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

<u>Purpose:</u> 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.

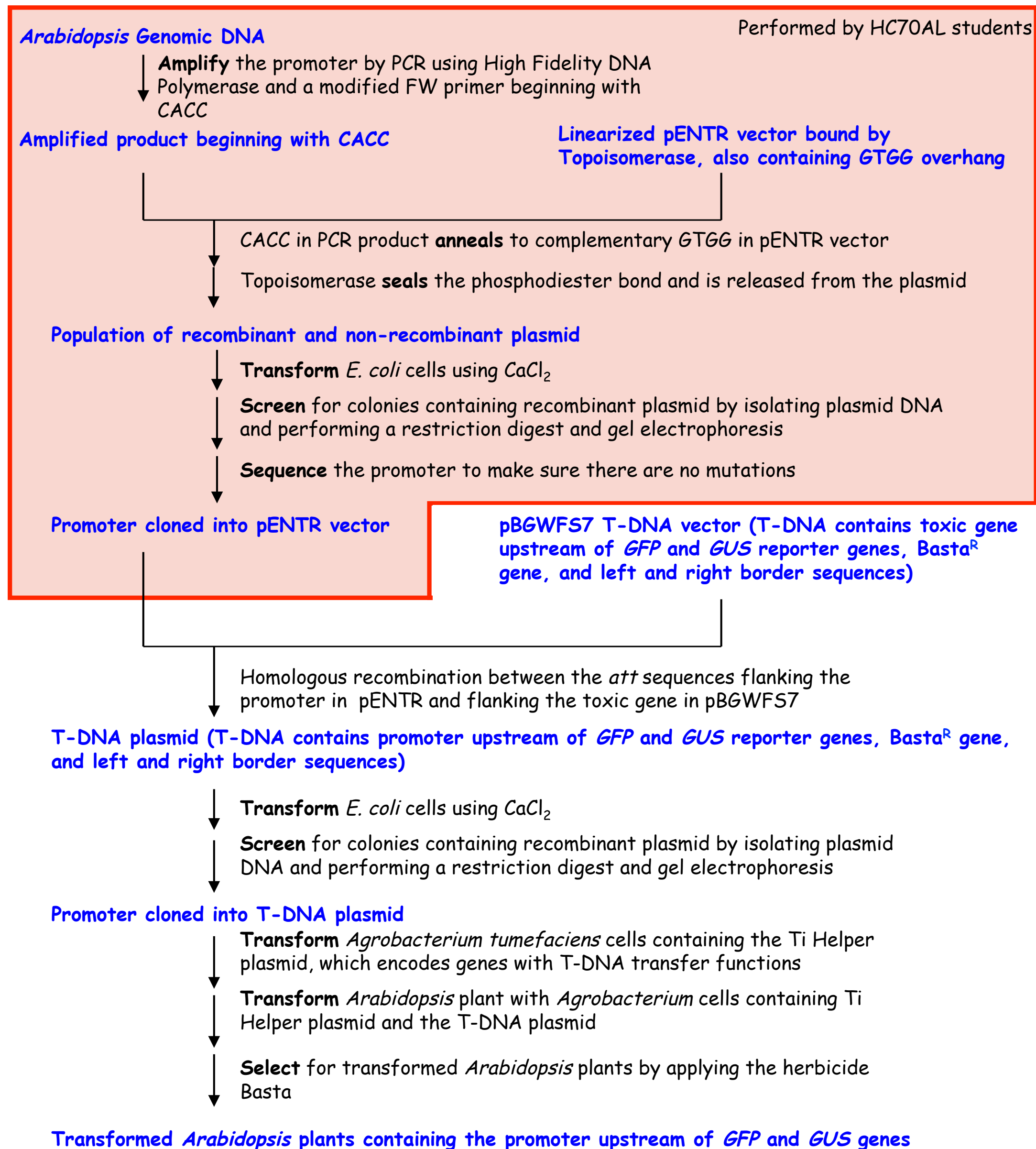
<u>References:</u>

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**

STRATEGY FOR DETERMINING THE ACTIVITY OF A GENE UPSTREAM REGION



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

<u>*Note:*</u> *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)

<u>Caution</u>: *Keep tube on ice at all times.*

<u>Note:</u> *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

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

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:

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

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**

Cycling parameters		**Number of Cycles**
Activation Enzyme step	98°C for 30 seconds	**1**
Denaturation step	98°C for 10 seconds	**30**
Annealing step	63°C (or T_m+3°C) for 20 seconds	
Extension step	72°C for 2 minutes (or 15-30 seconds/kb)	
Final Extension	72°C for 5 minutes	**1**

Hold	4°C for ∞	**1**

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.

Lane	Tube #	Sample	Expected Size (bp)
1	-	1 Kb Plus DNA Ladder	-
2	1	Genomic DNA	
3	2	PCR product amplified by TAs	
4	3	Sterile water	-

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.

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

    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**.

	Vial #1	Vial #2	Vial #3
Sample Name	**pENTR + Promoter**	**pENTR Only**	**pUC19**

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.*

	Vial #1	Vial #2	Vial #3
pENTR + Promoter	**2 μL**	-	-
pENTR Only	-	**2 μL**	-
pUC19	-	-	**1 μL**

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:

Plate #	Sample	Date	Initials	Volume of Cells
Kanamycin 1	pENTR + Promoter			10 µL
Kanamycin 2	pENTR + Promoter			50 µL
Kanamycin 3	pENTR Only			10 µL
Kanamycin 4	pENTR Only			50 µL
Ampicillin 1	pUC19			10 µL
Ampicillin 2	pUC19			50 µL

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

- Terrific broth (TB) medium containing 50 µg/mL Kanamycin

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.

