL SEQUENCES" instead of "PROCESS COMPLETE SET OF 96 SEQUENCES".
5. Select sequences to be downloaded, and click "DOWNLOAD SELECTED" or click on "SEQUENCE FILE TO DOWNLOAD".
6. Select "SAVE TO DISK" and choose "THE DESKTOP".
7. Open the saved file using a SEQUENCE VIEWER PROGRAM (CHROMAS on Windows or EDITVIEW on Mac).
8. Copy DNA sequences to a Microsoft Word file. Note: Name the files according to the name of gene of interest (for example, At5g09250).
9. Process the DNA sequences by "BLASTN" and "BLASTX" searches, respectively. **Note:** Blast search may take a few minutes or longer to complete depending on how busy is the NCBI server in Washington D.C (i.e. how many sequences have been processed by the NCBI server at the fraction of time).

10. Determine if the DNA sequence corresponds to the gene of interest.

11. Print out the Blast results as hard-copy records.

12. Save the Blast results in the **pdf** format so that you can upload them in your webbook.