



**A Gene Discovery Lab Manual For
Undergraduates:**

**Searching For Genes Required
To Make A Seed**

**Honors Collegium 70A Laboratory
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By

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EXPERIMENT 1 – INTRODUCTION TO GENERAL MOLECULAR BIOLOGY TECHNIQUES

STRATEGY

- I. MICROPIPETTING EXERCISE**
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- III. POLYMERASE CHAIN REACTION (PCR)**
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I. MICROPIPETTING EXERCISE

Purpose: To learn how to use micropipettors

Taken From: DNA Science: A First Course, Second Edition
Laboratory 1: Measurements, Micropipetting, and Sterile Techniques
pp. 327-328

Solutions Needed:

- Four Dye Solution Tubes Labeled I-IV
 - Solution I: Blue
 - Solution II: Red
 - Solution III: Yellow
 - Solution IV: Green

Apparatus Needed:

- Pipetman (P-10, P-20, P-200 & P-1000)
- Microcentrifuge tubes
- Rack for microcentrifuge tubes
- Pipet Tips (Regular = Not Filtered tips for PCR)

I. MICROPIPETTING EXERCISES

Taken From: DNA Science: A First Course, Second Edition
Laboratory 1: Measurements, Micropipetting, and Sterile Techniques
pp. 327-328

A. Small Volume Micropipettor Exercise

This exercise simulates setting up a reaction, using a micropipettor with a range of 0.5-10 μL or 1-20 μL .

1. Use a permanent marker (sharpie) to label three 1.5-mL tubes **A**, **B**, and **C**.
2. Use the **matrix below** as a checklist while adding solutions to each reaction tube.

Tube	Sol. I (Blue)	Sol. II (Red)	Sol. III (Yellow)	Sol. IV (Green)	Total Volume
A	4 μL	5 μL	1 μL	-	10 μL
B	4 μL	5 μL	-	1 μL	10 μL
C	4 μL	4 μL	1 μL	1 μL	10 μL

3. Set the micropipettor to **4 μL** and add **Solution I** to each reaction tube.
4. Use a *fresh tip* to add appropriate volume of **Solution II** to a clean spot inside reaction Tubes **A**, **B**, and **C**.
5. Use a *fresh tip* to add 1 μL of **Solution III** to Tubes **A** and **C**.
6. Use a *fresh tip* to add 1 μL of **Solution IV** to Tubes **B** and **C**.
7. Close lids. Pool and mix reagents by using one of the following methods:
 - a. Sharply tap the tube bottom on the bench top. Make sure that the drops have pooled into one drop at the bottom of the tube.

Or

- b. Place the tubes in a microfuge and apply a short, few-second pulse. Make sure that the **reaction tubes are placed** in a **balanced configuration** in the microfuge rotor. *Spinning tubes in an unbalanced position will damage the microfuge motor.*
8. A **total** of **10 μL** of reagents was added to each reaction tube. To check that the previous pipetting measurements were accurate, set the pipette to 10 μL and very carefully withdraw solution from each tube.
- a. Is the tip just filled? What does this suggest?
- Or
- b. Is a small volume of fluid left in tube? What does this suggest?
- Or
- c. After extracting all fluid, is an air space left in the tip end? What does this suggest? (The air can be displaced and actual volume determined simply by rotating volume adjustment to push fluid to very end of tip. Then, read the volume directly.)
9. How can you tell if the measurements were inaccurate? If several measurements were inaccurate, repeat this exercise to obtain a near-perfect result.

B. Large-Volume Micropipettor Exercise

This exercise simulates a bacterial transformation or plasmid preparation, for which a 100-1000- μL micropipettor is used. It is far easier to mismeasure when using a large-volume micropipettor. If the plunger is not released slowly, an air bubble may form or solution may be drawn into piston.

1. Use a permanent marker to label two 1.5-mL tubes **D** and **E**.
2. Use the matrix below as a checklist while adding solutions to each reaction tube.

Tube	Sol. I (Blue)	Sol. II (Red)	Sol. III (Yellow)	Sol. IV (Green)	Total Volume
D	100 μL	200 μL	150 μL	550 μL	1000 μL
E	150 μL	250 μL	350 μL	250 μL	1000 μL

3. Set the micropipettor to add appropriate volume of Solutions I-IV to reaction tubes **D** and **E**. Follow the same procedure as for the small-volume micropipettor add **Solutions I-IV** to each reaction tube.
4. A **total** of **1000 μL** of reactants was added to each tube. To check that the measurements were accurate, set the pipette to 1000 μL and very carefully withdraw solution from each tube.
 - a. Is the tip just filled? What does this suggest?
Or
 - b. Is a small volume of fluid left in tube? What does this suggest?
Or
 - c. After extracting all fluid, is an air space left in the tip end? (The air can be displaced and actual volume determined simply by rotating volume adjustment to push fluid to very end of tip. Then, read the volume directly.)

5. If several measurements were inaccurate, repeat this exercise to obtain a near-perfect result.

II. SERIAL DILUTION EXPERIMENT

Purpose: To test accuracy and precision of pipetting

Reference: Sambrook et al. (1989). Molecular Cloning Manual.

Introduction: *Diluting* is simply the **addition of a solution** (or plain solvent) to a **substance** in order to **decrease the concentration of the latter substance**. In this exercise, the **substance** is **DNA** and the **solution** is **TE Buffer**. By the end of this exercise, you will learn how to calculate the dilution factor and determine the accuracy of your pipetting techniques as determined by gel electrophoresis and spectrophotometer readings.

Solutions Needed:

- DNA Stock (Known Concentration)
- Diluted 1Kb DNA ladder solution
- TE Buffer
- Agarose solution
- 1X TAE buffer
- 10 mg/mL Ethidium Bromide (EtBr)
- 6X Loading Dye containing xylene cyanol and bromophenol blue dyes

Apparatus Needed:

- Microcentrifuge tubes
- Nanodrop Spectrophotometer
- Kimwipes
- P-10, P-20 Pipetman
- Gel casts
- Gel box
- Cables
- Gel combs
- Plastic (Saran) wrap
- Gel Document system (Bio-Rad)
- Plastic container for carrying the gel
- Pipet Tips (Regular = Not Filtered tips for PCR)

A. Serial Dilution of a DNA Stock

1. Label **3 1.5-mL microcentrifuge tubes** as:
“**Dil. #1**” for dilution #1
“**Dil. #2**” for dilution #2
“**Dil. #3**” for dilution #3
2. Pipet **15 μL** of **TE buffer** solution into each microcentrifuge tube in step 1. (Use the **P-20** pipetman)
3. Pipet **5 μL** of your DNA stock solution into the **Dil.#1** microcentrifuge tube. (Use the **P-10** or **P-20 pipetman**)
4. Vortex the content of the tube for 5 seconds. Then, spin the tube for **10 seconds** to ensure that all of your solution is on the bottom of the tube.
5. Pipet **5 μL** of DNA solution from the **Dil. #1** tube into the **Dil. #2** tube.
6. Vortex the content of the tube for 5 seconds. Then, spin the tube for **10 seconds** to ensure that all of your solution is on the bottom of the tube.
7. Pipet **5 μL** of DNA solution from the **Dil. #2** tube into the **Dil. #3** tube.
8. Vortex the content of the tube for **5 seconds**. Then, spin the tube for **10 seconds** to ensure that all of your solution is on the bottom of the tube.

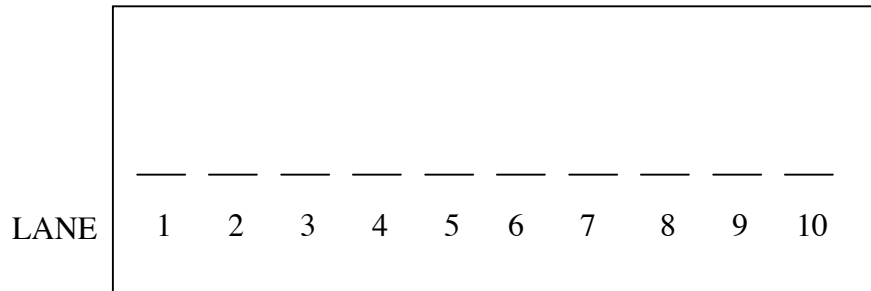
B. Determination of Pipetting Accuracy by Gel Electrophoresis

1. Label THREE microcentrifuge tubes with letters “**A**”, “**B**”, and “**C**”.
2. Pipet **10 μL** of DNA solution from dilutions to tubes A, B, and C:

<i>from</i>	Dil. #1	Dil. #2	Dil. #3
<i>to</i>	Tube A	Tube B	Tube C

3. Pipet **2 μL** of Loading Dye into tubes **A, B, C**. Mix by pipetting up and down for 5 times. The total volume for each solution is **12 μL** .

4. Load **10 μL** of diluted **1-kb DNA ladder solution** (50 ng of DNA/ μL ; see **Appendix 1C**) into the **first well** of a **1% agarose gel** (see **Appendix** below for Preparation of the Agarose Gel).
5. Load the contents in tubes A, B, and C into the wells next to the 1-kb ladder solution.
6. Add **10 μL** of **10 mg/mL of EtBr** to the running buffer at the **anode** (positively charged) of the gel box. (The **anode** is the **opposite** side from the wells)
Note: Ethidium bromide is positively charged. Therefore, it migrates towards the negative end of the gel box from anode to cathode. (Opposite direction from DNA migration). Remember that DNA is negatively charged; so, it migrates to the positive end of the gel. (DNA migrates from cathode to anode).
7. Put the lid of the gel box on the gel box and connect the electrodes to the power supply (**RED to RED** and **BLACK to BLACK**).
8. Record the **pattern of samples loaded** on the gel:



9. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue or BPB) has migrated one-half or two-thirds of the gel length. *Note: Use ~130 volts for TWO gels connected to the same power supply.*

Time power supply turned ON:

Time power supply turned OFF:

How long was the gel run? _____ minutes or _____ hour(s)

10. After 1-2 hours of running the gel, Turn **off** the power supply.

11. Remove the lid of the gel box. Put the gel in its gel cast into a small plastic container and bring the container to room 2828. ***Caution:*** *it is a MUST to put the gel into a plastic container so that the gel cannot slide off the gel cast, fall on the floor and be broken into pieces while walking to a different room (2828) for taking a picture of the gel.*
12. Take a picture of the gel using the BioRad Gel Document System in room 2828.
13. Label the picture using a text program of the Gel Document System (your TA will show you how).
14. Print out the picture.
15. (Optional) Label the picture by:
 - a. Putting a piece of white tape (on the picture) at a position immediately above the wells
 - b. Marking samples corresponding to all wells
16. Store the labeled picture a glassine envelope (obtain the envelopes from your TAs) that is pasted on a sheet of your note.

C. Determination of Pipetting Accuracy Using a Spectrophotometer

While running the gel, determine the **concentration** of DNA solutions in tubes labeled "DNA Stock", "Dil #1", "Dil #2", and "Dil #3" by using the **Nanodrop Spectrophotometer** (TAs will show how to use the instrument).

What is a spectrophotometer? (see Appendix 1B)

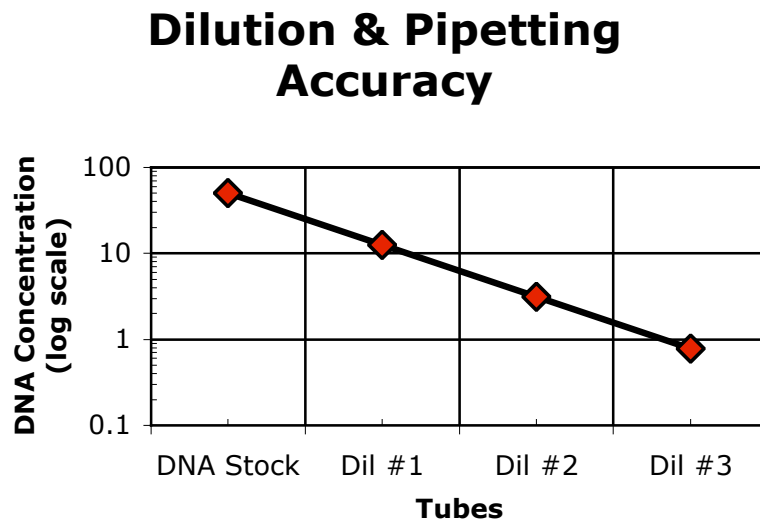
1. For each tube, read the concentration at least TWICE.
2. Record the DNA concentration (in **ng/ μ L**) from each tube.

D. Question and Summary

1. What did you expect to see on your gel?
2. How is your pipetting accuracy determined by gel electrophoresis?
3. Is the gel result showing what you expected? If not, what might be the problem?
4. What is the dilution factor in this exercise?
5. Given the stock DNA concentration, what is the expected DNA concentration in tubes "Dil #1", "Dil #2", and "Dil #3"?
6. Make a plot on log graph paper of the expected DNA concentration in question five (this will be your standard curve) as shown in the graph below:

The x-axis - Tubes (DNA stock, Dil #1, Dil #2, and Dil #3)

The y-axis - The expected DNA concentration



7. Add the DNA concentration reading you obtained from the spectrophotometer for each tube.
8. How does your DNA concentration reading deviate from the expected DNA concentration?

III. POLYMERASE CHAIN REACTION (PCR)

Purpose: To obtain a large amount of DNA molecules in a short time for other purposes, such as determination of DNA sequence by sequencing analysis, cloning into an appropriate vector for gene expression or promoter analysis.

Reagents and Apparatus Needed:

- 10x Ex-Taq buffer (Takara Mirus Bio) came with the Ex-Taq DNA polymerase
- dNTP mix (Takara Mirus Bio) came with the Ex-Taq DNA polymerase
- Ex-Taq DNA polymerase (Takara Mirus Bio)
- Sterile water
- 12 μ M Gene-specific Forward primer
- 12 μ M Gene-specific Reverse primer
- 0.2 ng/ μ L *Arabidopsis* Columbia-0 genomic DNA
- 1.5 mL sterile microcentrifuge tubes
- 0.2 mL sterile microcentrifuge (or PCR) tubes
- P-10, P-20, P-200 pipetman
- Filtered Pipet tips (0.1-10 μ L, 1-20 μ L and 1-200 μ L) for PCR
- Rack for 1.5 mL microcentrifuge tubes
- PCR Rack for 0.2 mL microcentrifuge tubes
- Gloves
- Black Ultra-fine sharpie pen
- Ice bucket or plastic container
- Microcentrifuge
- PCR machine (MyCycler, BioRad)

PROCEDURE

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 **10x Ex-Taq buffer** and **dNTP mix** on a microcentrifuge rack for 1.5 mL microcentrifuge tubes at **room temperature** for 5-10 minutes. Once the solutions are thawed out, put the tubes on ice until needed.
3. Thaw out **Forward** and **Reverse primer solutions** corresponding to a gene to be knocked out at room temperature as in step 2.
4. Obtain **THREE** 0.2-mL sterile PCR tubes and set them on a PCR rack for 0.2 mL microcentrifuge tubes.
5. Write on the **lids** of the tubes the number **1, 2, 3** and on the **side** of the tubes **Name of the gene, your Initial** and **Date** as follows: (*your TA will show you how to write on the tubes*)
 - Tube #1: **Name of the gene** (same name as primer's gene)
 - Tube #2: **Pos.** (Positive control serving as the positive control for PCR amplification)
 - Tube #3: **Neg.**(Negative control containing same components as in tube #1, but **NO** genomic DNA)
6. Obtain **ONE** 1.5 mL microcentrifuge tube and set it on a rack for 1.5 mL microcentrifuge tubes.
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 Master Mix (**Mmix**) solution for **4 reactions (3 samples + 1 extra)** as follows:

Master Mix:

	Mmix for ONE reaction	Mmix (for 4 rxns)
Sterile water	36.6 μ L	146.4 μ L
10x Ex-Taq buffer	5.0 μ L	20.0 μ L
dNTP mix	4.0 μ L	16.0 μ L
12 μM Gene-specific Forward primer	1.0 μ L	4.0 μ L
12 μM Gene-specific Reverse primer	1.0 μ L	4.0 μ L
Ex-Taq DNA polymerase (5 Units/μL)	0.4 μ L	1.6 μ L
Total volume	48.0 μL	192.0 μL

- a. Pipet into the **Mmix** tube the reagents with order from top down (example: water, 10x Ex-Taq buffer, dNTP mix, etc.)
- b. After pipetting all reagents into the **Mmix** tube, close the lid of the tube. Mix the contents by vortexing on the **vortex mixer** at **setting of 3-4** for **5 seconds**. 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 **48 μL** of the **Mmix** solution into PCR tubes (see table below)
 - b. pipetting **1-2 μL** of DNA or water to tubes #1-3(see table below)
 - c. Immediately, mix the contents by **pipetting up and down at least five times**

PCR reactions:

Components	Tube #1 (Gene Name)	Tube #2 (Positive)	Tube #3 (Negative)
Mmix	48 μL	48 μL	48 μL
<i>Arabidopsis</i> genomic DNA (Columbia-0 ecotype)	2 μL	-----	-----
Control template DNA (Positive control)	-----	1 μL	-----
Sterile water (negative control)	-----	1 μL	2 μL
Total volume	50 μL	50 μL	50 μL

10. Turn **ON** the PCR machine (MyCycler) by pressing and holding the "Standby" button for **1-2 seconds**. Wait for one minute for the machine to initializing.
11. Put the PCR tubes in the wells of the 96-well hot plate of the MyCycler.
12. Select the "**Protocol Library**" by pressing "**F1**" button.
13. Select "**Knockout**" protocol by pressing **yellow arrowheads** surrounding the "**ENTER**" button. Once it is selected, the "**Knockout**" protocol is highlighted. Press the "**ENTER**" button.

The **PCR profile** of the Knockout as following:

94°C, 3 min;

36 cycles of 94°C, 15 sec → 60°C, 30 sec → 72°C, 2 min;

72°C, 4 min;

4°C, infinity.

14. Under the "**Choose Operation**" window, "**Run Protocol**" is highlighted. Press the "**ENTER**" button to run the protocol.
15. Press "**F5**" button to "**Begin Run**" the protocol. *Note: it would take about 3 hours for the PCR amplification to be completed under the above specified PCR profile.*
16. Once the PCR amplification is completed, remove PCR tubes from the PCR machine and store them in the **refrigerator** until gel electrophoresis or leave them in the PCR machine until you have a chance to put them away later.

GEL ELECTROPHORESIS ANALYSIS OF PCR PRODUCT

1. Write the number (**1, 2, 3**) on the lids of **THREE 1.5-mL microcentrifuge tubes**.
2. Arrange **THREE** PCR tubes corresponding to **THREE** 1.5-mL microcentrifuge tubes.
3. Pipet into each of **THREE** 1.5-mL microcentrifuge tubes following:

	Tube #1	Tube #2	Tube #3
6x Loading dye	2 μ L	2 μ L	2 μ L
PCR Solution #1	10 μ L	-----	-----
PCR Solution #2	-----	10 μ L	-----
PCR Solution #3	-----	-----	10 μ L

Mix the contents by pipetting up and down for at least 5 times.

4. Load the samples along with diluted 1-kb DNA ladder on the 1% agarose gel.
5. Record the loading pattern of samples.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12

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

Time power supply turned ON:

Time power supply turned OFF:

How long was the gel run? _____ minutes or _____ hour(s)

7. Take a picture of the gel using the Gel Document system.

8. Analyze the size of the PCR product on the picture. *If the expected size is observed, proceed to purification of PCR product.*

What is the size (in bp) of the PCR product from gel electrophoresis? _____ **bp**

IV. PURIFICATION OF PCR PRODUCTS

Purpose: To purify DNA (PCR product) from free nucleotides, primers, salt, and enzyme for downstream applications such as sequencing reactions.