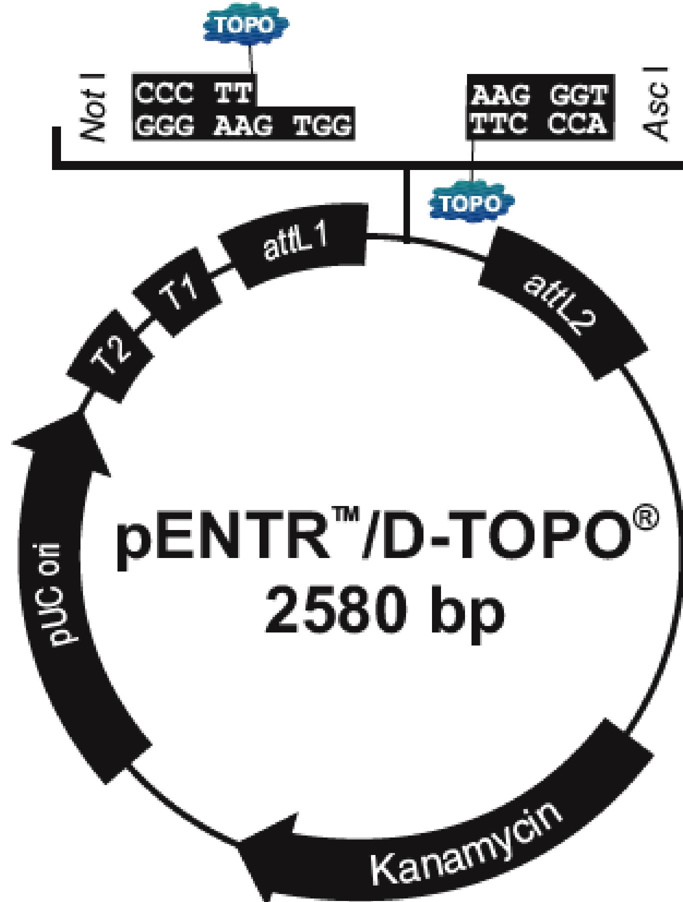
7. Pipet **another 1 mL** of liquid culture into the appropriate microcentrifuge tubes. Close the lids of the tubes.
8. Spin the tubes in a microcentrifuge at **FULL** speed for **30 seconds**.
9. Pour off the supernatant into a glass Erlenmeyer flask labeled "CULTURE WASTE." Dab off the extra liquid 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. <u>Caution:</u> *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. <u>Note:</u> *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. <u>Caution:</u> *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.* <u>Note:</u> *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
- *AscI* restriction enzyme (New England Biolabs, 10 units/μL)
- 10x NEB Buffer 4 (supplied with *AscI*)
- 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 ***AscI*** at **37°C** for **60 minutes**.

   Why AscI? *Check the presence of the* ***AscI*** *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

Components	**Standard Reaction**	**Final Concentration**	**Example** **500 ng DNA (200 ng/µL)**
DNA	**x** µL	-----	**2.5** µL
Sterile water	**y** µL	---	**15.0** µL
10x NEB buffer 4	**2.0 µL**	**1x**	**2.0** µL
AscI (10 U/µL, NEB)	**0.5 µL**	**0.25 U/µL**	**0.5** µL
Total Volume	**20.0 µL**	---	**20.0** µL

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 NEB buffer** is **1/10th** 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

Components	**Enz Mix** for **1 Reaction**	**Enz Mix** for ____**Reactions**
10x NEB Buffer 4	2.0 µL	____ µL
***AscI* (10 U/µL)**	0.5 µL	____ µL
Total Volume	**2.5 µL**	____ **µL**

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.

	Sample 1 ______**ng/µL**	**Sample 2** ______**ng/µL**	**Sample 3** ______**ng/µL**	**Sample 4** ______**ng/µL**
300 - 500 ng plasmid DNA	________ µL	________ µL	________ µL	________ µL
Sterile water	________ µL	________ µL	________ µL	________ µL
Enzyme Mix	2.5 µL	2.5 µL	2.5 µL	2.5 µL
Total volume	**20 µL**	**20 µL**	**20 µL**	**20 µL**

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.

Lane	Sample	Expected Sizes (bp)
1	100 bp DNA Ladder	-
2	Plasmid #1 x AscI	
3	Plasmid #2 x AscI	
4	Plasmid #3 x AscI	
5	Plasmid #4 x AscI	
6	1 Kb Plus DNA Ladder	-
7	Plasmid #1 Uncut	-
8	Plasmid #1 Uncut	-
9	Plasmid #1 Uncut	-
10	Plasmid #1 Uncut	-

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

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:

	ONE Reaction
DNA template *	**x** µL
Sterile water	**y** µL
20 µM Sequencing primer	1 µL
Big Dye v. 3 Solution	1 µL
5x Sequencing buffer	2 µL
Total volume	**10 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 **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.*

Master Mix (Mmix) for Sequencing Reactions:

Components	**Mmix** for **ONE reaction**	**Mmix** for **3 reactions**
250-500 ng of plasmid DNA	**x** μL	**x** (x 3) μL
Sterile water	**y** μL	**y** (x 3) μL
Big Dye v. 3	**1** μL	**3** μL
5x Sequencing buffer	**2** μL	**6** μL
Total Volume	**9 μL**	**27 μL**

   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.

Components	M13 Forward primer	M13 Reverse primer
Mmix	9 μL	9 μL
20 μM **M13 Forward** primer	1 μL	-
20 μM **M13 Reverse** primer	-	1 μL
Total volume	**10 μL**	**10 μL**

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

   USER: <<hc-lab>>
   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
   4°C ∞

   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.