EXPERIMENT 5 – CLONING THE UPSTREAM REGION OF THE GENE OF INTEREST

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.

Reference:

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

STRATEGY

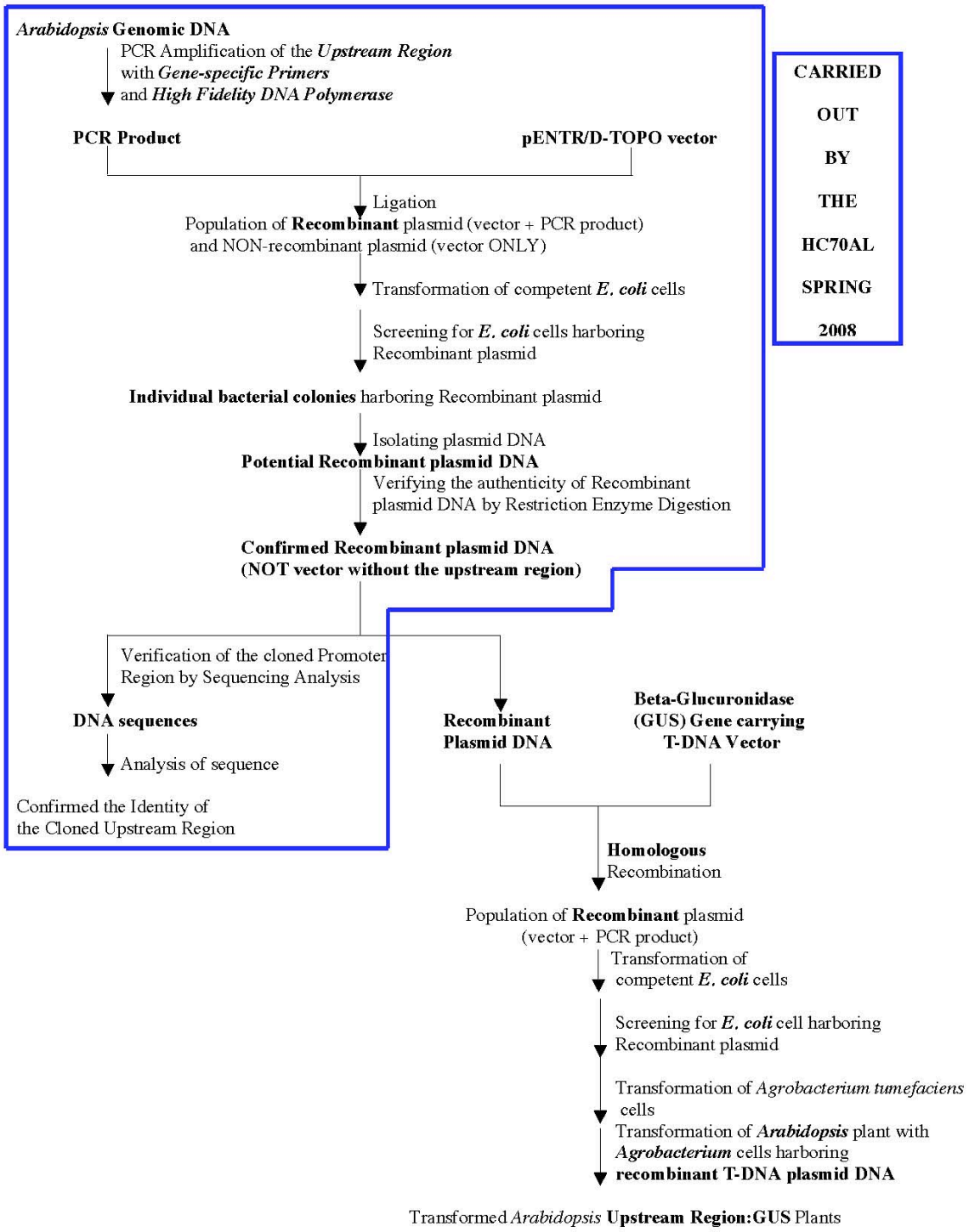
I. AMPLIFICATION OF THE UPSTREAM REGION OF THE GENE OF INTEREST USING HIGH FIDELITY (HIFI) DNA POLYMERASE