Reference: QIAquick Gel Extraction protocol (Qiagen)

Solutions Needed:

- QIAquick PCR Purification Kit (Qiagen, Cat. # 28104)
- PCR solutions

Materials Needed:

- 1.5 mL microcentrifuge tubes
- Microcentrifuge
- Nanodrop spectrophotometer

PROCEDURE

1. Write on the **lid** and **side** of a 1.5-mL microcentrifuge tube **your initial**.
2. Pipet **15 μ L** of the **PCR product solution** from the PCR tube containing the gene-specific DNA fragment into the 1.5-mL microcentrifuge tube.
3. Add **75 μ L** of **Buffer PB** (or **5 volumes of Buffer PB to 1 volume** of the PCR sample) to the tube in step 2. Mix by vortexing the tube for 5 seconds. Spin the tube in the microcentrifuge at **FULL speed** for **10 seconds** to bring all the solution down to the bottom of the tube. Set the tube back on the microcentrifuge rack.
4. Place a **QIAquick spin column** in a provided **2-mL collection tube**.
5. Apply the sample mixture in step 3 to the QIAquick column. Spin the column set in the microcentrifuge at **FULL speed** for **1 minute**. *This step allows the binding of DNA to the membrane*
6. Discard the **flow-through solution** in the collection tube. Put the QIAquick column back into the same collection tube.
7. Add **750 μ L** of **Buffer PE** to the QIAquick spin column and spin at **FULL speed** for **1 minute**.
8. Discard the flow-through solution in the collection tube. Put the QIAquick column back into the same collection tube.
9. Spin the column set at FULL speed for an **additional 1 minute** to get rid of residual ethanol in Buffer PE. *Caution: Residual ethanol from Buffer PE will NOT be completely removed unless the flow-through solution is discarded before this additional spin.*
10. While spinning, label on the **lid** and **side** of a **1.5-mL microcentrifuge tube** "**Purified PCR product**", **your initial**, and **date**.
11. Transfer the appropriate **QIAquick columns** in the **NEWLY** labeled microcentrifuge tubes. Discard the flow-through solutions and the collection tubes.
12. Pipet **30 μ L** of **Buffer EB** to the center of the QIAquick membrane. Let the column sit for **1 minute**, and then centrifuge at **FULL speed** for **1 minute**. *This step elutes the DNA from the QIAquick membrane.*

13. Determine DNA concentration using the Nanodrop spectrophotometer (measuring nucleic acids) or a DNA Fluorometer (measuring only DNA).

What is the concentration of purified PCR product? _____ **ng/μL**

What is the size (in bp) of the PCR product from gel electrophoresis? _____ **bp**

V. SEQUENCING REACTION WITH BIG DYE V. 3

Purpose: To determine a sequence of a desired DNA fragment, such as a PCR product of the gene of interest

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 μ M Sequencing primers (Gene-specific Forward and Reverse primers)
- 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- μ L** 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 μ L = the volume depends on **concentration** and **amount** of **DNA**

(see Table below)

y μ L = the **remaining volume** to bring the **total volume** to **20 μ L**

* **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.
- For **PCR product**, use the amount of DNA according to the **table** on the next page (Taken from Perkin-Elmer Big Dye Protocol). *Note: Use the **maximum** amount of DNA in the reaction if there is more than enough*

DNA available. For example, for **PCR product of 200 - 500 bp**, use **10 ng** of DNA.

Table: Amount of DNA Used in Sequencing Reactions Depending on Size of PCR Fragment

Size of PCR Product (bp)	Amount of DNA Used in Reactions
100 - 200	1 - 3 ng
200 - 500	3 - 10 ng
500 - 1000	5 - 20 ng
1000 - 2000	10 - 40 ng
> 2000	40 - 100 ng

For this exercise, there is **ONE DNA template**, i.e. the purified PCR product of the gene of interest; but, there are **TWO primers**, gene-specific forward and gene-specific 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 PCR product? _____ **ng/μL**

What is the size of the PCR product? _____ **bp**

What is the amount of DNA to be used? _____ **ng**

Sample calculations:

Size of PCR product is 400 bp and its concentration is 4.5 ng/μL

Want to use 10 ng of purified PCR product (see table above)

Hence, the amount of PCR to be used is $10 \text{ ng} / 4.5 \text{ ng}/\mu\text{L} = 2.2 \mu\text{L}$

What is the volume of PCR product 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
DNA template	x μ L	x (x 3) μ L
Sterile water	y μ L	y (x 3) μ L
Big Dye v. 3	2.0 μ L	6.0 μ L
Dye Dilution Mix (Sigma, S3938)	2.0 μ L	6.0 μ L
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 **gene-specific primer** into TWO labeled 0.2-mL PCR tubes.

Components	Gene-specific Forward primer	Gene-specific Reverse primer
Mmix	19 μ L	19 μ L
3 μ M Gene-specific Forward primer	1 μ L	-----
3 μ M Gene-specific Reverse primer	-----	1 μ L
Total volume	20 μL	20 μL

5. 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.

6. After the cycling reaction is finished, clean up sequencing reactions using Edge Biosystems spin columns (stored in the cold room) as following:

- Spin the pre-packed columns in a microcentrifuge at 3,000 rpm for 2 minutes at room temperature.
- Meanwhile, label a new set of 1.5 mL microcentrifuge tubes according to your reactions.
- Transfer the columns to new tubes.
- Pipet 20 μ L of sequencing reaction to appropriate columns.
- Spin the columns as in step a.
- Discard the columns.

6. 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.*

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

VI. RETRIEVING AND ANALYZING DNA SEQUENCES

Purpose: To verify that the sequence corresponds to that 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.

EXPERIMENT 2 – SCREENING SALK T-DNA MUTAGENESIS LINES (GENE ONE)

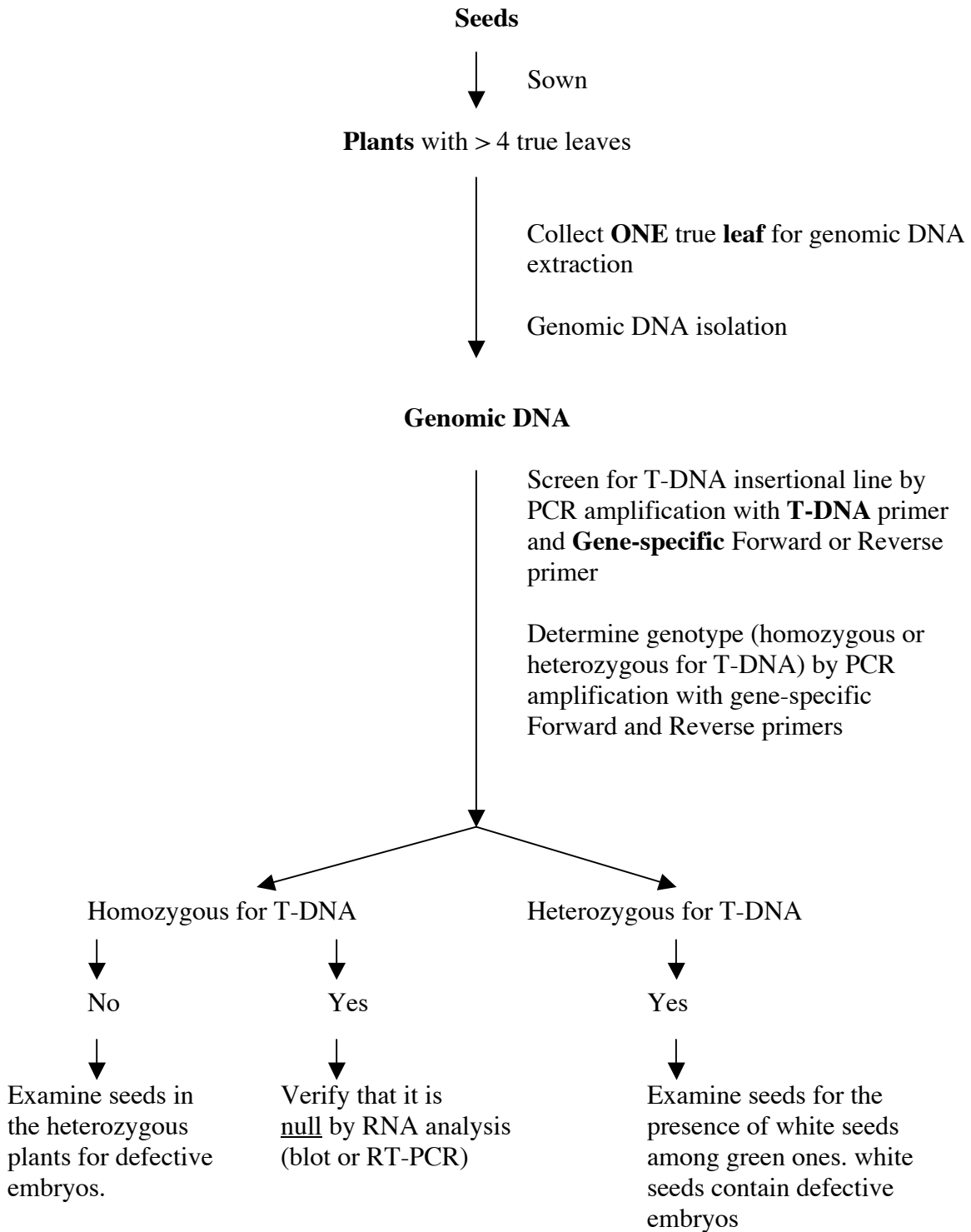
Purpose: To identify a knockout line for the gene of interest and characterize phenotype of mutant plant(s).

Reference: University of Wisconsin - Madison Knockout Facility

STRATEGY

- I. SOWING SEEDS AND GROWING PLANTS**
- II. ISOLATING GENOMIC DNA FROM LEAVES OF AT LEAST 2-3 WEEKS-OLD SEEDLINGS/PLANTS**
- III. IDENTIFYING PLANTS CONTAINING T-DNA INSERTED IN THE GENE OF INTEREST AND THEIR GENOTYPES**

STRATEGY



I. SOWING SEEDS AND GROWING PLANTS

Purpose: To generate seedlings/plants for genomic DNA extraction.

Caution: *Be extremely CAREFUL with seeds. Do NOT mix up labeled tags and actual seed lines.*

Materials Needed:

- Tubes of Seeds from the *Arabidopsis* Seed Stock Center
- A microcentrifuge rack
- White Xerox paper
- Black sharpie (Ultrafine or fine)
- Plastic tags
- A pair of pointed-end forceps
- Black plastic trays
- Black rectangular pots in sheets
- Clear plastic covers for black trays
- Soil in the Plant Growth Center (PGC)
- A growth chamber (Percival) with constant light in the PGC

PROCEDURE

1. Obtain **tubes of seeds** to be grown from the cold-room and put them on a microcentrifuge rack. *For example, S_112701, for gene At5g11240, and wildtype seeds and Columbia for Salk lines.*
2. If **plastic tags** are available in the lab, label them with a black sharpie.
 - a. For **knockout line:**
 - Gene name**
 - SALK line #**
 - Date**
 - Pot # 1-10** (for 1 flat with 11 pots)
 - b. For **wild-type:**
 - Columbia-0**
 - Date**
3. Bring the **items in steps 1 & 2**, along with **several sheets of white paper** and a **pair of tweezers**, to the **Plant Growth Center (PGC)**.
4. At the PGC, put all of these items on **the bench** that runs along the **East wall**. This bench does NOT have any soil on it. *Note: Do NOT put tubes of seeds or plants full of mature seeds near the bench of soil because the prepared soil will be contaminated with these seeds, which could in turn, result in false mutant phenotypes for other people's works.*
5. In the PGC, prepare ONE flat with **12 pots** of soil for every line of mutant seeds being planted.
 - a. Assemble each flat as follows:
 - i. Obtain a black plastic tray.
 - ii. Obtain a sheet of 12 rectangular plastic pots.
 - iii. Obtain a clear plastic cover.
 - iv. Set a sheet of 12 pots in one of the black plastic trays.
 - v. Fill the pots with soil (prepared by the PGC staff, Mr. Weimin Deng).
 - vi. Flatten the surface of the soil by scraping off excess soil with a metal plate.
 - b. Repeat step (a) for as many flats as needed.

- c. Remove **one pot** from the corner of the flat and put the soil back into the same amount of soil. So, there are **only 11 pots**. *The empty space will make it easier to put the water in.*
 - d. Bring the flat to the bench near the sink.
 - e. Make sure that the water hose is attached to the water pipeline labeled “**fertilizer-supplemented**”.
 - f. Fill each flat **2/3 of the way up** the tray with “fertilizer-supplemented” water.
 - g. Wait **15 minutes or until the surface of the soil appears darker** due to water sipping up from the bottom of the pots.
 - h. Cover the flat with **clear plastic cover** to prevent growth of air-borne molds and to protect from **strayed Arabidopsis seeds** from other plants.
6. Bring the flat over to the bench where the seeds and planting tools are located (or any other bench removed from the soil).
 7. Cut the **sheet of white paper** into quarters
 8. Fold **each quarter in half**, length-wise
 9. Gently pour out seeds from the microcentrifuge tube onto one of the folded pieces of paper.
 10. Bring the folded paper with seeds over each of the 12 pots. Lower one end of the paper near the soil surface. **Gently tap** the lower end of the paper to allow for one seed to slide down into the soil. The tweezers are a useful tool to guide one seed off of the paper to a precise location in the pot without dumping all of the seeds from the paper.
 11. Sow 2 seeds per pot, for 11 of the pots.
 12. Put the labeled tags for the **knockout line** into **each** of the **10 pots** containing knockout seeds.
 13. Put the seeds that were not used back into the **appropriate knockout seed** microcentrifuge **tube**.
 14. For pot **#11**, pour out wild-type seeds onto a **new** folded piece of white paper. Visually divide the pot into 4 quadrants, and sow a wild-type seed in each quadrant. **Four seeds of wild-type** should be sown in **pot #11**.
 15. Put a **wild-type labeled tag** into pot #11.

16. Cover the flat with the **clear plastic cover**.
17. Put the flat aside.
18. Repeat seed sowing for other knockout lines.
19. After all of the lines are sown, put the flats on a metal car and take the elevator to the lower level.
20. Put the flats on wired-racks in the cold-room (the first room on the right after entering the double doors across from the elevator).
CAUTION: Make sure the clear covers completely cover the flats so that no air-borne molds in the cold-room get in the soil.
21. Leave the flats in the cold-room for **2-3 days** to **vernalize seeds** and to enhance **synchronization of seed germination**.
22. After 2-3 days in the cold-room, transfer the flats to the white Percival growth chamber and leave them there for another 5-7 days.
NOTE: Keep the clear plastic covers on the flats.
23. After a total of 7-10 days after planting, bring the flats of seedlings with 2 cotyledons to the glasshouse #3
24. Put the flats of seedlings on a table.
NOTE: Choose a table that has no mature Arabidopsis plants bearing ripened seeds because these seeds could accidentally get in the soil of the seedling flats when the clear covers are removed.
25. Slide the clear covers off the trays by **0.5-1 inch** so that warm air under the covers will not cook the seedlings nor will the surface of the soil be too warm which is favorable for molds to grow.
26. Wait until most of seedlings in the flats have **4 true leaves**. Then remove the clear covers completely off the flats. Bring the clear covers to the washing room on the lower level of the PGC so that they will be washed by the PGC staff.
27. Map **positions of seedlings** in **each of 11 pots** on a sheet of "Plant Layout" chart.

28. Daily, **check water level** in the soil of the flats by feeling the wetness of the soil surface with your fingers. If the plants need to be watered, remove one pot at the corner and then put "fertilizer-supplemented" water in.

NOTE: Do NOT overwater the plants because overwatering may cause stress to plants, resulting in false mutant phenotype that will not appear in the next generation. Bigger plants need more water than smaller ones. Therefore, you need to check water level in the soil more often daily with big plants.

GENOTYPING ARABIDOPSIS PLANTS
PLANT LAYOUT CHART

Gene ID: At__ g _____ SALK line#: _____ Date: _____
Primers for PCR: _____
Size of PCR product: _____

Pot #	Pot #
Pot #	Pot #
Pot #	Pot #
Pot #	Pot #
Pot #	Pot #
Pot #	Pot #
Pot #	Pot #

II. ISOLATING GENOMIC DNA FROM LEAVES OF AT LEAST 2-3 WEEKS-OLD SEEDLINGS/PLANTS

Purpose: To isolate genomic DNA from seedlings' leaves for identifying plants containing a T-DNA insert within the gene of interest.

Recommendation:

Instead of isolating genomic DNA from all 22-24 seedlings at once, you can **start** with the **FIRST 6 seedlings/plants (5 from the knockout line and 1 from Wild type)**. Once you are familiar with the method of isolating genomic DNA, you can isolate genomic DNA from the remaining seedlings, including Wild type.

Materials and Reagents Needed:

- Seedlings/plants (knockout lines and wild type)
- Sterile 1.5-mL microcentrifuge tubes
- PCR (aerosol-barrier) pipet tips
- Microcentrifuge-tube racks
- Microcentrifuge
- P-10, P-20, P-200 and P-1000 pipetman
- 80% ethanol solution
- A box of Kimwipes
- One or two pairs of latex gloves
- Two pairs of pointed-end tweezers (forceps)
- A pen
- A plant layout chart
- The key to the Plant Growth Center
- A squirt bottle of 100% ethanol solution
- TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0)
- Extraction Buffer (0.2 M Tris-HCl, pH 9.0; 0.4 M LiCl; 25 mM EDTA; 1% SDS)
- Isopropanol
- Glass beakers labeled as "Waste solution"
- Agarose
- 1X TAE buffer
- Gel apparatus and power supply
- 55-60°C water bath
- 6X Loading dye
- 50 ng/μL 1-kb DNA ladder solution
- 1X TNE (high salt solution) diluted from 10X stock
- 1 mg/mL Hoesch dye H33258 solution stored in the coldroom
- TKO Mini Fluorometer (Hoefer Scientific Instruments)

PROCEDURE

Attention: You will need to **assess the quality of isolated genomic DNA** later (at **step 37**); therefore, to use time efficiently you need to prepare a 0.7% agarose gel before you start the extraction of genomic DNA (see **Agarose Gel Electrophoresis Appendix**). While the agarose mixture is cooled in the 55-60°C water bath for at least 30 minutes, you go to the Plant Growth Center to collect leaves. After 30 minutes or so, add 5 µL of 10 mg/mL Ethidium Bromide (EtBr) solution to the agarose mixture, swirl to mix the EtBr, pour the gel with a 20-tooth comb, and let the agarose mixture to solidify.

1. Put **6** sterile 1.5-mL microcentrifuge tubes on a microcentrifuge-tube rack.
2. Label number 1-6 on lids of the tubes.

Tube #1 - 5: seedlings/plants #1 - 5 of **Knockout** lines

Tube #6: **1** seedling/plant from **Wild type** (Columbia-0)

3. Pipet **100 µL** of **Extraction Buffer** into each tube.

Note: I (Anhthu) found that it is **not** necessary to keep tubes of **Extraction Buffer on ice** during collection of the leaf samples if genomic DNA will be isolated from samples within one hour.

4. Gather together the following items on a plastic tray or container:

- A pair of latex gloves
- Two pairs of tweezers
- A box of Kimwipes tissues
- A squirt bottle of 100% Ethanol solution
- A "Plant Layout" chart
- Several sheets of white Xerox paper
- A ruler with Metric system (mm and/or cm)
- A pen
- The Nikon 5400 digital Camera
- The key to the Plant Growth Center

5. Go to the **Plant Growth Center (PGC)** and locate your flat with plants.
6. Use the "**Plant Layout Chart**" to mark the **locations of the plants** you will collect samples from. The **order of plants** should **correspond** to the **labeled tags** that were numbered when the seeds were planted.

Note: **NOT** all of the seeds will have germinated.

7. Use a piece of Kimwipes to clean the tweezers with 95-100% ethanol solution.

Note: The tweezers must be cleaned after collection each leaf to avoid cross-contamination, and two sets of tweezers are used per plant.

8. Remove one **small leaf** from the **first** plant.
9. Place the leaf on the white paper and measure it with the ruler. *The leaf should be between 0.5 cm and 1.0 cm in length.*
10. Take a picture of the leaf to document the size used to extract DNA.
11. Place this leaf in the microcentrifuge tube #1 containing the extraction buffer.
12. Repeat this process with other plants.
Note: MAKE SURE TO CLEAN THE TWEEZERS BETWEEN LEAF SAMPLES!
13. Go back to the lab.
14. Homogenize or macerate the collected leaf in the extraction buffer by crushing them with a **blue micropestle** until no more chunks of plant tissue observed in the mixture.
Note: Do NOT dispose the micro-pestle, but follow step 15.
15. Rinse the **micropestle** with **300 µL** of Extraction buffer. The **total volume** of Extraction Buffer in the microcentrifuge tube is now **400 µL**.
16. Vortex the **homogenate** for 20 seconds.
17. Set the tube **on ice**.
18. Repeat steps 14-17 for **other tubes**.
19. Centrifuge tubes of homogenates at **room temperature** for **5 minutes** at **FULL speed**.
20. Meanwhile, label a set of **microcentrifuge tubes** with **Gene Name** and **tube #**.
21. Pipet **350 µL** of **isopropanol** to each of labeled tubes.
Note: Make sure that the number on tubes being centrifuged corresponds to the number on the tubes on the rack.
22. After centrifugation, transfer tubes from the microcentrifuge onto a microcentrifuge-tube rack.
23. Organize tubes such that the **numbers** on the **lids** of **NEW tubes match** with **numbers** on **the lids** of **tubes containing homogenates**.
24. Pipet **350 µL** of **supernatant (homogenate)** from the centrifuged tubes to the corresponding tubes containing isopropanol. Close the lids of the tubes.
Note: AVOID pipetting plant debris on the bottom of the tubes as much as possible. However, it is okay if you accidentally transfer some plant debris into the isopropanol tube.
25. Mix the isopropanol and homogenate by inverting the tube **5-10 times**.

26. Incubate the mixture at **room temperature** for **5 minutes** to precipitate **nucleic acids** (*both genomic DNA and total RNA*).
27. Centrifuge tubes at **room temperature** for **10 minutes** at **FULL** speed.
28. Pour off the supernatant into a **glass beaker** labeled as "**Waste solution**".
Note: DNA is now in your pellet along with RNA. Therefore, be extremely careful when pouring off isopropanol because the pellets are sometimes loose.
29. Add **1 mL** of **80% ethanol** solution to each pellet. Close the lid of the tube and invert five times. *This step is to wash off any residual amount of salts (in the extraction buffer) and isopropanol.*
30. Centrifuge the tubes at **room temperature** for **5 minutes**.
31. Pour off the supernatant into a **glass beaker** labeled as "**Waste solution**". Dab the tubes on Kimwipes tissues to remove as much ethanol as possible.
Note: Be extremely careful when pouring off the ethanol solution because the pellet is loose.
32. Put the tubes on a microcentrifuge-tube rack with their lids opened allowing ethanol to be evaporated.
33. Dry pellets either in a **Speedvac** at room temperature for **5-10 minutes** (TAs will show you how to do this step) or leaving on the **bench** at **room temperature for 60 minutes**.
34. *After drying the pellets*, resuspend each pellet by adding **100 µL** of **TE** buffer, closing the lids of the tubes, and **raking** the tubes over the microcentrifuge-rack for **10-15 times** or **vortexing** the tubes for a few minutes until no visible of pellets.
35. Spin tubes in a microcentrifuge for **1 minute** to bring down liquid and any contaminants to the bottom of the tubes.
36. Store DNA solutions at **4°C** (on ice or refrigerator) until used.
*Note: (a) Keep DNA solution cold as much as possible to prevent degradation of DNA because this is a crude extraction of genomic DNA, and there may be a tiny trace amount of endonuclease present in the DNA solution. (b) Before using DNA solution for PCR amplification after a long period of storage (more than 12 hours), spin tubes of DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed to bring down water condensation on the lid as well as any contaminated plant debris and/or carbohydrates in the solutions to the bottom of the tubes.*

Attention: At this step, you need to assess the **quality** and **quantity** of isolated genomic DNA by **gel electrophoresis** (see **step 37**) and **fluorometer reading** (see **step 38**), respectively.

37. Analyze the **quality** of isolated genomic DNA by **gel electrophoresis** as follows:

a. Prepare a **0.7%** agarose gel with a **20-tooth** comb (**0.7g** of **agarose** in **100 mL** of **1X TAE buffer**; see **Agarose Gel Electrophoresis Appendix** for preparing the agarose gel).

Note: The agarose gel can be prepared before the collection of leaves for the extraction of genomic DNA

b. Label the **numbers (1-6)** and **your initial** on the lids of 6 microcentrifuge tubes and set tubes on the microcentrifuge rack.

c. Pipet **10 µL** of **isolated genomic DNA** solutions into each of labeled tubes.

d. Add **2 µL** of **6x Loading dye solution** to each tube and mix the contents by pipetting up and down for 5 times.

e. Load **10 µL** of **diluted 1-kb DNA ladder solution** along with 12 µL of DNA mixtures prepared in steps c and d.

f. Record **loading patterns** of samples

1	2	3	4	5	6	7	8	9	10	11	12	13	14	

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

Starting time:

Ending time:

h. Take a picture of the gel using the Bio-Rad Gel Documentation system.

What do you observe on the gel?

What is the size of genomic DNA?

38. Determine DNA concentration of isolated DNA solutions using a **Fluorometer** and **Hoesch** dye. (Your TAs will demonstrate how to use the Fluorometer).

Note: Hoesch dye is sensitive to light; therefore, the 1 mg/mL Hoesch dye solution is stored in a 14-mL tube wrapped with aluminum foil at 4°C. The tube of 1 mg/mL Hoesch dye solution and a microcentrifuge tube containing a standard DNA solution of 100 ng/μL are stored in a 1-liter plastic container on the first left shelf in the cold room. Return the plastic bottle containing the Hoesch dye solution and the standard DNA solution to the cold room as soon as you finish with it.

Record **concentration of DNA** solution in the **table** below:

Samples	DNA Concentration (ng/μL)
Plant #1	
Plant #2	
Plant #3	
Plant #4	
Plant #5	
Plant #6	

Question: Why do you use the Fluorometer instead of the Nanodrop spectrophotometer to determine DNA concentration for these DNA solutions?

Answer: Two following reasons:

a. Because the **major components** in the DNA solutions are **ribosomal RNAs** and **tRNAs**, the concentration of DNA determined by the Nanodrop or any other spectrophotometer reflects mostly the concentration of RNAs. Thus, you do not know the DNA concentration of your DNA solutions.

b. **Property of Hoesch dye H33258** allows us to estimate DNA concentration of the DNA samples containing RNAs (see explanation taken from the **Instruction Manual** for TKO 100 Dedicated Mini Fluorometer - Hoefer Scientific Instruments)

Table: Excitation and Emission Spectra of Hoesch Dye H33258

	Excitation Spectrum peaks at	Emission Spectrum peaks at
Absence of DNA	356 nm	492 nm
Presence of DNA	365 nm	458 nm

The fluorescence enhancement provided by using the Hoesch H33258 dye has been shown to be **highly specific for DNA**, binding preferentially to A-T rich regions (Brunk et al., 1979; Labarca and Paigen, 1980). The dye binds twice as well to **double-stranded DNA** as to **single-stranded DNA**, but does not appear to intercalate (Brunk et al., 1979).

RNA enhances the fluorescence of H33258 to a much smaller extent than DNA. Under high salt conditions, in which chromatin proteins are fully dissociated from DNA leading to the increase the fluorescence enhancement of the DNA/dye complex, RNA enhancement is usually well below 1% of that produced by the same concentration by weight of DNA (Labarca and Paigen, 1980). For this reason, the presence of RNA in the sample does not interfere with the quantitation of DNA. Because RNA does not compete with DNA for binding with H33258, it is, therefore, extremely useful for estimating the DNA content of samples containing RNA. Thus, the Hoesch Dye allows us to measure the concentration of solely the DNA present in a given solution.

References:

- Brunk, C. F., Jones, K.C., and James, T.W. (1979). Assay for nanogram quantities of DNA in cellular homogenates. *Anal. Biochem.* 92: 497-500.
- Labarca, C. and Paigen, K. (1980). A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* 102: 344-352.

39. Dilute **5 μL** of **original DNA solutions** to a **final concentration** of **0.2 $\text{ng}/\mu\text{L}$** with TE buffer. Label on the lids and sides of microcentrifuge tubes with the following information: **0.2 $\text{ng}/\mu\text{L}$, plant#, your initial, and date**. Keep all tubes of DNA solutions **on ice**.

Note: Dilution of DNA solutions would serve two purposes: (a) contaminants, such as carbohydrates that bind nonspecifically to nucleic acids and proteins, in DNA solutions will be diluted out. Therefore, a tiny amount of contaminants in PCR reactions will not interfere with the amplification of targeted DNA. (b) ONLY small amount of Arabidopsis genomic DNA (~0.4 ng) is needed for the PCR amplification. How to make a dilution?

Use the basic formula that is widely used in general chemistry lab. That is,

$$V_i \cdot C_i = V_f \cdot C_f$$

where,

V_i = **initial volume** (the volume of original DNA solution is **5 μL**)

C_i = **initial concentration** (reading from the Fluorometer; example: **8 $\text{ng}/\mu\text{L}$**)

V_f = **final volume** (depends on the initial concentration)

C_f = **final concentration** (**0.2 $\text{ng}/\mu\text{L}$**)

then,

$$V_f = (V_i \cdot C_i) / C_f = (5 \mu\text{L} \times 8 \text{ ng}/\mu\text{L}) / (0.2 \text{ ng}/\mu\text{L}) = 200 \mu\text{L} \text{ of total volume}$$

What is the volume of TE to be used in dilution?

$$V_{\text{TE}} = V_f - V_i = 200 \mu\text{L} - 5 \mu\text{L} = 195 \mu\text{L} \text{ of TE}$$

Record **volume of TE** and **final volume** in the **table** below

	Volume of isolated genomic DNA	Volume of TE	Final Volume
Plant #1			
Plant #2			
Plant #3			
Plant #4			
Plant #5			
Plant #6			

III. IDENTIFYING PLANTS CONTAINING T-DNA INSERTED IN THE GENE OF INTEREST AND THEIR GENOTYPES

Purpose: To identify plants containing T-DNA insert and determine genotypes of T-DNA tagged plants as heterozygous and/or homozygous for T-DNA.

Materials and Reagents Needed:

- 12 μ M Gene-specific Forward primer
- 12 μ M Gene-specific Reverse primer
- 12 μ M **Lb1** primer (Left Border (LB) region of T-DNA from **SALK** Lines)
- 10X Ex-Taq buffer
- dNTP Mix
- Ex-Taq DNA polymerase
- Sterile water
- 1-kb DNA ladder
- PCR Machine (Applied Biosystems GeneAmp 9700 or BioRad MyCycler)
- 0.2 mL PCR tubes
- 1.5 mL microcentrifuge tubes
- P-10, P-20, P-200 Pipetman
- PCR rack for 0.2 mL PCR tubes
- Rack for 1.5 mL microcentrifuge tubes
- Filtered Pipet tips for PCR
- Ice bucket
- Gloves
- Microcentrifuge
- Agarose
- Gel apparatus and power supply
- Bio-Rad Gel Documentation System

PROCEDURE

Note: There are **6** plants to be characterized and **2 controls** (genomic DNA isolated by TA + No DNA template), prepare a **master mix** for **8 + 1 extra = 9 reactions**.

1. Label on the lids and sides **8 PCR tubes** and put them on a PCR rack sitting on ice.
2. Prepare a **master mix** for **9 PCR reactions** in a 1.5 mL microcentrifuge tube labeled as "**Mmix**" sitting on ice.

Note: The reaction volume is **reduced** from **50 μ L** in previous reactions to **25 μ L**.

	Mmix for ONE reaction	Mmix for 9 reactions
Sterile water	16.5 μ L	148.5 μ L
10x Ex-Taq buffer	2.5 μ L	22.5 μ L
dNTP mix	2.0 μ L	18.0 μ L
12 μ M Gene-specific Forward primer	0.5 μ L	4.5 μ L
12 μ M Gene-specific Reverse primer	0.5 μ L	4.5 μ L
12 μ M LBB1 primer (for SALK lines)	0.5 μ L	4.5 μ L
Ex-Taq DNA polymerase (5 U/ μ L)	0.5 μ L	4.5 μ L
Total Volume	23.0 μL	207.0 μL

3. Mix the contents by flicking the tube five times or vortexing for the tube containing the master mix for **5 seconds**. Spin the tube in a microcentrifuge for **10 seconds**. Put the tube back **on ice**.
4. Pipet **23 μ L** of the **Mmix** into each of **8 PCR tubes**.
5. Pipet **2 μ L** of **0.2 ng/ μ L** genomic DNA extracted from each of **6** seedlings/plants into PCR tubes #1-6. Pipet up and down for five times to mix the contents. Put the first tube back on ice and work on the remaining tubes.
6. Pipet **2 μ L** of **0.2 ng/ μ L** genomic DNA extracted (by TAs) from **wild type** (Col-0) seedlings into each of tubes **#7**. Pipet up and down for five times to mix the contents.

7. Pipet **2 μ L** of **sterile water** to tube **#8 (negative control)** without DNA template).
Pipet up and down for five times to mix the contents.
8. Spin PCR tubes in the microcentrifuge for PCR tubes for **5 seconds** to bring the liquid to the bottom of the tubes.
9. Put the tubes on the **wells** of the PCR machine.
10. Perform PCR with the "**KNOCKOUT**" program with the following profile:
 - 1 cycle of Hot start or 96°C for 3 minutes
 - 36 cycles of 94°C, 15 seconds -> 60°C, 30 seconds -> 72°C, 2 minutes
 - 1 cycle of 72°C, 4 minutes
 - 4°C, ∞
11. Prepare a **1% agarose gel** in 1X TAE buffer with a **20-tooth** comb.
12. Label **8 1.5-mL microcentrifuge** tubes and set them on a rack.
13. Add **2 μ L** of **loading dye** to each tube.
14. Pipet **10 μ L** of **PCR solutions** to each tube.
15. Load samples on the **1% agarose gel** along with **10 μ L** of **diluted DNA ladder** solution on each side of the loaded samples. Record sample loading pattern below:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

16. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) is two-thirds of the gel.
17. Stop the gel electrophoresis.
18. Take a picture of the gel.
19. Analyze data.
 - Do you observe PCR fragments?
 - What are the sizes of these fragments?
 - Do the sizes agree with expected sizes for the gene of interest and T-DNA insertion?

20. After determining the genotypes of T-DNA insertion plants, put small piece of tape on each of a number of wooden sticks corresponding to the number of T-DNA tagged plants (homozygous or heterozygous for T-DNA). Write the **number** that corresponding to the **plant #** on the Plant Layout chart and either homozygous or heterozygous.
21. Go to the Plant Growth Center, put the wooden sticks next to the identified T-DNA tagged plants.
22. Observe T-DNA tagged plants for abnormal phenotypes.

DETERMINATION OF T-DNA INSERTION SITE

Purpose: To verify the location of T-DNA insertion site in the gene of interest indicated by the SALK Institute Genomic Analysis Laboratory website.

Note:

1. *Although the results of PCR reactions should confirm the size of the so-called "T-DNA fragment", which contains a portion of the plant gene and T-DNA region, it is a **good scientific practice** to verify the exact location of T-DNA insert site.*
2. *Depending on the PCR results on the **first screen** of 5 SALK plants, you can use one of the following procedure to purify PCR products.*

*a. if plants of **heterozygote for T-DNA** and wild type are identified, then the "T-DNA fragment" must be purified from a gel agarose slice (see **QIAquick Gel Extraction procedure**) below. Because you already learned how to purify PCR products using QIAquick PCR Purification kit in the Experiment ONE, **for this experiment** you will learn how to purify PCR product via **Gel Electrophoresis** even though you may obtain a homozygote for T-DNA.*

*b. if a plant of **homozygote for T-DNA** is identified, then the "T-DNA fragment" can be purified directly from the PCR solution as carried out in the Experiment ONE (see **QIAquick PCR Purification procedure**) below.*

A. PURIFICATION OF PCR PRODUCTS

QIAquick Gel Extraction Procedure

Reference: Qiagen QIAquick Gel Extraction protocol

Reagents and Materials Needed:

- PCR solutions
- Agarose
- QIAquick Gel Extraction Kit (Qiagen, Cat. # 28704)
- Isopropanol
- PCR solutions of super pools containing knockout lines
- 6X Loading dye
- 10 mg/mL Ethidium Bromide solution
- 1X TAE buffer
- Gel apparatus and a power supply
- Razor blade
- 50°C water bath
- 1.5-mL microcentrifuge tubes
- Microcentrifuge
- Scale
- Metal waste container for sharp objects

PROCEDURE

1. Prepare a **1% agarose** gel with a **20-tooth comb**.
2. Add **4 μ L** of **6X loading dye** to each tube of **\sim 25- μ L** PCR solutions.
3. Load the samples on the gel.
4. Record loading pattern below:

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

5. Run the gel at **105 volts** for 1.5 - 2 hours.
6. Take a picture of the gel.
7. Verify the presence of **expected size PCR product**.

8. Label on the **lids** of **TWO** microcentrifuge tubes "**T-DNA**", "**WT**", and **your initial**.
9. Place a **NEW** piece of plastic wrap on an UltraViolet (UV) box, then place your gel on the plastic wrap.
10. Put on a UV shield to protect your eyes and face.
11. Turn **on** the **UV box**. *Note: Turn **off** the UV box as soon as you are done with excising DNA band(s).*
12. Excise desired fragment from the DNA gel using a razor blade. *Note: Trim off excess agarose surrounding the DNA band as much as possible (your TAs will demonstrate).*
13. Place the agarose slice in the **appropriate 1.5-mL microfuge tube**. Repeat this step for more than one DNA fragments.
14. Take a picture of the gel **after removing excised agarose slice(s)**. *This step serves as a record of DNA fragment(s) being collected.*
15. Centrifuge the gel fragment for **1 minute**.
16. Estimate the **gel volume** in the microfuge tube using a **scale**. Write the **weight** on the side of the tube. *Note: **0.1 g** of the **agarose slice** is **equivalent to 100 μ L**.*
17. Add **3 gel volumes** of **buffer QG** to tube containing agarose slice. *For example, if the **weight of the agarose slice** is **0.15 g**, then its **gel volume** is **150 μ L**. Therefore, add **450 μ L** of **buffer QG** to the tube.*
18. Incubate tube at **50 °C** in a **water bath** for **10 minutes** or until the gel slice has dissolved. *To help dissolve gel, you may vortex the tube for 5 seconds during incubation. This step solubilizes the agarose completely. Make sure the color of the mixture is yellow.*
19. Add **1 gel volume** of **isopropanol** to the mixture and mix by **vortexing** for **5 seconds** or **inverting** the tube for **5-10 times**. *This increases the yield of DNA fragments.*
20. During incubation, obtain **spin columns (purple)** in their **collection tubes**. Label on the **side** of the **spin columns** and **collection tubes** "**T-DNA PCR**", "**WT PCR**", and **your initial**.
21. Pipet the **mixture** from **step 19** to the appropriate **spin columns (purple)**. *Do NOT pipet **more than 800 μ L** of the mixture into the column. If the total volume is **more than 800 μ L**, repeat steps 21-23.*

22. Centrifuge the tube for **1 minute**.
23. Separate the **spin column** from the **collection tube** and then **pour off** the **flow-through solution** in collection tube. Put the spin column back in the collection tube. *This step allows DNA binding to the membrane. Keep collection tube for use in steps 24-26.*
24. Add **500 μ L** of **buffer QG** to the **spin column** and centrifuge for **1 minute**. Discard the flow-through solution. *This step removes all traces of agarose.*
25. Add **750 μ L** of **buffer PE** and let the tube stand for **2-5 minutes**. Centrifuge the tube for **1 minute**. *This step washes the column.*
26. Discard the **flow-through solution** and centrifuge **1 minute** to remove all the **ethanol** from the column.
27. While spinning the tubes, label on the lids and sides of NEW 1.5-mL microcentrifuge tubes "**T-DNA PCR**", "**WT PCR**", **your initial**, and **date**.
28. After spinning, transfer the **spin columns** in the **appropriate labeled microcentrifuge tubes**. *Note: Make sure that the labels on the spin columns corresponding to those on the microcentrifuge tubes.*
29. Add **30 μ L** of **buffer EB** to the **center of the membrane**. Let the columns stand for **1 minute**, and then centrifuge for **1 minute**. *This step elutes the DNA from the membrane. DNA is in the microcentrifuge tube.*
30. Discard the collection tube.
31. Determine DNA concentration using a Nanodrop spectrophotometer (measuring nucleic acids) or a DNA Fluorometer (measuring only DNA).

What is the concentration of purified PCR product? _____ **ng/ μ L**

What is the size (in bp) of the PCR product from gel electrophoresis? _____ **bp**

QIAquick PCR Purification Procedure

Materials and Reagents Needed:

- QIAquick PCR Purification Kit (Qiagen, Cat. # 28104)
- PCR solutions
- 1.5 mL microcentrifuge tubes
- Microcentrifuge
- Nanodrop spectrophotometer

Note: This procedure is used when you run 10 μ L of PCR products on the gel and identify homozygote for T-DNA or wild type.