II. CLONING THE AMPLIFIED PROMOTER REGION INTO A PLASMID VECTOR

- **A. LIGATING THE PCR PRODUCT AND A 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**

III. STRATEGY OF PROMOTER ACTIVITY ANALYSIS

STRATEGY FOR DETERMINING THE ACTIVITY OF A GENE'S UPSTREAM REGION



Materials Needed:

High quality *Arabidopsis* genomic DNA (12 ng/μL)
PCR product of the promoter region tested with iProof 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
pENTR/D-TOPO Cloning kit (Cat.# K2400-20, Invitrogen)
One Shot TOP10 Competent cells (Included in the TOPO Cloning kit)
pUC19 plasmid (10 pg/μL) (Included in the TOPO Cloning kit)
S.O.C. medium
Sterile toothpicks
Terrific Broth (TB) medium
50 mg/mL Kanamycin
LB + Kanamycin plates
LB + Ampicillin plates for pUC19 plasmid (control for transformation efficiency)
37^{o}C Air incubator
Orbital shaker in the 37^{o}C air incubator
42^{o}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 UPSTREAM 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 $MgCl_2$** 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

	Mmix for 1 Reaction	**Mmix for 4 Reactions**	**Final Concentration**
Sterile water	28.5 µL	114.0 µL	
5x iProof HF Buffer, 7.5 mM $MgCl_2$	10.0 µL	40.0 µL	1x
dNTP mix (2.5 mM each dNTP)	1.0 µL	4.0 µL	0.20 mM
12 µM Gene-specific Forward primer	1.0 µL	4.0 µL	0.24 µM
12 µM Gene-specific Reverse primer	1.0 µL	4.0 µL	0.24 µM
iProof DNA polymerase (2.0 Units/µL)	0.5 µL	2.0 µL	0.2 Unit
Total volume	**42.0 µL**	**168.0 µL**	

   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:

	Tube #1	**Tube #2 (Positive Control)**	**Tube #3 (Negative Control)**
Mmix	**42 µL**	**42 µL**	**42 µL**
~100 ng *Arabidopsis* genomic DNA (12 ng/µL)	**8 µL**	0 µL	0 µL
Positive control DNA template (PCR product made by using iProof DNA polymerase)	0 µL	**1 µL**	0 µL
Sterile water	0 µL	**7 µL**	**8 µL**
Total volume	**50 µL**	**50 µL**	**50 µL**

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.**

Cycling parameters	**Up to 3 kbp** of PCR product	**Number of Cycles**
Activation Enzyme step	98ºC for 30 seconds	**1**
Denaturation step	98ºC for 10 seconds	**30**
Annealling step	63ºC (or T_m+3ºC) for 30 seconds	
Extension step	72ºC for 75 seconds (or 15-30 seconds/kb)	
Final Extension	72ºC for 5 minutes	**1**

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 pENTR/D-TOPO VECTOR

1. Thaw **on ice** a tube of the **pENTR/D-TOPO** vector solution.
2. Label each of TWO 1.5-mL microcentrifuge tubes "**pENTR + Gene Name**" and **"Date"**; "**pENTR Only**" and "**Date"**. Place the labeled tubes on ice.
3. a. Pipet the following reagents into the labeled tube as listed in the **Table** below:

Reagent	**pENTR + Gene Name**	**pENTR Only (Negative Control)**
Freshly prepared **PCR** product	**2.0 µL**	**0.0 µL**
Salt Solution	**1.0 µL**	**1.0 µL**
Sterile water	**2.5 µL**	**4.5 µL**
pENTR/D-TOPO	**0.5 µL**	**0.5 µL**
Total Volume	**6.0 µL**	**6.0 µL**

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^{o}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^{o}C overnight****.*

B. TRANSFORMATION OF *E. COLI* COMPETENT CELLS WITH THE LIGATION MIXTURE

1. **Thaw on ice** THREE vials of One Shot *E. coli* competent cells for transformation for **a few minutes**. *Note: Remove tubes of competent cells from the -70°C freezer and place then on ice just a few minutes before you need them.*
2. **Write** on the CAP and SIDE of each vial as follows:

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

3. **Pipet** the **TOPO ligation mixtures** or **control plasmid DNA** (pUC19) into the vial 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 + Gene	**2 µL**	**0 µL**	**0 µL**
pENTR Only	**0 µL**	**2 µL**	**0 µL**
pUC19	**0 µL**	**0 µL**	**1 µL**

4. **Incubate** the cell mixture **on ice** for **10-20 minutes**.
5. **Heat-shock** the cells for **30 seconds** in the **42°C waterbath** without shaking.
6. Immediately, **transfer** the tube **back on ice**. Leave it **on ice** for **2 minutes**.
7. **Transfer** the tube to **a rack** for microcentrifuge tubes at room temperature.
8. In the bacterial hood, **pipet 250 µL** of room temperature **S.O.C medium** to the cell mixture. **Cap** the tube **tightly**.
9. **Shake** the tubes **horizontally** at **150-200 rpm** an orbital shaker in a **37°C** incubator for **45-60 minutes**.

10. Meanwhile, **label** on the **bottom** of **6 prewarmed** (37^{o}C) plates (**4 Kanamycin** plates with TWO lines on the side of the lids for **pENTR samples** and **2 Ampicillin** plates with ONE line on the side of the lids for **pUC19**) the following:

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

11. **Spread 10 µL** and **50 µL** of **each** transformation mixture on the appropriate labeled plates.

    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

12. Incubate the plates in the **37^{o}C** incubator overnight (14-16 hours).
13. **Next day**, count the number of colonies. Seal the plates with pieces of parafilm and then store them at **4^{o}C** (cold room or fridge) until inoculation step.

C. SCREENING FOR *E. COLI* CELLS HARBORING THE RECOMBINANT PLASMID AND ISOLATING PLASMID DNA

INOCULATION OF A LIQUID 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 + Kanamycin solution in each tube with **individual** colonies by using a sterile toothpick or pipet tip to pick a single colony on the "**pENTR+Gene**" plate and drop that toothpick/pipet tip in the tube #1.
5. Repeat step 4 for FIVE other tubes.
6. Incubate the tubes at 37^{o}C overnight by
   a. transferring all 6 tubes to a **wired rack** on an **orbital shaker** in the **37^{o}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.*

Attention: if the culture tubes sit in the fridge or coldroom for more than ONE hour, vortex the tubes for 5 seconds to mix the content before transfer it to the microcentrifuge tube.

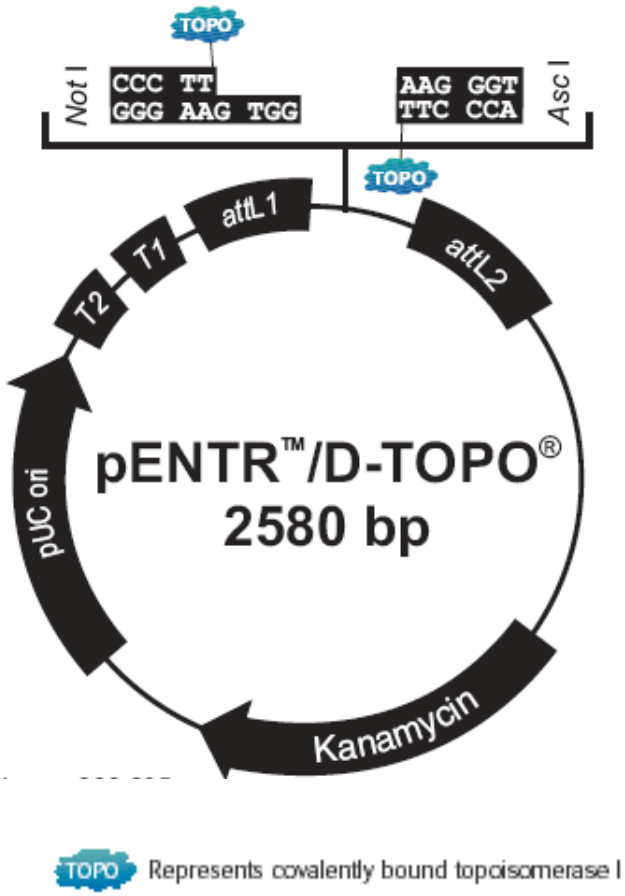
3. **Carefully,** pour the liquid culture into the microcentrifuge tube. Close the lids of the tubes.
4. Spin tubes in a microcentrifuge at **FULL** speed for **2 minutes**.
5. Pour off the supernatant into a glass Erlenmyer flask labeled as "CULTURE WASTE". Dab off the extra liquid on a piece of paper 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 QIAprep 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: **pENTR-**"**gene name**"; **Number**, **your initial**, **date**. **(Tubes will be used at step 27).**
17. After 10 minutes of spinning, transfer **800 µL** of the **supernatant** from step 14 to the QIAprep column by pipetting. *Caution: Make sure that the **numbers** on the **lids** of tubes and the **SIDE** of QIAprep 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 QIAprep 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 microcentrrifuge 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 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
AscI restriction enzyme (New England Biolabs or NEB, 10 units/μL)
10 NEB buffer #4 (supplied with *AscI*)
37°C water bath
Agarose
Gel Apparatus
Gel Document system