1. Write on the **lids** and **sides** of **1.5-mL microcentrifuge** tubes "**T-DNA**" or "**WT**", and **your initial**.
2. Pipet **15 μ L** of the **PCR product solution** from the PCR tube containing the T-DNA fragment or gene-specific DNA fragment into the 1.5-mL microcentrifuge tube.
3. Add **75 μ L** of **Buffer PB** (or **5 volumes** of **Buffer PB** to **1 volume** of the **PCR sample**) to the tube in step 2. Mix by vortexing the tube for 5 seconds. Spin the tube in the microcentrifuge at **FULL speed** for **10 seconds** to bring all the solution down to the bottom of the tube. Set the tube back on the microcentrifuge rack.
4. Place a **QIAquick spin column** in a provided **2-mL collection tube**.
5. Apply the sample mixture in step 3 to the QIAquick column. Spin the column set in the microcentrifuge at **FULL speed** for **1 minute**. *This step allows the binding of DNA to the membrane*
6. Discard the **flow-through solution** in the collection tube. Put the QIAquick column back into the same collection tube.
7. Add **750 μ L** of **Buffer PE** to the QIAquick spin column and spin at **FULL speed** for **1 minute**.
8. Discard the **flow-through solution** in the collection tube. Put the **QIAquick column back** into the same collection tube.

9. Spin the column set at FULL speed for an **additional 1 minute** to get rid of residual ethanol in Buffer PE. *Caution: Residual ethanol from Buffer PE will NOT be completely removed unless the flow-through solution is discarded before this additional spin.*
10. While spinning, label on the **lids** and **sides** of **1.5-mL microcentrifuge** tubes "**Purified T-DNA PCR**" or "**Purified WT PCR**", **your initial**, and **date**.
11. Transfer the appropriate **QIAquick columns** in the **NEWLY labeled microcentrifuge tubes**. Discard the flow-through solutions and the collection tubes.
12. Pipet **30 μ L** of **Buffer EB** to the center of the QIAquick membrane. Let the column sit for **1 minute**, and then centrifuge at **FULL speed** for **1 minute**. *This step elutes the DNA from the QIAquick membrane.*
13. Determine DNA concentration using the Nanodrop spectrophotometer (measuring nucleic acids) or a DNA Fluorometer (measuring only DNA).

What is the concentration of purified PCR product? _____ **ng/ μ L**

What is the size (in bp) of the PCR product from gel electrophoresis? _____ **bp**

SEQUENCING REACTION WITH BIG DYE V. 3

Purpose: To determine the exact location of T-DNA insertion site in the gene of interest from the SALK T-DNA knockout line.

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 μ M LBb1 primer (for T-DNA)
- 3 μ M Gene-specific Forward primer
- 3 μ M Gene-specific 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- μ L** 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 μ L = the volume depends on **concentration** and **amount** of **DNA**

(see Table below)

y μ L = the **remaining volume** to bring the **total volume** to **20 μ L**

* **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.
- For **PCR product**, use the amount of DNA according to the **table** on the next page (Taken from Perkin-Elmer Big Dye Protocol). *Note: Use the **maximum** amount of DNA in the reaction if there is more than enough*

DNA available. For example, for **PCR product of 200 - 500 bp**, use **10 ng** of DNA.

Table: Amount of DNA Used in Sequencing Reactions Depending on Size of PCR Fragment

Size of PCR Product (bp)	Amount of DNA Used in Reactions
100 - 200	1 - 3 ng
200 - 500	3 - 10 ng
500 - 1000	5 - 20 ng
1000 - 2000	10 - 40 ng
> 2000	40 - 100 ng

For this exercise, there is **ONE DNA template**, i.e. the purified PCR product of the T-DNA fragment; but, there are **TWO primers**, LBb1 (T-DNA) primer and a gene-specific primer (either forward or reverse) depending on the orientation of the T-DNA Left Border (LB) inserted in the gene of interest (based on your analysis of the SALK line). The **sequencing reaction with the gene-specific primer** serves as a **control for the master mix** of Big Dye and Dye Dilution mix. 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 PCR product? _____ **ng/μL**

What is the size of the PCR product? _____ **bp**

What is the amount of DNA to be used? _____ **ng**

Sample calculations:

Size of PCR product is 400 bp and its concentration is 4.5 ng/μL

Want to use 10 ng of purified PCR product (see table above)

Hence, the amount of PCR to be used is $10 \text{ ng} / 4.5 \text{ ng}/\mu\text{L} = 2.2 \mu\text{L}$

What is the volume of PCR product solution to be used? _____ **μL**

PROCEDURE

7. Get ice from the icemaker in room 2911 or 3906.
8. 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.
9. Label on the **lid** and **side** of a **1.5-mL microcentrifuge tube** as “**Mmix**” and **your initial**. Set the tube on ice.
10. 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 2 reactions
DNA template	x μ L	x (x 3) μ L
Sterile water	y μ L	y (x 3) μ L
Big Dye v. 3	2.0 μ L	6.0 μ L
Dye Dilution Mix (Sigma, S3938)	2.0 μ L	6.0 μ L
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 **gene-specific primer** into TWO labeled 0.2-mL PCR tubes.

Components	LBb1 primer	either	or
		Gene-specific <u>Forward</u> primer	Gene-specific <u>Reverse</u> primer
Mmix	19 µL	19 µL	19 µL
3 µM LB1 primer	1 µL	-----	-----
3 µM Gene-specific Forward primer	-----	1 µL	-----
3 µM Gene-specific Reverse primer	-----	-----	1 µL
Total volume	20 µL	20 µL	20 µL

11. 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.

12. After the cycling reaction is finished, clean up sequencing reactions using Edge

Biosystems spin columns (stored in the cold room) as following:

- g. Spin the pre-packed columns in a microcentrifuge at 3,000 rpm for 2 minutes at room temperature.
- h. Meanwhile, label a new set of 1.5 mL microcentrifuge tubes according to your reactions.
- i. Transfer the columns to new tubes.
- j. Pipet 20 µL of sequencing reaction to appropriate columns.
- k. Spin the columns as in step a.
- l. Discard the columns.

6. 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.*
7. 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.

13. From any computers in the lab, Log in to the UCLA Sequencing Retrieval System via <http://www.genetics.ucla.edu/webseq/>

14. Enter in the USER NAME field: **goldberg_r**

15. Enter in the PASSWORD field: **embryo**

16. 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".

17. Select sequences to be downloaded, and click "DOWNLOAD SELECTED" or click on "SEQUENCE FILE TO DOWNLOAD".

18. Select "SAVE TO DISK" and choose "THE DESKTOP".

19. Open the saved file using a SEQUENCE VIEWER PROGRAM (CHROMAS on Windows or EDITVIEW on Mac).

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

21. 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).
22. Determine if the DNA sequence corresponds to the gene of interest.
23. Print out the Blast results as hard-copy records.
24. Save the Blast results in the **pdf** format so that you can upload them in your webbook.

EXPERIMENT 3 – GENE EXPRESSION STUDY IN *ARABIDOPSIS THALIANA* (GENE ONE)

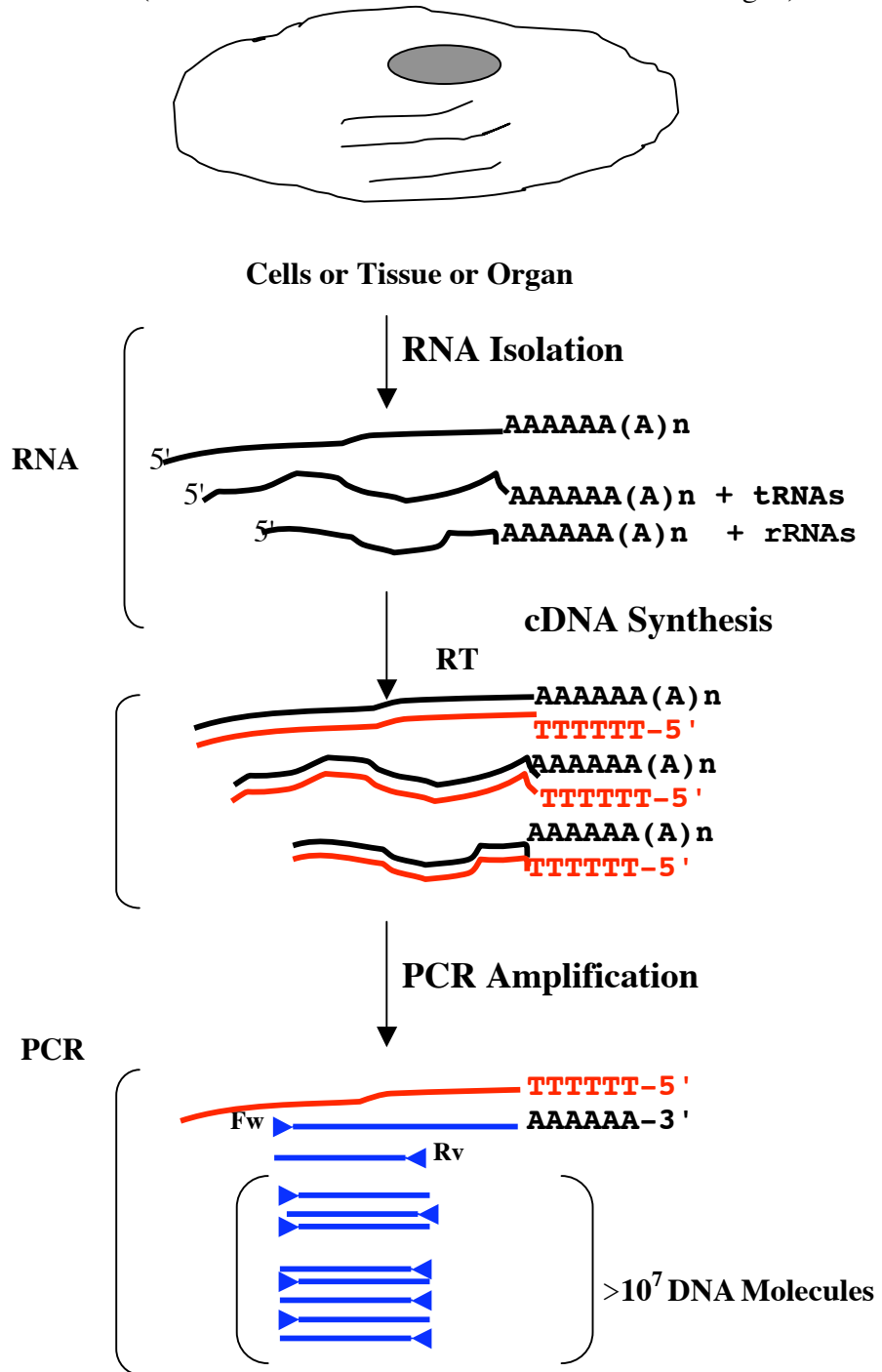
Purpose: To determine mRNA accumulation patterns of genes encoding transcription factors in Arabidopsis leaves and seeds.

OVERVIEW OF RT-PCR

STRATEGY

- I. ISOLATION OF TOTAL RNA USING QIAGEN RNEASY PLANT MINI KIT**
- II. SYNTHESIZING FIRST STRAND cDNA USING REVERSE TRANSCRIPTASE (REVERSE TRANSCRIPTION or RT)**
- III. CARRYING OUT PCR AMPLIFICATION (RT-PCR) ANALYSIS**

OVERVIEW OF RT-PCR (Based on RT-PCR Technical Note from Invitrogen)



I. ISOLATION OF TOTAL RNA USING QIAGEN RNEASY PLANT MINI KIT

Purpose: To extract total RNA from tissues/organs for gene expression study.

Reference:

- Qiagen's RNeasy Plant Mini Kit Protocol (accompanied the kit).
- Sambrook et al. (1989). Molecular Cloning Manual Volume 1.
- Ambion's DNase-Free Technical Bulletin

FREQUENT ASKED QUESTIONS

PROCEDURE

A. RNA ISOLATION

B. REMOVING CONTAMINATED GENOMIC DNA FROM TOTAL RNA SOLUTION USING RNase-FREE DNase I

C. DETERMINING QUALITY OF ISOLATED TOTAL RNA BEFORE AND AFTER DNase I-TREATMENT VIA GEL ELECTROPHORESIS

Materials and Reagents Needed:

- Plant organs: leaves and seeds from Arabidopsis.
- Qiagen RNeasy Plant Mini Kit: (Cat. # 74903 for 20 extractions or 74904 for 50 extractions) containing extraction buffer, PE buffer, RNase-free water.
- DiEthyl PyroCarbonate (DEPC). *Note: DEPC is suspected to be carcinogen and corrosive. Therefore, it is handled with care! DEPC inhibits RNase.*
- One to Three Gel apparatus (gel boxes, 20-tooth combs, gel trays and casts) treated with DEPC water and air-dried on a drying rack overnight.
- **500 mL** of freshly prepared 0.05% **DEPC-treated water (non-autoclaved)** for cleaning up pipetman, microcentrifuge rotor and chamber, racks for microcentrifuge tubes, SpeedVac rotor and chamber, vortex mixer.
- β -mercaptoethanol. *Caution: work in the fume hood because this chemical has very bad odor.*
- RNA ladder (Invitrogen)
- Formamide (Ultrapure grade, Invitrogen)
- 37% formaldehyde (Fisher)
- Ambion DNase I kit (stored at -20°C)
- Sevag (24 chloroform :1 isoamyl alcohol, v/v)
- Buffer-saturated phenol (Invitrogen)
- 3M NaOAc, pH 5.2 or 6.0
- Ice-cold 100% and 80% ethanol solutions dedicated for RNA work
- Loading dye for RNA samples
- 10X MOPS buffer
- **Autoclaved DEPC-treated (DEPC'd) water**
- Agarose dedicated for RNA work
- 5 mg/mL ethidium bromide. *Caution: this chemical is suspected carcinogen.*
- Liquid Nitrogen. *Caution: It is very cold (at least -100°C). Avoid getting frost-bite.*
- Black ultra-fine sharpie
- RNase-free spatulas
- RNase-free 14-mL disposable centrifuge tubes
- RNase-free 1.5 mL microcentrifuge tubes
- Racks for microcentrifuge tubes
- RNase-free filtered PCR tips for P-20, P-200, P-1000
- White Revco storage boxes
- Gloves (small, medium, large, or extra-large)
- Microcentrifuges
- Razor blades
- Kimwipes
- Plastic wrap
- SpeedVac
- Spectrophotometer (Nanodrop)
- Vortex
- Ice bucket or plastic container
- 55°C Water bath
- 37°C Heat block

FREQUENT ASKED QUESTIONS

(Taken from Qiagen RNeasy Plant Mini Handbook June 2001)

1. What is the maximum amount of starting material?

100 mg

2. Is the yield of total RNA the same for the same amount of starting material for different plant species?

No, the yield varies for different plant species.

3. Which lysis buffer can be used for plant materials?

- Buffer **RLT** (Guanidine Isothiocyanate) is used for all tissues except endosperm and tissues containing endosperms (e.g., seeds).
- Buffer **RLC** (Guanidine Hydrochloride) is used for seeds with endosperm

4. Is total RNA isolated with RNeasy kit free of genomic DNA?

No, most (but not all) of DNA is eliminated. Therefore, if total RNA will be used for downstream application such as Reverse-transcription-PCR (RT-PCR), then DNase I-treatment must be carried out for the total RNA.

5. What is the role of QIAshredder homogenizer?

It simultaneously **removes insoluble material** and **reduces the viscosity of the lysates** by disrupting gelatinous material.

Cautions:

- **All steps of the RNeasy protocol should be carried out at room temperature.**
During the procedure, **work quickly.**
- **All centrifugation steps are carried out at 20-25°C.** Ensure that the centrifuge does not cool less than 20°C.
- **Keep** all reagents, glassware, plasticware, and equipment **RNase-free.**
- **Use aerosol-barrier pipet tips throughout the procedure.**
- **Change GLOVES frequently!**

PROCEDURE

Attention: Before isolating RNA, use Kimwipes wetted with freshly prepared non-autoclaved DEPC treated water to clean all equipments (pipetman sets, pipetman stand, microcentrifuge-tube racks, microcentrifuges and its rotors, speedvac, test-tube racks, pens and sharpies) to be used in isolating RNA.

A. RNA ISOLATION

1. Label on the WHITE area on the side of TWO RNase-free **14-mL disposable centrifuge tubes "Leaf" or "Seed" and your initial**. Chill them on either **crushed dry ice** or a styrofoam floater in a styrofoam box containing **liquid nitrogen** (filling up to one-third of the styrofoam).
2. Chill **RNase-free** spatulas in a Dewar flask containing liquid nitrogen.
3. Remove bottles/tubes containing **frozen ground organs** from a **-80 °C freezer** and set them on **crushed dry-ice** in a styrofoam container or in **liquid nitrogen**.
4. (Option) Add **small amount of liquid nitrogen** to the bottles containing **ground organs** (leaves, seeds) to ensure that the frozen powder is not partially thawed out.
5. Use a **chilled spatula** to transfer small amount (**~100 mg**) of **frozen ground material** from the bottle to a chilled **14-mL centrifuge tube**. **Keep** the tube on dry ice or in liquid nitrogen. Proceed with other tubes for all organs.

<u>Organs</u>	<u>Type of Lysis Buffer used</u>
➤	RLT or RLC (circle one)
➤	RLT or RLC (circle one)
➤	RLT or RLC (circle one)
➤	RLT or RLC (circle one)
➤	RLT or RLC (circle one)
➤	RLT or RLC (circle one)

6. Aliquot an **appropriate volume (= # of organs x 500 µL)** of **lysis buffer** to a **14-mL disposable centrifuge tube** standing on a test-tube rack. (TA will prepare lysis buffer and β-mercaptoethanol).

7. Add **10 μL** of **β -mercaptoethanol** for every **1 mL** of **lysis buffer** in a fume hood.
Mix well by vortexing for 5 seconds. Put the tube back on the rack.

Note: β -mercaptoethanol is toxic and has a bad odor. It is kept in the fume hood in room LS 2828.

<u>Volume of lysis buffer (mL)</u>	<u>Volume of β-mercaptoethanol (μL)</u>
_____ RLT	_____
_____ RLC	_____

8. Remove the chilled tube containing **$\sim 100\text{mg}$** of ground organ powder from the styrofoam container and set on the rack at room temperature. Briefly, tap the tube on the bench to loosen frozen powder.
9. **Immediately**, pipet **450 μL** of **lysis buffer containing β -mercaptoethanol** into the 14-mL tube containing **$\sim 100\text{ mg}$** of starting organ. Cap the tube. **Immediately**, vortex the tube **vigorously** for at least **1-2 minutes**. Then set the tube back on a tube rack. *The lysate should appear clear with no lumps of ground organ powder. (Optional) A short incubation time (1-3 minutes) at 56°C may help to disrupt the tissue. But NOT appropriate for an organ, such as seeds or old leaves, rich in starch.*
10. **Repeat steps 8-9** for all organs.
11. Label **QIAshredder (purple)** spin columns placed in **2-mL collection tubes**.
12. Pipet the lysate directly onto a QIAshredder spin column.
13. Centrifuge at **FULL speed** (13,200 rpm) for **2 minutes**.
14. Meanwhile, label on the lids of TWO 1.5-mL RNase-free microcentrifuge tubes "**Leaf**" or "**Seed**" and **your initial**. Set the labeled tubes on a microcentrifuge-tube rack at room temperature.
15. Transfer **$\sim 450\ \mu\text{L}$** of the **supernatant (= volume of sample)** of the **flow-through solution** to a **NEW RNase-free 1.5-mL microcentrifuge tube** without disturbing the cell-debris pellet.
16. Add **0.5 volume** (or **225 μL**) of **room temperature 96-100% ethanol** to the **clear lysate**. **Immediately**, mix the mixture by pipetting **up and down for 10 times**.

17. Apply ~**675 μ L** of the **mixture** (including any precipitate) to an **RNeasy (pink) mini column** placed in a **2-mL collection tube**. Close the lid of the tube **gently**.
18. Centrifuge for **15 seconds** at **10,000 rpm** (or FULL speed).
19. Remove the **spin column** from the collection tube with one hand and hold it while **pouring** off the **flow-through solution** in the **collection tube**. Put the column back on the collection tubes.
Note: if the sample volume is >700 μ L, load aliquots successively onto the RNeasy column and centrifuge as before.
20. Add **700 μ L** of **buffer RW1** to the RNeasy column. Close the lid of the tube.
21. Centrifuge for **15 seconds** at **10,000 rpm** to wash the column.
22. Transfer the column(s) to **NEW 2-mL collection tube(s)**.
23. Discard the flow-through solution and collection tubes.
24. Pipet **500 μ L** of **buffer RPE** onto each RNeasy column.
25. Centrifuge for **15 seconds** at **10,000 rpm** to wash the column.
26. Discard the flow-through solution as in step 18.
27. Add another **500 μ L** of **buffer RPE** to the column.
28. Centrifuge for **1 minute** at **10,000 rpm** to wash the RNeasy silica-gel membrane again.
29. Discard the flow-through solution as in step 18.
30. Spin the column **again** for **1 minute** to ensure that ethanol is removed completely from the membrane. *Caution: This step is **crucial** because if residual ethanol is still on the membrane, it will be eluted with RNA in steps 32-35. If this is the case, RNA solution will float up when it is loaded on an agarose gel.*
31. While spinning at step 29, label on the **lid** and **side** of RNase-free 1.5-mL microcentrifuge tubes "**Name of Sample RNA**", "**your initial**", and "**date**".
32. Transfer the **spin columns** to these **NEW labeled tubes**.
33. Pipet 30 μ L of **RNase-free water** (supplied with the kit) or DEPC-treated water directly onto the center of the silica-gel membrane of the RNeasy columns.
34. Wait for **1 minute** to allow water to evenly absorbed in the membrane.

35. Centrifuge for **1 minute** at **10,000 rpm** to elute RNA.
36. Repeat steps 32-34 with **another 20 µL** of **RNase-free water**.
37. Mix the content in the tubes by gently flicking. Put tubes **on ice**. *Note: From this step on, **KEEP RNA solution ON ICE** to prevent RNA degradation.*
38. Determine the **total volume** of **RNA solution** using a P-200 pipetman. The volume should be **~48 µL**.
39. Determine **RNA concentration** and **total amount** using a **spectrophotometer**.
*Note: (a) If **Nanodrop** spectrophotometer is used, there is **NO** need to make a dilution of RNA solution, and the concentration is directly given in **ng/µL**;(b) however, if **Beckman** or **other brand** spectrophotometer is used, dilute a small volume of RNA solution to 1/50 dilution (i.e., 4 µL RNA solution in 200 µL total volume), and the calculation is determined as shown below:*

Calculations:

$$[\text{RNA}] = (\text{OD}_{\text{A}260} \text{ reading}) (\text{Dilution factor}) (40 \mu\text{g}/\text{mL} \cdot \text{OD}) = \mathbf{X \mu\text{g}/\text{mL}} \text{ or}$$

$$= \mathbf{X \mu\text{g}/\mu\text{L}}$$

$$\text{Total amount of RNA} = (\text{X } \mu\text{g}/\mu\text{L}) (\text{Volume of RNA solution in } \mu\text{L}) = \mathbf{Y \mu\text{g}}$$

Records of organs and their RNA concentration and total amount

Organs	[RNA] (µg/µL)	Estimated Total Amount (µg)
Leaves		
Seeds		

Attention: *To be safe, only **HALF** of the volume of the RNA solution is treated with **RNase-free DNase I**; the remaining volume of RNA solution is kept on ice or stored in the -20°C RNA freezer until gel electrophoresis step to determine quality of RNA before DNase I treatment.*

B. REMOVING CONTAMINATING GENOMIC DNA FROM TOTAL RNA SOLUTION USING RNase-FREE DNase I

Reference: Modification from the Ambion protocol accompanied the DNase-Free components (Cat # 1906).

Important Note: This protocol is suitable for removing up to 1 µg of DNA from RNA in a 25-100 µL reaction volume.

PROCEDURE

1. Add **0.1 volume** of **10X DNase I buffer** and **1 µL** of **2 Units/µL DNase I** (Ambion) to the RNA solution. **One unit** of **DNase I** is defined as the amount of enzyme that degrades **1 µg of DNA** in **10 minutes** at **37°C** (Ambion).

	RNA solution			
DEPC'd water	1.0 µL			
RNA sample	25.0 µL			
10X DNase I buffer	3.0 µL			
DNase I (2 Units/µL)	1.0 µL			
Total volume	30.0 µL			

2. Mix the solutions gently by flicking the tubes. Spin briefly (**10 seconds**).
3. Incubate at **37°C** in a **heat block** for **20-30 minutes**. Spin tubes for **10 seconds** in a microcentrifuge.
4. To inactivate DNase I, add **0.1 volume** (or **3.0 µL**) of the **DNase inactivation reagent (slurry)** to the sample. Mix well by **flicking the tube**.
Note: Make sure the slurry is WHITE. If the DNase inactivation reagent is CLEAR, revertex the mixture for a few seconds.
5. Incubate the tube at **room temperature** for **2 minutes**. Flick the tube **once more during the incubation** to re-disperse the **DNase inactivation reagent**.

6. At the meantime, label on the **lids** and **sides** of **NEW RNase-free microcentrifuge tubes** "**Purified Leaf RNA**" or "**Purified Seed RNA**", "**your initial**", and "**date**".
7. Spin the tube at **~10,000 rpm** for **1 minute** to pellet the **DNase inactivation reagent**.
8. **Carefully**, Pipet **~30 μL** of the **RNA solution** (*AVOID pipetting the PELLET!*) and transfer it into NEW labeled tubes. *Note: It is okay if tiny amount of the pellet is carried over in the RNA solution.*
9. Keep RNA tubes **on ice**.
10. Determine RNA concentration using a Nanodrop or Beckman spectrophotometer

Calculations (if Using Beckman spectrophotometer):

$$[\text{RNA}] = (\text{OD}_{\text{A}260} \text{ reading}) (\text{Dilution factor}) (40 \mu\text{g}/\text{mL} \cdot \text{OD}) = X \mu\text{g}/\text{mL}$$

$$\text{Total amount} = (X \mu\text{g}/\text{mL}) (\text{volume of RNA solution}) = Y \mu\text{g}$$

Note: 1 μg = 1,000 ng; therefore, you need to convert ng/ μL to $\mu\text{g}/\mu\text{L}$

Organs	[RNA] ($\mu\text{g}/\mu\text{L}$)	Estimate Total Amount (μg)
Purified Leaf RNA		
Purified Seed RNA		

11. Store the RNA solution at **-20°C for up to 1 week** or **-70°C for up to 6 months**. **Alternatively**, precipitate RNA by adding **0.1 volume** (or **3.0 μL**) of **3 M NaOAc, pH 5.2** and **3 volumes** (or **90 μL**) of **ice-cold 100% ethanol** (dedicated for RNA work); mix well by **inverting 10 times** and keep in the **-70°C freezer** until use.

C. DETERMINING QUALITY OF ISOLATED TOTAL RNA BEFORE AND AFTER DNase I-TREATMENT VIA GEL ELECTROPHORESIS

1. Prepare a **1.3%** Agarose Formaldehyde (AF) midi gel (10 cm x 17 cm)

	<u>100 mL</u>
Agarose	1.3 g
DEPC'd water	80.0 mL
10X MOPS buffer	10.0 mL

2. Microwave for **2-5 minutes**. Swirl to ensure that all agarose granules are melted.
3. Cool the agarose mixture in a **55°C water bath** for **at least 30 minutes**.
4. In a fume hood, add **9.8 mL** of **37% formaldehyde solution** to the warm agarose mixture using a sterile (RNase-free) disposable 10-mL pipet. **Immediately**, swirl **gently** to mix the mixture. *Caution: Avoid creating many bubbles.*
5. Cast the gel and allow agarose to solidify for ~30 minutes in a **fume hood** (room LS 2828).
6. Prepare **700 mL** of **1X MOPS running buffer** (**70 mL** of **10X MOPS buffer** + **630 mL** of **DEPC'd water**) in a **1-L Erlenmyer flask**.

Note: 10X MOPS buffer is prepared as follows:

<u>10X MOPS buffer</u>	<u>1 Liter</u>	<u>Final Concentration</u>
MOPS	46.2 g	0.2 M or 200 mM
Sodium Acetate (trihydrates)	10.9 g	0.08 M or 80 mM
0.5 M EDTA, pH 8.0	20 mL	0.01 M or 10 mM
DEPC'd water	800 mL	

- Adjust **pH to 7.0** with a **NaOH solution**.
- Bring the **final volume** to **1 Liter** with DEPC'd water.
- Filter the buffer using a **Nalgene filtering unit**.
- Autoclave for **15 minutes**. The buffer turns **yellow**.
- Store on a **reagent shelf dedicated for RNA** work at room temperature.

7. Prepare RNA samples for loading on the gel

How many RNA samples will be analyzed on the gel? **5**

2 RNA (leaves and seeds) samples **before** DNase I treatment

2 RNA (leaves and seeds) samples **after** DNase I treatment

1 RNA ladder

a. Label on the lids of 5 RNase-free microcentrifuge tubes **numbers "1-5"** and **your initial**. Set tubes **on ice**.

b. Label **one** RNase-free microcentrifuge tube as **Mmix** and **your initial**. Set the tube on ice.

➤ Prepare a **master mix (Mmix)** (for **6 = 5** Samples + **1** extra; see table below).

Mix the contents well.

	Mmix for 1 RNA sample	Mmix for 6 RNA samples
10X MOPS buffer	1.5 μL	9.0 μL
37% formaldehyde	2.6 μL	15.6 μL
Formamide (Ultrapure)	7.5 μL	45.0 μL
5 mg/mL Ethidium Bromide	1.0 μL	6.0 μL
Total Volume	12.6 μL	75.6 μL

➤ Aliquot **12.6 μL** of the **Mmix** into each of the labeled RNase-free microcentrifuge tubes in **step (a)** and add **3.4 μL** of **RNA** to each tube as shown on the table below

	Tube #1	Tube #2	Tube #3	Tube #4	Tube #5
Mmix	12.6 μL	12.6 μL	12.6 μL	12.6 μL	12.6 μL
Leaf RNA <u>before</u> DNase I	3.4 μL	-----	-----	-----	-----
Leaf RNA <u>after</u> DNase I	-----	3.4 μL	-----	-----	-----
Seed RNA <u>before</u> DNase I	-----	-----	3.4 μL	-----	-----
Seed RNA <u>after</u> DNase I	-----	-----	-----	3.4 μL	-----
RNA Ladder	-----	-----	-----	-----	3.4 μL
Total Volume	16.0 μL	16.0 μL	16.0 μL	16.0 μL	16.0 μL

- Mix the contents by flicking the tubes several times or pipetting gently up and down for 5 times.
- Heat samples to **70 °C** for **10 minutes to denature secondary structure of RNA**.
Quench tubes **on ice** for **2 minutes**.
- Add **2 μ L** of **10X loading dye** for **RNA samples** to each tube. Centrifuge tubes briefly to bring water condensation from the lids down.
 - Preparation of the 10X loading buffer is shown below:

<u>10X Loading Dye</u>	<u>Volume</u>	<u>Stock</u>
50% Glycerol	250 μ L	100%
1 mM EDTA	1 μ L	0.5 M EDTA, pH8.0
Bromophenol blue	~10 mg (a pinch to get blue color)	
DEPC'd H ₂ O	249 μ L	

8. Load samples on the gel. **Record** a pattern of the loaded samples **below**.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

9. Run the gel at **~70 volts** for **1-2 hours** in the **fume hood**.

Starting time:

Ending time:

*Note: To reduce the brightness of the ethidium bromide band migrated in the gel, the top of the gel is excised after the ethidium bromide band ran into it (usually 15 minutes after turning on the power supply). However, it is **okay** if you forget to excise the top of the gel after the ethidium bromide band ran into it.*

10. Take a picture of the gel. Paste the picture below.

What do you see in the picture?

What are the RNA fragments?

What are the sizes of RNA fragments?

Is there any difference in brightness between different samples before and after

DNase I treatment?

What is the reason for the difference?

II. SYNTHESIZING FIRST STRAND cDNA USING REVERSE TRANSCRIPTASE

Purpose: To generate cDNA template for PCR analysis.

Reference:

- Instruction Manual for iScript cDNA Synthesis Kit (Bio-Rad, Cat.#170-8890).
The **iScript reverse transcriptase** is RNase H⁺, resulting in greater sensitivity than RNase H⁻ enzyme. **iScript** is a modified MMLV-derived reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA.
The enzyme is provided pre-blended with RNase inhibitor.
The unique blend of oligo(dT) and random (6-bases, 8-bases, 10-bases) primers in the iScript Reaction Mix works exceptionally well with a wide range of targets. This blend is optimized for the production of targets <1kb in length.
iScript cDNA Synthesis Kit produces excellent results in both real-time and conventional RT-PCR. *Caution: when using >1 µg of total RNA, the reaction volume should be scaled up. For examples, 40 µL reaction for 2 µg, 100 µL reaction for 5 µg to ensure optimum synthesis efficiency.*

Note:

- For **every** RNA sample, set up one reaction **with** Reverse Transcriptase (+ RT) and one reaction **without** Reverse Transcriptase (- RT). The -RT sample serves as a negative control for the PCR amplification step because without first strand cDNA template, there will be NO PCR product with expected size observed. However, if a PCR product is observed in the - RT sample, then RNA sample is contaminated with genomic DNA.
- Work with master mixes as often as possible to prevent FALSE negative results due missing components.

Materials and Reagents Needed:

- 5x iScript Reaction mix (came with the iScript RT, Bio-Rad, stored in a **-20°C RNA Freezer**, room 2918)
- Nuclease-free water (came with the iScript RT, Bio-Rad, stored in a **-20°C RNA Freezer**, room 2918)
- iScript Reverse transcriptase (iScript RT, Bio-Rad, stored in a **-20°C RNA Freezer**, room 2918)
- Total RNA samples (stored in a **-20°C RNA Freezer**, room 2918)
- DEPC'd water
- 42°C and 85°C dry baths (or heating blocks)
- RNase-free 1.5 mL microcentrifuge tubes
- Aerosol-barrier PCR pipet tips
- Pipetman sets
- Microcentrifuge-tube rack

PROCEDURE

1. Write down **concentration** of purified total RNA samples to be used.

Note: 1 μg = 1,000 ng. Therefore, the concentration determined by using the

Nanodrop spectrophotometer as ng/ μL needs to be converted into $\mu\text{g}/\mu\text{L}$.

	Leaves	Seeds
RNA concentration	_____ $\mu\text{g}/\mu\text{L}$	_____ $\mu\text{g}/\mu\text{L}$

2. Determine a **volume** for **1 μg** of Total RNA to be added to **RT reactions**.

Volume of 1 μg RNA = (Amount of RNA) / (concentration of RNA).

Example: If Leaf RNA has a concentration of 0.5 $\mu\text{g}/\mu\text{L}$, then the volume of 1 μg

RNA will be (1 μg) / (0.5 $\mu\text{g}/\mu\text{L}$) = 2 μL

	Leaves	Seeds
Volume	_____ μL	_____ μL

3. Use the following table as the guide to fill in volumes for total RNA and DEPC'd water.

➤ **The volume of DEPC'd water is the difference between the Total Reaction Volume and the volumes of other components.**

Components	RNA +RT	RNA -RT
1 μg Total RNA	X μL	X μL
DEPC'd (or nuclease-free) water	Y μL	Y μL
+ RT Mix	5.0 μL	----- μL
- RT Mix	----- μL	5.0 μL
Total Reaction Volume	20.0 μL	20.0 μL

X μL = volume of RNA sample; Y μL = volume of DEPC'd water

4. Get a bucket full of **ice** from an icemaker in **room 2911** or **3906**.
5. Label on the lids of RNase-free 1.5 mL microcentrifuge tubes as "**Name of the plant organ**" and either "**+RT**" or "**-RT**". *For example, Leaf +RT and Leaf -RT*. Keep tubes **on ice**.
6. Thaw out the following tubes of **5x iScript Reaction Mix** and **Nuclease-free Water** at room temperature. Once the solutions are **thawed out**, keep the tubes **on ice**.
7. Prepare **two** tubes of **Master mixes (+RT Mix and -RT Mix)** as follows:
 - Determine a number of RT reactions to be set up.

Note: # RT reactions = # of RNA samples + 1 Extra

Example: # RT reactions = 3 = Leaves + Seeds + 1 Extra
 - Write on the **lid** of each of RNase-free microcentrifuge tubes as "**+RT mix**" and "**-RT mix**". Keep tubes **on ice**.
 - Remove a tube of iScript Reverse transcriptase from a **-20 °C RNA freezer** (dedicated for RNA Work, in room LS 2918). Keep the **tube on ice** at all time to prevent degradation of enzymes such as RNase-inhibitor and reverse transcriptase in this tube.
 - Pipet the **following components** into appropriate tubes as shown below.

Master Mixes:

Components	+RT Mix for ONE Reaction	+RT Mix for 3 Reactions	-RT Mix for ONE Reaction	-RT Mix for 3 Reactions
DEPC'd (or nuclease-free) water	---- μL	---- μL	1.0 μL	3.0 μL
5x iScript Reaction mix	4.0 μL	12.0 μL	4.0 μL	12.0 μL
iScript Reverse transcriptase	1.0 μL	3.0 μL	---- μL	---- μL
Total volume	5.0 μL	15.0 μL	5.0 μL	15.0 μL

- Mix the contents by pipetting up and down **five times** or flicking the tube several times. Repeat for all reaction tubes.

- Spin the tubes in a microcentrifuge for **10 seconds**. Put tubes on ice.
8. Using the "**+RT & -RT**" **chart** written up earlier in **step 3**, pipet into **+RT** and **-RT** tubes (labeled in **step 5**) the following components:
 - DEPC'd or Nuclease-free water
 - Total RNA
 - **+RT Mix** into **+RT** tubes
 - **-RT Mix** into **-RT** tubes
 - Mix the contents in each tube by pipetting **gently** up and down **five times**. Keep tubes on ice until the last component is added into the last RT tube.
 9. Transfer all +RT and -RT tubes from the ice bucket to a rack for microcentrifuge tubes on the bench.
 10. Incubate reaction tubes at **25°C** (or room temperature) for **5 minutes**. *This step is to allow oligo(dT) and random primers annealing to messenger RNA in the reactions.*
 11. Incubate reaction tubes at **42°C** for **30 minutes** on a **dry bath** (or heating block). *This step is to synthesize first strand cDNAs.*
 12. **After 30 minutes at 42°C**, inactivate **reverse transcriptase**, *which is known to interfere with Taq DNA polymerase in the PCR amplification step*, by heating the mixture at **85°C** for **5 minutes**.
 13. Chill the tubes **on ice** for at least **2 minutes**.
 14. Centrifuge the tubes at room temperature for **1 minute** to bring down water condensation on the lids of the tubes. *Note: The RT reactions are ready for PCR amplification step.*
 15. Store RT reactions in a **-20°C freezer** if they are not used for the same day. Otherwise, keep them **on ice** while setting up the PCR amplification step.

III. CARRYING OUT PCR AMPLIFICATION (RT-PCR) ANALYSIS

Purpose: To amplify DNA template corresponding to mRNA that is either absent or present at different levels in different plant organs throughout plant development.