PROCEDURE

1. Digest **300 - 500 ng** of plasmid DNA with the restriction enzyme ***Asc I*** at **37°C** for **45-60 minutes**.

(**Why *Asc I*?** Check the presence of the ***Asc I***site in the **Multiple Cloning Site** of the pENTR/D-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.*

***Table 1. Setting up** the **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
Asc I (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 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 **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 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.*

<u>Note:</u> 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 Asc I enzyme (see the Table below) for seven reactions (6 samples + 1 extra reaction).

a. Label on the lid of a microcentrifuge tube as "**Enz Mix"** → Prepare the **Enzyme Mix** for the **number of plasmid DNA samples + 1 Extra reaction** to be analyzed (use the **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
***Asc* I (10 U/µL)**	0.5 µL	µL
Total Volume	**2.5 µL**	**µL**

c. Determine the volume of plasmid DNA and volume of water so that you have the volume of **17.5 µL** and **fill in** the **Table** below.

Components	**Sample 1 (ng/µL)**	**Sample 2 (ng/µL)**	**Sample 3 (ng/µL)**	**Sample 4 (ng/µL)**	**Sample 5 (ng/µL)**	**Sample 6 (ng/µL)**
300 - 500 ng plasmid DNA	µL	µL	µL	µL	µL	µL
Sterile water	µL	µL	µL	µL	µL	µL
Total volume	**17.5 µL**	**17.5 µL**	**17.5 µL**	**17.5 µL**	**17.5 µL**	**17.5 µL**

d. Label on the lids of microcentrifuge tubes **sample number**, ***Asc* I**, 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.

e. 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.

f. 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 **10 µL or 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.

____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**

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 M13 Forward primer
- 3 μM M13 Reverse 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:

	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:

Components	**Mmix** for **ONE reaction**	**Mmix** for **3 reactions**
250-500 ng of DNA template	**x** μL	**x** (x 3) μL
Sterile water	**y** μL	**y** (x 3) μL
Big Dye v. 3	**2.0** μL	**6.0** μL
Dye Dilution Mix (Sigma, S3938)	**2.0** μL	**6.0** μL
Total Volume	**19.0 μL**	**57.0 μL**

- 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 **specific primer** into TWO labeled 0.2-mL PCR tubes.

Components	**M13 <u>Forward</u> primer**	**M13 <u>Reverse</u> primer**
Mmix	**19 µL**	**19 µL**
3 µM M13 <u>Forward</u> primer	**1 µL**	**0 µL**
3 µM M13 <u>Reverse</u> primer	**0 µL**	**1 µL**
Total volume	**20 µL**	**20 µL**

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, ∞

<u>or</u> **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. <u>*Note:*</u> *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/
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.