Materials and Reagents Needed:

- Reverse transcription (+RT & -RT) reactions
- 10X Ex-Taq buffer (Takara Mirus Bio)
- dNTP mix (Takara Mirus Bio)
- **12 μ M Gene-specific RT-PCR Fw primers**
- **12 μ M Gene-specific RT-PCR Rv primers**
- **12 μ M Tubulin Fw primers**
- **12 μ M Tubulin Rv primers**
- Sterile water
- Ex-Taq DNA polymerase (Takara Mirus Bio, 5 U/ μ L)
- Agarose
- 1X TAE buffer
- 1 KB DNA ladder (Invitrogen)
- 6X Loading buffer
- 0.2 mL PCR tubes
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube racks
- PCR Machine (Bio-Rad MyCycler or PE 9700)
- Gel apparatus
- Power supplies

PROCEDURE

1. Get ice from the icemaker in room 2911 or 3906.
2. Determine **how many RT reactions**, including **+RT's** and **-RT's**, will be amplified.
3. Make a **Table** with information such as **tube #**, **plant organ(s)**, and **+RT's/-RT's** (see the **example** Table below)

Tube #	1	2	3	4	5 (Positive)	6 (Negative)
Organ & RT	Leaf +RT	Leaf -RT	Seed +RT	Seed -RT	Genomic DNA	Sterile Water

4. Label on the **lids** and **sides of FIVE 0.2 mL PCR tubes** with **Number** and **your initial**.
5. Put the labeled tubes on a PCR rack sitting **on ice**.
6. Prepare a **master mix** in a **1.5-mL microcentrifuge tube** for the **number of PCR solutions** being carried out **plus 1 extra solution volume** as followings: (**How many reactions are carried out?**)

	Mmix for 1 Reaction	Mmix for 7 Reactions
10X Ex-Taq buffer	5.0 μL	35.0 μL
dNTP mix	4.0 μL	28.0 μL
12 μM RT-PCR Gene-specific Fw primer	1.0 μL	7.0 μL
12 μM RT-PCR Gene-specific Rv primer	1.0 μL	7.0 μL
*12 μM Control (Tubulin) Fw primer	1.0 μL	7.0 μL
*12 μM Control (Tubulin) Rv primer	1.0 μL	7.0 μL
Sterile water	34.5 μL	241.5 μL
Ex-Taq DNA Polymerase (5 U/μL)	0.5 μL	3.5 μL
Total Volume	48.0 μL	336.0 μL

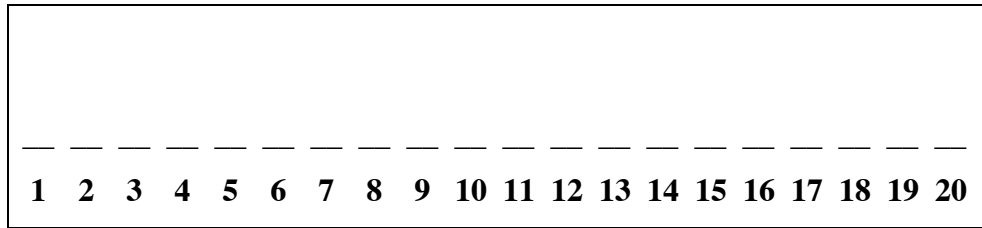
* *Control primers* are used to ensure that the absence of Gene-specific PCR product in **+RT samples** is **NOT** due to technical mistakes.

7. Pipet **48 μL** of the **master mix** to the labeled tubes and **2 μL** of **appropriate RT** to each of the tubes shown on the table below. Mix the contents by pipetting **gently** up and down for five times.

Tube #	1	2	3	4	5 (Positive)	6 (Negative)
Mmix	48 μL	48 μL	48 μL	48 μL	48 μL	48 μL
Leaf +RT	2 μL	---- μL	---- μL	---- μL	---- μL	---- μL
Leaf -RT	---- μL	2 μL	---- μL	---- μL	---- μL	---- μL
Seed +RT	---- μL	---- μL	2 μL	---- μL	---- μL	---- μL
Seed -RT	---- μL	---- μL	---- μL	2 μL	---- μL	---- μL
0.2 ng/μL Genomic DNA	---- μL	---- μL	---- μL	---- μL	2 μL	---- μL
Water	---- μL	---- μL	---- μL	---- μL	---- μL	2 μL
Total Volume	50 μL	50 μL	50 μL	50 μL	50 μL	50 μL

8. Carry out PCR reactions with the **RT-PCR program** containing the following profile: 1 cycle of 96°C, 3 min. → 40 cycles of 94°C, 10 sec./60°C, 30 sec./72°C, 45 sec. → 1 cycle of 72°C, 4 min. → 4°C, ∞.
9. Prepare **100 mL** of **1.5% agarose gel** in **1X TAE** buffer as usual (Use a **20-tooth** comb).
- Note: The percentage of agarose gels depends on the difference in size of two PCR products. If there is at least 100-bp difference between two PCR products, then use a 1% agarose gel. However, if there is 50-100 bp difference between two PCR products, then use 1.5-2% agarose gel. For example, the size of PCR products is 0.6 kb and 0.55 kb for the control and gene A, respectively. The 2.0% agarose gel resolves these two PCR products as two discreet DNA bands whereas the 1.0% agarose gel shows these two PCR products as a single DNA band.*
10. Label 1.5 mL microcentrifuge tubes according to the PCR solutions being performed.

11. Add to the labeled 1.5 mL microcentrifuge tubes **20 μ L** of **PCR solution** and **3 μ L** of **6X loading dye**. *Note: 20 μ L of PCR solution was loaded so that you can see the PCR products clearly*
12. Load samples on a 1.5% agarose gel along with **10 μ L** of **50 ng/ μ L 1-Kb ladder** solution. Record RNA loading pattern.



13. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) is about two-thirds of the gel.

Starting time:

Ending time:

14. Take a picture of the gel.
15. Analyze the data.

How many DNA fragments do you see on the gel?

Is there different brightness between the fragments from one organ to the other?

What are the sizes of DNA fragments?

What is the size of the PCR fragment corresponding to mRNA of the gene of interest?

What is a conclusion on gene expression of the gene of interest for the tested plant organs, leaves and seeds?

Are the RT-PCR results correlated to the GeneChip data?

iScript™ cDNA Synthesis Kit

25 x 20 µl reactions 170-8890

100 x 20 µl reactions 170-8891

For Research purposes only

Store at -20 °C (not frost-free)

iScript cDNA Synthesis kit provides a sensitive and easy-to-use solution for two-step RT-PCR. This kit includes just three tubes - comprehensive of the reagents required for successful RT-PCR.

The iScript reverse transcriptase is RNase H+, resulting in greater sensitivity than RNase H- enzymes. iScript is a modified MMLV-derived reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The enzyme is provided pre-blended with RNase inhibitor. The unique blend of oligo (dT) and random hexamer primers in the iScript Reaction Mix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets < 1kb in length.

iScript cDNA Synthesis Kit produces excellent results in both real-time and conventional RT-PCR.

Storage and Stability

Store the iScript™ cDNA Synthesis Kit at -20 °C in a constant temperature freezer. When stored under these conditions the kit components are stable for a minimum of one year after ship date. Nuclease-free water can be stored at room temperature.

Kit Contents

Reagent	Volume
25 reaction kit	
5x iScript Reaction Mix	100µl
Nuclease-free water	1.5ml
iScript Reverse Transcriptase	25µl
100 reaction kit	
5x iScript Reaction Mix	400µl
Nuclease-free water	1.5ml
iScript Reverse Transcriptase	100µl

Reaction Set Up

Component	Volume per reaction
5x iScript Reaction Mix	4 μ L
iScript Reverse Transcriptase	1 μ L
Nuclease-free water	x μ L
<u>RNA template (100fg to 1μg Total RNA)*</u>	<u>x μL</u>
Total Volume	20 μ L

Reaction Protocol

Incubate complete reaction mix:

5 minutes at 25°C

30 minutes at 42°C

5 minutes at 85°C

Hold at 4°C (optional)

Reagents and Materials Not Supplied

Pipette tips, aerosol barrier tips

Nuclease-free tubes

RNA purification kit

Recommendations for optimal results using the iScript cDNA Synthesis Kit:

The maximum amount of the cDNA reaction that is recommended for downstream PCR is one-tenth of the reaction volume, typically 2 μ L.

*When using larger amounts of input RNA (>1 μ g) the reaction should be scaled up e.g. 40 μ L reaction for 2 μ g, 100 μ L reaction for 5 μ g to ensure optimum synthesis efficiency.

Practice of the patented polymerase chain reaction (PCR) process requires a license. The iCycler iQ system includes a licensed thermal cycler and may be used with PCR licenses available from PE Corporation. Its use with

authorized reagents also provides a limited PCR license in accordance with the label rights accompanying such

reagents. Some applications may require licenses from other parties.

Bio-Rad Laboratories

2000 Alfred Nobel Drive, Hercules, CA 94547

510-741-1000 4106228 Rev A

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.

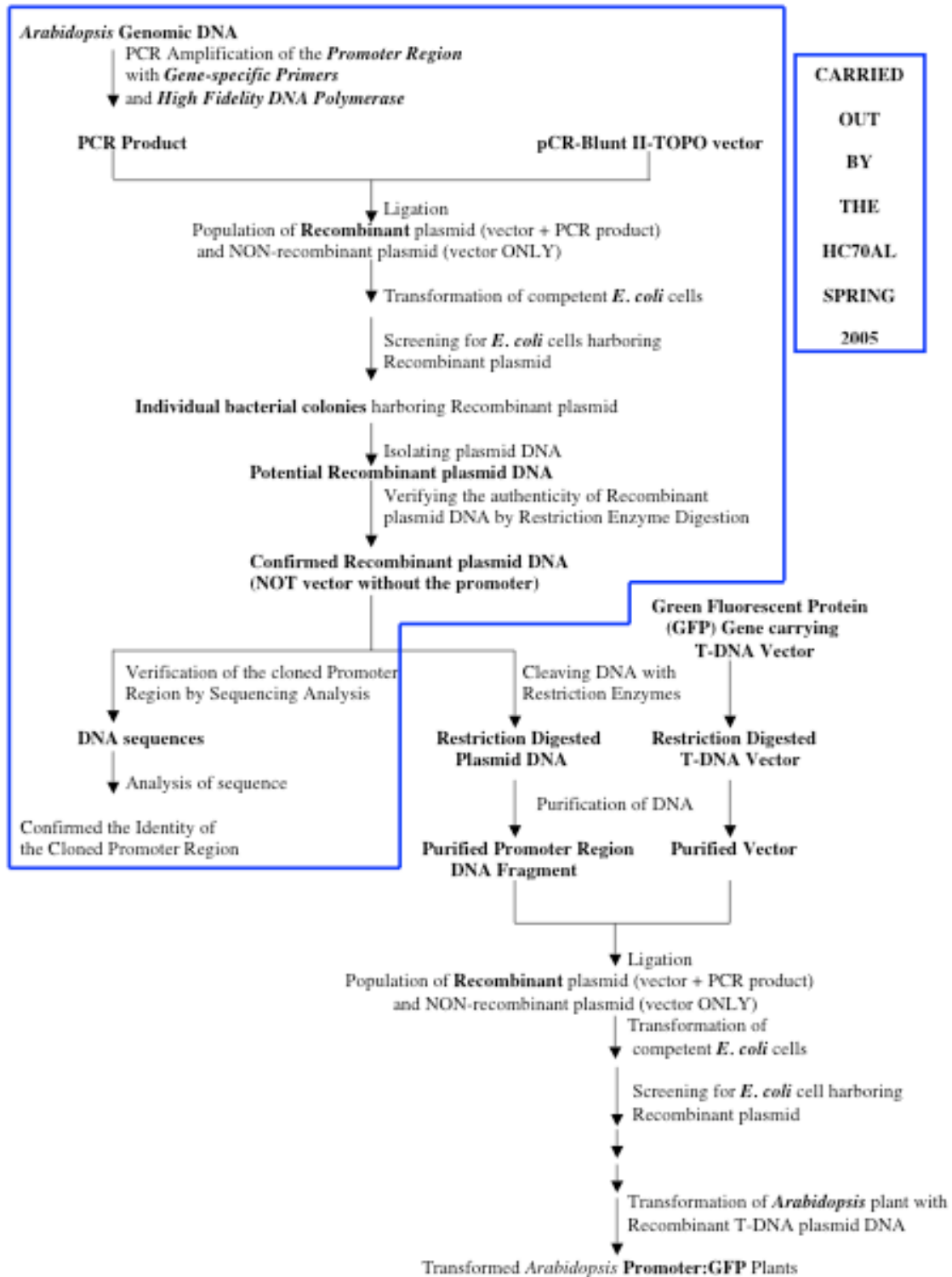
Reference:

1. KOD HiFi DNA Polymerase Technical Note (Novagen) (see Attachment 1)
2. TOPO Cloning Instruction Manual (Invitrogen) (see Attachment 2)
3. QIAprep Miniprep Handbook (see Attachment 3)

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



Materials Needed:

High quality *Arabidopsis* genomic DNA (12 ng/ μ L)
PCR product of the promoter region tested with Ex-Taq DNA Polymerase
Filter tips for PCR
KOD Hot Start DNA Polymerase kit (Cat.# 71086-3, Novagen)
Sterile water
12 μ M Promoter Gene-specific Forward primer
12 μ M 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 **10x Buffer for KOD Hot Start DNA Polymerase, 25 mM MgSO₄ and dNTPs (2 mM each)** 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 **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)** as follows:

Master Mix (*Note: Amplification of targets greater than 3 kbp may require more DNA polymerase*)

	Mmix for 1 Reaction	Mmix for 4 Reactions	Final Concentration
Sterile water	27.0 μ L	108.0 μ L	
10x Buffer for KOD Hot Start DNA polymerase	5.0 μ L	20.0 μ L	1x
dNTP mix (2 mM each dNTP)	5.0 μ L	20.0 μ L	0.2 mM
25 mM MgSO₄	2.0 μ L	8.0 μ L	1.0 mM
12 μM Gene-specific Forward primer	1.0 μ L	4.0 μ L	0.24 μ M
12 μM Gene-specific Reverse primer	1.0 μ L	4.0 μ L	0.24 μ M
Hot Start DNA polymerase (1.0 Units/μL)	1.0 μ L	4.0 μ L	0.2 Unit
Total volume	42.0 μL	168.0 μL	

- Pipet into the tube the reagents with order from top down (*example: water, 10x Buffer, dNTP mix, etc.*).
- 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	Tube #3
Mmix	42 μL	42 μL	42 μL
~100 ng <i>Arabidopsis</i> genomic DNA (12 ng/μL)	8 μL	0 μL	0 μL
Positive control DNA template (PCR product using Ex-Taq 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 and holding the "**Standby**" button for **TWO** seconds.
- 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 "**KOD HiFi**" protocol by pressing yellow arrowheads surrounding the "**ENTER**" button. Once it is selected, the "**KOD HiFi**" protocol is highlighted. Press the "**ENTER**" button.

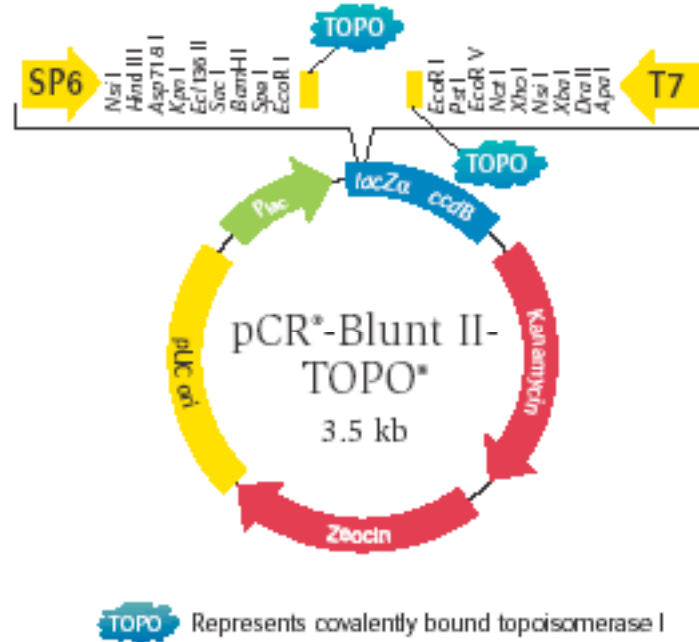
The **PCR profile for Genomic DNA templates.**

Cycling parameters	Up to 3 kbp of PCR product
Activation Enzyme step	94°C for 2 minutes
Number of cycle	1
Denaturation step	94°C for 15 seconds
Annealling step	55°C (or $T_m - 5^\circ\text{C}$) for 30 seconds
Extension step	72°C for 75 seconds
Number of cycles	30

- 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:

Reagent	
Freshly prepared PCR product	2.0 μL
Salt Solution	1.0 μL
Sterile water	2.5 μL
pCR-Blunt II – TOPO	0.5 μL
Total Volume	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°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 μ L** 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 μ L** 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 μ L or 50 μ L
Your Initial
10. Spread 10-50 μ L of transformation mixture on TWO **prewarmed** (37°C) selective (Kanamycin) plates.
Plate #1: 10 μ L of transformation mixture + **20 μ L** of S.O.C medium (for even spreading of a small volume)
Plate #2: 50 μ L 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.

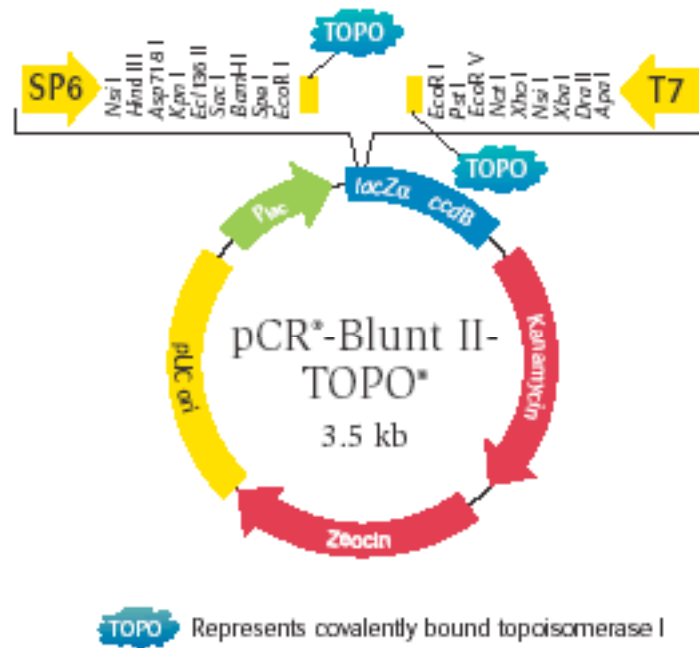
Immediately, invert the tube to mix the solution as in **step 11**. The solution appears cloudy. *Note: Do NOT vortex the mixture!*
13. Repeat step 12 for other tubes (one by one) Spin tubes in the microcentrifuge at **FULL** speed for **10 minutes**.

14. 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.
15. 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 26**).
16. After 10 minutes of spinning, transfer 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.*
17. Spin the columns in their collection tubes at full speed for 30 seconds.
18. Lift the column off the collection tube and discard the flow-through liquid into a glass **BEAKER**.
19. Put the column back in its collection tube.
20. Pipet **500 µL** of **Buffer PB** to each column. Spin the columns at **FULL** speed in the microcentrifuge for 30 seconds.
21. Lift the column off the collection tube and discard the flow-through liquid into a glass **BEAKER**.
22. 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.*
23. Lift the column off the collection tube and discard the flow-through liquid into a glass **BEAKER**.
24. 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. Also, ethanol will inhibit enzyme activity in later steps.*
25. Transfer the QIAprep columns in **NEWLY** labeled tubes. Discard the collection tubes. *Note: make sure the numbers on the columns and microcentrifuge tubes matched.*

26. Pipet **50 μ L** of **Buffer EB (10 mM Tris-HCl, pH 8.5)** to the center of each QIAprep column.
27. Let the columns stand for **1 minute**. Note: it is okay to incubate **longer than 1 minute**.
28. Spin the tubes with columns at **FULL** speed for **1 minute**. Steps 28 and 29 are for eluting plasmid DNA off the column.
29. After spinning, discard the columns. **Save the eluted plasmid DNA** in the microcentrifuge tubes.
30. Determine **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 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/mL)
- 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

Components	Standard Reaction	Final Concentration	<u>Example</u> 500 ng DNA (@200 ng/μL)
DNA	x μL	-----	2.5 μL
Sterile water	y μL	---	15.0 μL
*10x React buffer #2 (Invitrogen)	2.0 μL	1x	2.0 μL
<i>EcoRI</i> (10 U/μL, Invitrogen)	0.5 μL	0.25 U/μL	0.5 μL
Total Volume	20.0 μL	---	20.0 μL

Explanation of volumes

- ✓ $x \mu\text{L}$ = Volume of Plasmid DNA depends on the **amount** (in ng) of DNA to be digested and the **concentration** of plasmid DNA (in ng/μL)
- ✓ Volume of 10x 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 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!

Components	Sample A (300 ng/μL)	Sample B (200 ng/μL)	Sample C (100 ng/μL)	Final Concentration
500 ng of DNA	1.7 μL	2.5 μL	5.0μL	25 ng/μL
Sterile water	8.3 μL	7.5 μL	5.0 μL	---
2x Enzyme mix	10.0 μL	10.0 μL	10.0 μL	1x
Total volume	20.0 μL	20.0 μL	20.0 μL	

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?

Components	Enz Mix for 1 Reaction	Enz Mix for #__Reactions
10x React buffer #3	2.0 μL	____ μL
Sterile water	7.5 μL	____ μL
<i>EcoRI</i> (10 U/μL)	0.5 μL	____ μL
Total Volume	10.0 μL	____ μL

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.

Components	Sample 1 (ng/ μL)	Sample 2 (ng/ μL)	Sample 3 (ng/ μL)	Sample 4 (ng/ μL)
500 ng of DNA	____ μL	____ μL	____ μL	____ μL
Sterile water	____ μL	____ μL	____ μL	____ μL
2x Enzyme mix	10.0 μL	10.0 μL	10.0 μL	10.0 μL
Total volume	20.0 μL	20.0 μL	20.0 μL	20.0 μL

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 1 hour.

2. Meantime, prepare a 1% agarose gel in 1X TAE buffer with 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.
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.

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 μ M T7 primer
- 3 μ M 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:

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

either or

Components	T7 primer	SP6 primer
Mmix	19 µL	19 µL
3 µM T7 primer	1 µL	-----
3 µM SP6 primer	-----	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, ∞

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/>
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.

EXPERIMENT 5 - IDENTIFYING FEATURES OF MUTANT EMBRYO USING NOMARSKI MICROSCOPY (GENE ONE)

Purpose: To introduce Differential Interference Contrast (DIC) or Nomarski Interference Contrast (NIC) microscopy technique as a tool to identify features of defective embryos as illustrated in a T-DNA knockout mutant *dicer-like*, *lec1*, *raspberry3*, and *titan*.

Reference: The protocol was written by Dr. Miguel Aguilar in Professor Robert L. Fischer laboratory at University of California, Berkeley.

Materials Needed:

- Siliques containing seeds with a wide range of embryo stages (globular to mature green) from Arabidopsis
 - a. Wild type
 - b. T-DNA knockout mutant *raspberry3* or *titan* or *dicer-like* or *lec1*
 - c. homozygote or heterozygote mutant
- Absolute ethanol
- Acetic acid
- Chloral Hydrate (C-8383, Sigma-Aldrich)
- Glycerol (Gibco/Invitrogen)
- Fine point forceps
- 30-gauge hypodermic needles
- Microcentrifuge tubes (1.5 mL)
- Microcentrifuge tube rack
- Disposable transfer pipets or Pasteur pipets
- Double-distilled water
- Pipetman P-200 and P-1000
- Pipette tips
- Rulers with METRIC scale (cm)
- Fine-point scissors or razor blades
- Coverslips
- Microscope Slides

Equipment Needed:

- Dissecting microscopes (we got 10 extra dissecting microscopes from our Biology department storage)
- A microscope equipped with Normarski optical parameter (Leica CTR5000)

PROCEDURE

Each student collects the followings from Wild type, one of the known mutants, and his/her homozygous/heterozygous mutant:

(a) 5 siliques containing white seeds with embryo stages of globular to heart or torpedo. Note: silique length is in a range of 0.5 cm to 1.5 cm.

(b) all seeds from One silique containing mature green embryos.

A. Fixing Plant Materials

1. Prepare 5 mL of fixative solution of ethanol: acetic acid (9:1, v/v) in a 14-mL sterile Falcon tube using disposable 5-mL pipets.

Absolute ethanol	4.5 mL
<u>Acetic acid</u>	<u>0.5 mL</u>
Total volume	5.0 mL

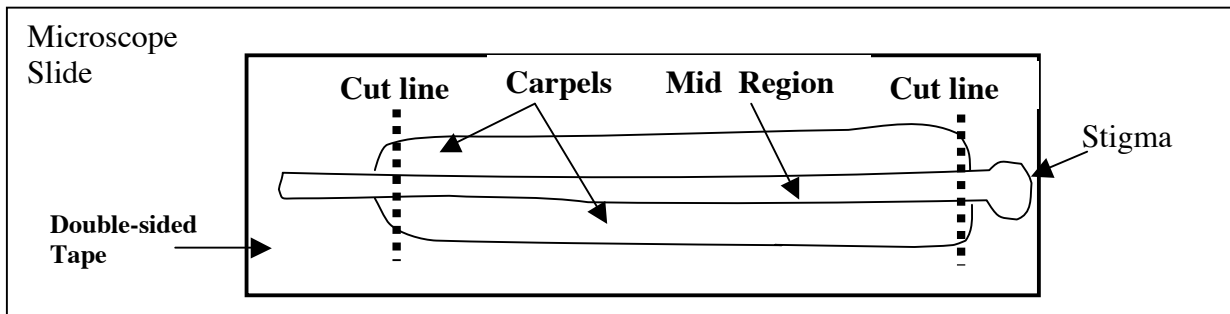
- Close the tube with its cap.
 - Invert the tube to mix the content.
2. Pipet **0.75 mL** of the **fixative solution** into **SIX** 1.5 mL microcentrifuge tubes sitting on the microcentrifuge rack at room temperature.
 3. Label on the lid and side of each tube in step 2 with your initial, number (1-6), and information (mutant/wild type, siliques/seeds).
 4. Label on the lids and sides of **THREE** microcentrifuge tubes with information (Wild type, known mutant, or your homozygote/heterozygote mutant). *Note: siliques with different lengths from wild type, known mutant, and homozygote/heterozygote mutant plants will be collected into each of these three tubes.*
 5. Bring the **tubes in step 4** and the **Plant Chart** with information about plant number and the genotype of those plants to the Plant Growth Center (or greenhouse).

6. Measure and collect **3 siliques** for **each** of **two different lengths** (one short and one long) in a **range of 0.5-1.5 cm** from the wild type, a known T-DNA knockout (*raspberry 3*, *dicer-like*, *lec1*, or *titan*), and homozygous/heterozygous mutant.

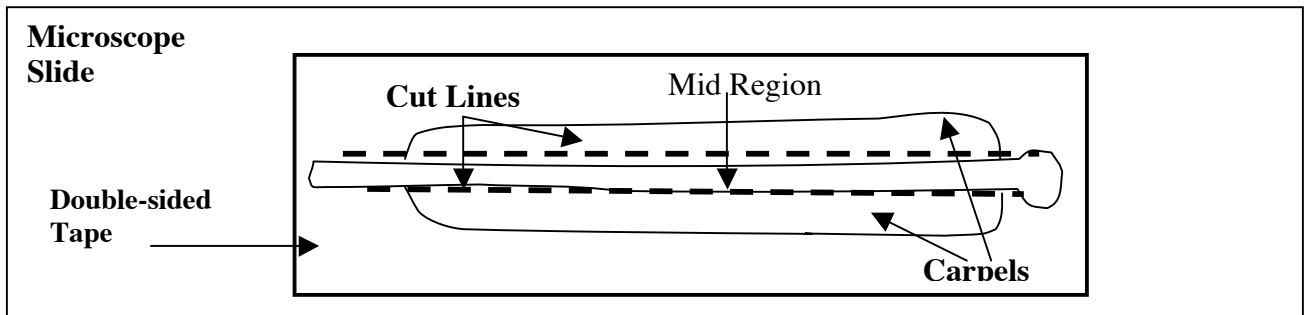
Note: collect the same length of siliques for wild type, known mutant, and homozygous/heterozygous mutant.

What are the genotype and phenotype of the mutant plants?

7. Collect **siliques** and **seeds** and put them in the fixative solution as follows:
- For **siliques with length < 0.7 cm**, Place a **piece of double-sided tape** on a **microscope slide** --> Carefully, use a **fine-point forceps** to place a silique on the tape --> **Excise the top and bottom of the silique** at its **ends of carpels** to allow solutions to penetrate the seeds and embryos during fixation, washing, and clearing steps (see a diagram below) --> **Immediately**, use the fine-point forceps to transfer the cut silique into the Fixative solution. Repeat the cutting of siliques for other siliques.



- For **siliques with length > 0.7 cm**, including those containing seeds with mature green embryos, **dissect** the seeds out of the siliques as illustrated below (NOT drawn to scale): Place a **piece of double-sided tape** on a **microscope slide** --> Carefully, use a **fine-point forceps** to place a **silique** on the tape and **rearrange** the silique such that the **mid region** is facing you --> Use a **28G or 30G hypodermic needle attached to a 1cc syringe** to **slice the carpels** along the **mid region** as shown in the diagram, then Use the fine-point forceps to **tear off the carpels** to expose the seeds --> Use either the forceps or the needle to **collect and transfer seeds** into the **Fixative solution**.



8. Fix seeds and siliques in the fixative solution for 2 hours to overnight.
Note: It is recommended to fix the seeds from siliques with length > 0.7 cm overnight to ensure that fixative solution penetrates the seeds and their embryos.
9. Next day, CAREFULLY pipet off **650 μ L** of the fixative solution using a **P-1000** pipetman and then the **remaining volume** with a **P-200** pipetman. *Note: Do not let the seeds and siliques dried out.*
10. **Immediately**, pipet 0.5 mL of 90% ethanol solution into the tube using a P-1000 pipetman. *The 90% ethanol solution will remove chlorophyll from the embryos.*

90% ETHANOL SOLUTION

Absolute ethanol	4.5 mL
<u>Double-distilled water</u>	<u>0.5 mL</u>
Total volume	5.0 mL

11. Incubate seeds and siliques in the ethanol solution for 0.5 - 1 hour.
Note: It is safe to leave the materials in the ethanol solution longer than 1 hour (or indefinite).
12. Replace the 90% ethanol solution with 70% ethanol similar to steps 9 & 10.

70% ETHANOL SOLUTION

Absolute ethanol	3.5 mL
<u>Double-distilled water</u>	<u>1.5 mL</u>
Total volume	5.0 mL

13. Incubate seeds and siliques in the ethanol solution for 0.5 - 1 hour.
Note: it is safe to leave the materials in the ethanol solution longer than 1 hour (or indefinite).

B. Observation of Seeds and Embryos

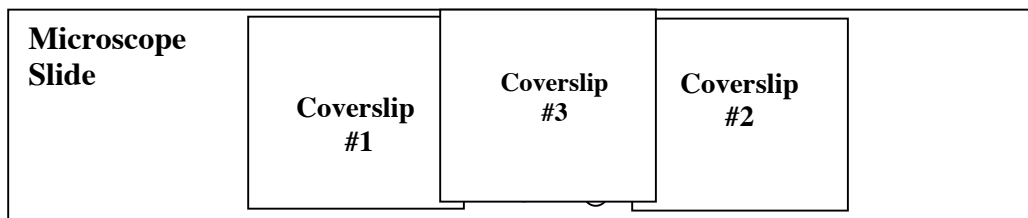
Note: At least ONE hour before observation of the seeds and their embryos. Seeds must be submerged in the clearing solution. (a) The older the silique, the longer it takes to clear and sink. For young seeds, clearing is usually fast, i.e., seeds can be observed under a microscope within 30 minutes. (b) Tissues CANNOT be stored in the CLEARING solution for more than TWO days because they will lose their structures quickly.

1. Prepare a *fresh* CLEARING solution of chloral hydrate/glycerol/water (8:1:2, w/v/v) in a 14-mL Falcon tube (*Note: The TA will prepare this solution before the lab class begins*)

CLEARING SOLUTION

Chloral hydrate	8 g
Glycerol	1 mL
Water	<u>2 mL</u>
Total volume	3 mL

2. Replace the 70% ethanol solution with the CLEARING solution. Wait for 30-60 minutes or until the seeds and siliques SINK to the bottom of the tubes.
3. Set a new glass microscope slide on the bench.
4. Gently, pipet the clearing solution up and down for 5 times to mix up the seeds using a P-200 pipetman.
5. Dispense **100-150 μ L** of the clearing solution with seeds in the center of the microscope slide.
6. Carefully, place two squared coverslips, one on each side of the solution. Then, place a third coverslip over the clearing solution (see below)



7. Observe the seeds under Nomarski optic using Leica CTR5000 microscope.
8. Take pictures of the embryos.

Note: For siliques, you need to dissect seeds out of the silique on a microscope slide under a dissecting microscope. Then, proceed steps 5-8.

EXPERIMENT 6 – SCREENING SALK T-DNA MUTAGENESIS LINES (GENE TWO)

Purpose: To identify a knockout line for the gene of interest and characterize phenotype of mutant plant(s).

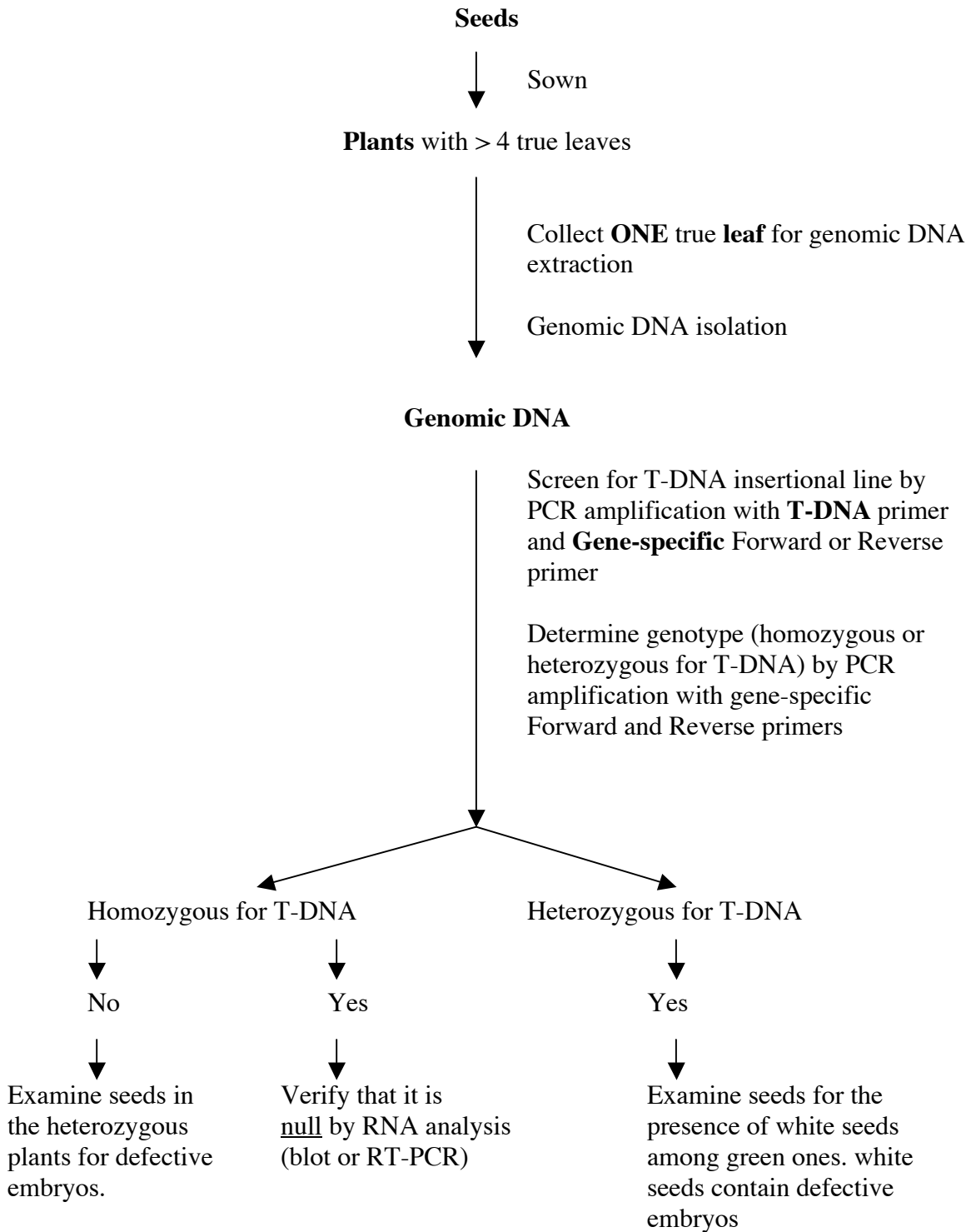
Reference: University of Wisconsin - Madison Knockout Facility

Attention: *By now, you have experience with the genotyping procedure. You can isolate genomic DNA from 12 or more plants. So, you use this protocol as the guide and adjust the volumes of reactions according to the number of plants that you are analyzing.*

STRATEGY

- I. SOWING SEEDS AND GROWING PLANTS**
- II. ISOLATING GENOMIC DNA FROM LEAVES OF AT LEAST 2-3 WEEKS-OLD SEEDLINGS/PLANTS**
- III. IDENTIFYING PLANTS CONTAINING T-DNA INSERTED IN THE GENE OF INTEREST AND THEIR GENOTYPES**

STRATEGY



I. SOWING SEEDS AND GROWING PLANTS

Purpose: To generate seedlings/plants for genomic DNA extraction.

Caution: *Be extremely CAREFUL with seeds. Do NOT mix up labeled tags and actual seed lines.*

Materials Needed:

- Tubes of Seeds from the *Arabidopsis* Seed Stock Center
- A microcentrifuge rack
- White Xerox paper
- Black sharpie (Ultrafine or fine)
- Plastic tags
- A pair of pointed-end forceps
- Black plastic trays
- Black rectangular pots in sheets
- Clear plastic covers for black trays
- Soil in the Plant Growth Center (PGC)
- A growth chamber (Percival) with constant light in the PGC

PROCEDURE

1. Obtain **tubes of seeds** to be grown from the cold-room and put them on a microcentrifuge rack. *For example, S_112701, for gene At5g11240, and wildtype seeds and Columbia for Salk lines.*
2. If **plastic tags** are available in the lab, label them with a black sharpie.
 - a. For **knockout line:**
 - Gene name**
 - SALK line #**
 - Date**
 - Pot # 1-10** (for 1 flat with 11 pots)
 - b. For **wild-type:**
 - Columbia-0**
 - Date**
3. Bring the **items in steps 1 & 2**, along with **several sheets of white paper** and a **pair of tweezers**, to the **Plant Growth Center (PGC)**.
4. At the PGC, put all of these items on **the bench** that runs along the **East wall**. This bench does NOT have any soil on it. *Note: Do NOT put tubes of seeds or plants full of mature seeds near the bench of soil because the prepared soil will be contaminated with these seeds, which could in turn, result in false mutant phenotypes for other people's works.*
5. In the PGC, prepare ONE flat with **12 pots** of soil for every line of mutant seeds being planted.
 - a. Assemble each flat as follows:
 - i. Obtain a black plastic tray.
 - ii. Obtain a sheet of 12 rectangular plastic pots.
 - iii. Obtain a clear plastic cover.
 - iv. Set a sheet of 12 pots in one of the black plastic trays.
 - v. Fill the pots with soil (prepared by the PGC staff, Mr. Weimin Deng).
 - vi. Flatten the surface of the soil by scraping off excess soil with a metal plate.
 - b. Repeat step (a) for as many flats as needed.

- c. Remove **one pot** from the corner of the flat and put the soil back into the same amount of soil. So, there are **only 11 pots**. *The empty space will make it easier to put the water in.*
 - d. Bring the flat to the bench near the sink.
 - e. Make sure that the water hose is attached to the water pipeline labeled “**fertilizer-supplemented**”.
 - f. Fill each flat **2/3 of the way up** the tray with “fertilizer-supplemented” water.
 - g. Wait **15 minutes or until** the **surface of the soil appears darker** due to water sipping up from the bottom of the pots.
 - h. Cover the flat with **clear plastic cover** to prevent growth of air-borne molds and to protect from **strayed Arabidopsis seeds** from other plants.
6. Bring the flat over to the bench where the seeds and planting tools are located (or any other bench removed from the soil).
 7. Cut the **sheet of white paper** into quarters
 8. Fold **each quarter in half**, length-wise
 9. Gently pour out seeds from the microcentrifuge tube onto one of the folded pieces of paper.
 10. Bring the folded paper with seeds over each of the 12 pots. Lower one end of the paper near the soil surface. **Gently tap** the lower end of the paper to allow for one seed to slide down into the soil. The tweezers are a useful tool to guide one seed off of the paper to a precise location in the pot without dumping all of the seeds from the paper.
 11. Sow 2 seeds per pot, for 11 of the pots.
 12. Put the labeled tags for the **knockout line** into **each** of the **10 pots** containing knockout seeds.
 13. Put the seeds that were not used back into the **appropriate knockout seed** microcentrifuge **tube**.
 14. For pot **#11**, pour out wild-type seeds onto a **new** folded piece of white paper. Visually divide the pot into 4 quadrants, and sow a wild-type seed in each quadrant. **Four seeds of wild-type** should be sown in **pot #11**.
 15. Put a **wild-type labeled tag** into pot #11.

16. Cover the flat with the **clear plastic cover**.
17. Put the flat aside.
18. Repeat seed sowing for other knockout lines.
19. After all of the lines are sown, put the flats on a metal car and take the elevator to the lower level.
20. Put the flats on wired-racks in the cold-room (the first room on the right after entering the double doors across from the elevator).
CAUTION: Make sure the clear covers completely cover the flats so that no air-borne molds in the cold-room get in the soil.
21. Leave the flats in the cold-room for **2-3 days** to **vernalize seeds** and to enhance **synchronization of seed germination**.
22. After 2-3 days in the cold-room, transfer the flats to the white Percival growth chamber and leave them there for another 5-7 days.
NOTE: Keep the clear plastic covers on the flats.
23. After a total of 7-10 days after planting, bring the flats of seedlings with 2 cotyledons to the glasshouse #3
24. Put the flats of seedlings on a table.
NOTE: Choose a table that has no mature Arabidopsis plants bearing ripened seeds because these seeds could accidentally get in the soil of the seedling flats when the clear covers are removed.
25. Slide the clear covers off the trays by **0.5-1 inch** so that warm air under the covers will not cook the seedlings nor will the surface of the soil be too warm which is favorable for molds to grow.
26. Wait until most of seedlings in the flats have **4 true leaves**. Then remove the clear covers completely off the flats. Bring the clear covers to the washing room on the lower level of the PGC so that they will be washed by the PGC staff.
27. Map **positions of seedlings** in **each of 11 pots** on a sheet of "Plant Layout" chart.

28. Daily, **check water level** in the soil of the flats by feeling the wetness of the soil surface with your fingers. If the plants need to be watered, remove one pot at the corner and then put "fertilizer-supplemented" water in.

NOTE: Do NOT overwater the plants because overwatering may cause stress to plants, resulting in false mutant phenotype that will not appear in the next generation. Bigger plants need more water than smaller ones. Therefore, you need to check water level in the soil more often daily with big plants.

GENOTYPING ARABIDOPSIS PLANTS
PLANT LAYOUT CHART

Gene ID: At__ g _____ SALK line#: _____ Date: _____
Primers for PCR: _____
Size of PCR product: _____

Pot #	Pot #
Pot #	Pot #
Pot #	Pot #
Pot #	Pot #
Pot #	Pot #
Pot #	Pot #

II. ISOLATING GENOMIC DNA FROM LEAVES OF AT LEAST 2-3 WEEKS-OLD SEEDLINGS/PLANTS

Purpose: To isolate genomic DNA from seedlings' leaves for identifying plants containing a T-DNA insert within the gene of interest.

Recommendation: *By now, you have experience with the genotyping procedure. You can isolate genomic DNA from 12 or more plants. So, you use this protocol as the guide and adjust the volumes of reactions according to the number of plants that you are analyzing.*

Materials and Reagents Needed:

- Seedlings/plants (knockout lines and wild type)
- Sterile 1.5-mL microcentrifuge tubes
- PCR (aerosol-barrier) pipet tips
- Microcentrifuge-tube racks
- Microcentrifuge
- P-10, P-20, P-200 and P-1000 pipetman
- 80% ethanol solution
- A box of Kimwipes
- One or two pairs of latex gloves
- Two pairs of pointed-end tweezers (forceps)
- A pen
- A plant layout chart
- The key to the Plant Growth Center
- A squirt bottle of 100% ethanol solution
- TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0)
- Extraction Buffer (0.2 M Tris-HCl, pH 9.0; 0.4 M LiCl; 25 mM EDTA; 1% SDS)
- Isopropanol
- Glass beakers labeled as "Waste solution"
- Agarose
- 1X TAE buffer
- Gel apparatus and power supply
- 55-60°C water bath
- 6X Loading dye
- 50 ng/μL 1-kb DNA ladder solution
- 1X TNE (high salt solution) diluted from 10X stock
- 1 mg/mL Hoesch dye H33258 solution stored in the coldroom
- TKO Mini Fluorometer (Hoefer Scientific Instruments)

PROCEDURE

Attention: You will need to **assess the quality of isolated genomic DNA** later (at **step 37**); therefore, to use time efficiently you need to prepare a 0.7% agarose gel before you start the extraction of genomic DNA (see **Agarose Gel Electrophoresis Appendix**). While the agarose mixture is cooled in the 55-60°C water bath for at least 30 minutes, you go to the Plant Growth Center to collect leaves. After 30 minutes or so, add 5 µL of 10 mg/mL Ethidium Bromide (EtBr) solution to the agarose mixture, swirl to mix the EtBr, pour the gel with a 20-tooth comb, and let the agarose mixture to solidify.

1. Put **6** sterile 1.5-mL microcentrifuge tubes on a microcentrifuge-tube rack.
2. Label number 1-6 on lids of the tubes.

Tube #1 - 5: seedlings/plants #1 - 5 of **Knockout** lines

Tube #6: **1** seedling/plant from **Wild type** (Columbia-0)

3. Pipet **100 µL** of **Extraction Buffer** into each tube.

Note: I (Anhthu) found that it is **not** necessary to keep tubes of **Extraction Buffer on ice** during collection of the leaf samples if genomic DNA will be isolated from samples within one hour.

4. Gather together the following items on a plastic tray or container:

- A pair of latex gloves
- Two pairs of tweezers
- A box of Kimwipes tissues
- A squirt bottle of 100% Ethanol solution
- A "Plant Layout" chart
- Several sheets of white Xerox paper
- A ruler with Metric system (mm and/or cm)
- A pen
- The Nikon 5400 digital Camera
- The key to the Plant Growth Center

5. Go to the **Plant Growth Center (PGC)** and locate your flat with plants.
6. Use the "**Plant Layout Chart**" to mark the **locations of the plants** you will collect samples from. The **order of plants** should **correspond** to the **labeled tags** that were numbered when the seeds were planted.

Note: **NOT** all of the seeds will have germinated.

7. Use a piece of Kimwipes to clean the tweezers with 95-100% ethanol solution.

Note: The tweezers must be cleaned after collection each leaf to avoid cross-contamination, and two sets of tweezers are used per plant.

8. Remove one **small leaf** from the **first** plant.
9. Place the leaf on the white paper and measure it with the ruler. *The leaf should be between 0.5 cm and 1.0 cm in length.*
10. Take a picture of the leaf to document the size used to extract DNA.
11. Place this leaf in the microcentrifuge tube #1 containing the extraction buffer.
12. Repeat this process with other plants.
Note: MAKE SURE TO CLEAN THE TWEEZERS BETWEEN LEAF SAMPLES!
13. Go back to the lab.
14. Homogenize or macerate the collected leaf in the extraction buffer by crushing them with a **blue micropestle** until no more chunks of plant tissue observed in the mixture.
Note: Do NOT dispose the micro-pestle, but follow step 15.
15. Rinse the **micropestle** with **300 μ L** of Extraction buffer. The **total volume** of Extraction Buffer in the microcentrifuge tube is now **400 μ L**.
16. Vortex the **homogenate** for 20 seconds.
17. Set the tube **on ice**.
18. Repeat steps 14-17 for **other tubes**.
19. Centrifuge tubes of homogenates at **room temperature** for **5 minutes** at **FULL speed**.
20. Meanwhile, label a set of **microcentrifuge tubes** with **Gene Name** and **tube #**.
21. Pipet **350 μ L** of **isopropanol** to each of labeled tubes.
Note: Make sure that the number on tubes being centrifuged corresponds to the number on the tubes on the rack.
22. After centrifugation, transfer tubes from the microcentrifuge onto a microcentrifuge-tube rack.
23. Organize tubes such that the **numbers** on the **lids** of **NEW tubes match** with **numbers** on **the lids** of **tubes containing homogenates**.
24. Pipet **350 μ L** of **supernatant (homogenate)** from the centrifuged tubes to the corresponding tubes containing isopropanol. Close the lids of the tubes.
Note: AVOID pipetting plant debris on the bottom of the tubes as much as possible. However, it is okay if you accidentally transfer some plant debris into the isopropanol tube.
25. Mix the isopropanol and homogenate by inverting the tube **5-10 times**.

26. Incubate the mixture at **room temperature** for **5 minutes** to precipitate **nucleic acids** (*both genomic DNA and total RNA*).
27. Centrifuge tubes at **room temperature** for **10 minutes** at **FULL** speed.
28. Pour off the supernatant into a **glass beaker** labeled as "**Waste solution**".
Note: DNA is now in your pellet along with RNA. Therefore, be extremely careful when pouring off isopropanol because the pellets are sometimes loose.
29. Add **1 mL** of **80% ethanol** solution to each pellet. Close the lid of the tube and invert five times. *This step is to wash off any residual amount of salts (in the extraction buffer) and isopropanol.*
30. Centrifuge the tubes at **room temperature** for **5 minutes**.
31. Pour off the supernatant into a **glass beaker** labeled as "**Waste solution**". Dab the tubes on Kimwipes tissues to remove as much ethanol as possible.
Note: Be extremely careful when pouring off the ethanol solution because the pellet is loose.
32. Put the tubes on a microcentrifuge-tube rack with their lids opened allowing ethanol to be evaporated.
33. Dry pellets either in a **Speedvac** at room temperature for **5-10 minutes** (TAs will show you how to do this step) or leaving on the **bench** at **room temperature for 60 minutes**.
34. *After drying the pellets*, resuspend each pellet by adding **100 µL** of **TE** buffer, closing the lids of the tubes, and **raking** the tubes over the microcentrifuge-rack for **10-15 times** or **vortexing** the tubes for a few minutes until no visible of pellets.
35. Spin tubes in a microcentrifuge for **1 minute** to bring down liquid and any contaminants to the bottom of the tubes.
36. Store DNA solutions at **4°C** (on ice or refrigerator) until used.
*Note: (a) Keep DNA solution cold as much as possible to prevent degradation of DNA because this is a crude extraction of genomic DNA, and there may be a tiny trace amount of endonuclease present in the DNA solution. (b) Before using DNA solution for PCR amplification after a long period of storage (more than 12 hours), spin tubes of DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed to bring down water condensation on the lid as well as any contaminated plant debris and/or carbohydrates in the solutions to the bottom of the tubes.*

Attention: At this step, you need to assess the **quality** and **quantity** of isolated genomic DNA by **gel electrophoresis** (see **step 37**) and **fluorometer reading** (see **step 38**), respectively.

37. Analyze the **quality** of isolated genomic DNA by **gel electrophoresis** as follows:

a. Prepare a **0.7%** agarose gel with a **20-tooth** comb (**0.7g** of **agarose** in **100 mL** of **1X TAE buffer**; see **Agarose Gel Electrophoresis Appendix** for preparing the agarose gel).

Note: The agarose gel can be prepared before the collection of leaves for the extraction of genomic DNA

b. Label the **numbers (1-6)** and **your initial** on the lids of 6 microcentrifuge tubes and set tubes on the microcentrifuge rack.

c. Pipet **10 µL** of **isolated genomic DNA** solutions into each of labeled tubes.

d. Add **2 µL** of **6x Loading dye solution** to each tube and mix the contents by pipetting up and down for 5 times.

e. Load **10 µL** of **diluted 1-kb DNA ladder solution** along with 12 µL of DNA mixtures prepared in steps c and d.

f. Record **loading patterns** of samples

1	2	3	4	5	6	7	8	9	10	11	12	13	14

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

Starting time:

Ending time:

h. Take a picture of the gel using the Bio-Rad Gel Documentation system.

What do you observe on the gel?

What is the size of genomic DNA?

38. Determine DNA concentration of isolated DNA solutions using a **Fluorometer** and **Hoesch** dye. (Your TAs will demonstrate how to use the Fluorometer).

Note: Hoesch dye is sensitive to light; therefore, the 1 mg/mL Hoesch dye solution is stored in a 14-mL tube wrapped with aluminum foil at 4°C. The tube of 1 mg/mL Hoesch dye solution and a microcentrifuge tube containing a standard DNA solution of 100 ng/μL are stored in a 1-liter plastic container on the first left shelf in the cold room. Return the plastic bottle containing the Hoesch dye solution and the standard DNA solution to the cold room as soon as you finish with it.

Record **concentration of DNA** solution in the **table** below:

Samples	DNA Concentration (ng/μL)
Plant #1	
Plant #2	
Plant #3	
Plant #4	
Plant #5	
Plant #6	

Question: Why do you use the Fluorometer instead of the Nanodrop spectrophotometer to determine DNA concentration for these DNA solutions?

Answer: Two following reasons:

a. Because the **major components** in the DNA solutions are **ribosomal RNAs** and **tRNAs**, the concentration of DNA determined by the Nanodrop or any other spectrophotometer reflects mostly the concentration of RNAs. Thus, you do not know the DNA concentration of your DNA solutions.

b. **Property of Hoesch dye H33258** allows us to estimate DNA concentration of the DNA samples containing RNAs (see explanation taken from the **Instruction Manual** for TKO 100 Dedicated Mini Fluorometer - Hoefer Scientific Instruments)

Table: Excitation and Emission Spectra of Hoesch Dye H33258

	Excitation Spectrum peaks at	Emission Spectrum peaks at
Absence of DNA	356 nm	492 nm
Presence of DNA	365 nm	458 nm

The fluorescence enhancement provided by using the Hoesch H33258 dye has been shown to be **highly specific for DNA**, binding preferentially to A-T rich regions (Brunk et al., 1979; Labarca and Paigen, 1980). The dye binds twice as well to **double-stranded DNA** as to **single-stranded DNA**, but does not appear to intercalate (Brunk et al., 1979).

RNA enhances the fluorescence of H33258 to a much smaller extent than DNA. Under high salt conditions, in which chromatin proteins are fully dissociated from DNA leading to the increase the fluorescence enhancement of the DNA/dye complex, RNA enhancement is usually well below 1% of that produced by the same concentration by weight of DNA (Labarca and Paigen, 1980). For this reason, the presence of RNA in the sample does not interfere with the quantitation of DNA. Because RNA does not compete with DNA for binding with H33258, it is, therefore, extremely useful for estimating the DNA content of samples containing RNA. Thus, the Hoesch Dye allows us to measure the concentration of solely the DNA present in a given solution.

References:

Brunk, C. F., Jones, K.C., and James, T.W. (1979). Assay for nanogram quantities of DNA in cellular homogenates. *Anal. Biochem.* 92: 497-500.

Labarca, C. and Paigen, K. (1980). A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* 102: 344-352.

39. Dilute **5 μ L** of **original DNA solutions** to a **final concentration** of **0.2 ng/ μ L** with **TE** buffer. Label on the lids and sides of microcentrifuge tubes with the following information: **0.2 ng/ μ L, plant#, your initial, and date**. Keep all tubes of DNA solutions **on ice**.

Note: Dilution of DNA solutions would serve two purposes: (a) contaminants, such as carbohydrates that bind nonspecifically to nucleic acids and proteins, in DNA solutions will be diluted out. Therefore, a tiny amount of contaminants in PCR reactions will not interfere with the amplification of targeted DNA. (b) ONLY small amount of Arabidopsis genomic DNA (~0.4 ng) is needed for the PCR amplification. How to make a dilution?

Use the basic formula that is widely used in general chemistry lab. That is,

$$V_i \cdot C_i = V_f \cdot C_f$$

where,

V_i = initial volume (the volume of original DNA solution is **5 μ L**)

C_i = initial concentration (reading from the Fluorometer; example: **8 ng/ μ L**)

V_f = final volume (depends on the initial concentration)

C_f = final concentration (**0.2 ng/ μ L**)

then,

$$V_f = (V_i \cdot C_i) / C_f = (5 \mu\text{L} \times 8 \text{ ng}/\mu\text{L}) / (0.2 \text{ ng}/\mu\text{L}) = 200 \mu\text{L of total volume}$$

What is the volume of TE to be used in dilution?

$$V_{\text{TE}} = V_f - V_i = 200 \mu\text{L} - 5 \mu\text{L} = 195 \mu\text{L of TE}$$

Record **volume of TE** and **final volume** in the **table** below

	Volume of isolated genomic DNA	Volume of TE	Final Volume
Plant #1			
Plant #2			
Plant #3			
Plant #4			
Plant #5			
Plant #6			

III. IDENTIFYING PLANTS CONTAINING T-DNA INSERTED IN THE GENE OF INTEREST AND THEIR GENOTYPES

Purpose: To identify plants containing T-DNA insert and determine genotypes of T-DNA tagged plants as heterozygous and/or homozygous for T-DNA.

Materials and Reagents Needed:

- 12 μ M Gene-specific Forward primer
- 12 μ M Gene-specific Reverse primer
- 12 μ M **Lbb1** primer (Left Border (LB) region of T-DNA from **SALK** Lines)
- 10X Ex-Taq buffer
- dNTP Mix
- Ex-Taq DNA polymerase
- Sterile water
- 1-kb DNA ladder
- PCR Machine (Applied Biosystems GeneAmp 9700 or BioRad MyCycler)
- 0.2 mL PCR tubes
- 1.5 mL microcentrifuge tubes
- P-10, P-20, P-200 Pipetman
- PCR rack for 0.2 mL PCR tubes
- Rack for 1.5 mL microcentrifuge tubes
- Filtered Pipet tips for PCR
- Ice bucket
- Gloves
- Microcentrifuge
- Agarose
- Gel apparatus and power supply
- Bio-Rad Gel Documentation System

PROCEDURE

Note: There are **6** plants to be characterized and **2 controls** (genomic DNA isolated by TA + No DNA template), prepare a **master mix** for **8 + 1 extra = 9 reactions**.

1. Label on the lids and sides **8 PCR tubes** and put them on a PCR rack sitting on ice.
2. Prepare a **master mix** for **9 PCR reactions** in a 1.5 mL microcentrifuge tube labeled as "**Mmix**" sitting on ice.

Note: The reaction volume is **reduced** from **50 μ L** in previous reactions to **25 μ L**.

	Mmix for ONE reaction	Mmix for 9 reactions
Sterile water	16.5 μ L	148.5 μ L
10x Ex-Taq buffer	2.5 μ L	22.5 μ L
dNTP mix	2.0 μ L	18.0 μ L
12 μ M Gene-specific Forward primer	0.5 μ L	4.5 μ L
12 μ M Gene-specific Reverse primer	0.5 μ L	4.5 μ L
12 μ M LBB1 primer (for SALK lines)	0.5 μ L	4.5 μ L
Ex-Taq DNA polymerase (5 U/ μ L)	0.5 μ L	4.5 μ L
Total Volume	23.0 μL	207.0 μL

3. Mix the contents by flicking the tube five times or vortexing for the tube containing the master mix for **5 seconds**. Spin the tube in a microcentrifuge for **10 seconds**. Put the tube back **on ice**.
4. Pipet **23 μ L** of the **Mmix** into each of **8 PCR tubes**.
5. Pipet **2 μ L** of **0.2 ng/ μ L** genomic DNA extracted from each of **6** seedlings/plants into PCR tubes #1-6. Pipet up and down for five times to mix the contents. Put the first tube back on ice and work on the remaining tubes.
6. Pipet **2 μ L** of **0.2 ng/ μ L** genomic DNA extracted (by TAs) from **wild type** (Col-0) seedlings into each of tubes **#7**. Pipet up and down for five times to mix the contents

7. Pipet **2 μ L** of **sterile water** to tube **#8 (negative control)** without DNA template).
Pipet up and down for five times to mix the contents.
8. Spin PCR tubes in the microcentrifuge for PCR tubes for **5 seconds** to bring the liquid to the bottom of the tubes.
9. Put the tubes on the **wells** of the PCR machine.
10. Perform PCR with the "**KNOCKOUT**" program with the following profile:
 - 1 cycle of Hot start or 96°C for 3 minutes
 - 36 cycles of 94°C, 15 seconds -> 60°C, 30 seconds -> 72°C, 2 minutes
 - 1 cycle of 72°C, 4 minutes
 - 4°C, ∞
11. Prepare a **1% agarose gel** in 1X TAE buffer with a **20-tooth** comb.
12. Label **8 1.5-mL microcentrifuge** tubes and set them on a rack.
13. Add **2 μ L** of **loading dye** to each tube.
14. Pipet **10 μ L** of **PCR solutions** to each tube.
15. Load samples on the **1% agarose gel** along with **10 μ L** of **diluted DNA ladder** solution on each side of the loaded samples. Record sample loading pattern below:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

16. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) is two-thirds of the gel.
17. Stop the gel electrophoresis.
18. Take a picture of the gel.
19. Analyze data.
 - Do you observe PCR fragments?
 - What are the sizes of these fragments?
 - Do the sizes agree with expected sizes for the gene of interest and T-DNA insertion?

20. After determining the genotypes of T-DNA insertion plants, put small piece of tape on each of a number of wooden sticks corresponding to the number of T-DNA tagged plants (homozygous or heterozygous for T-DNA). Write the **number** that corresponding to the **plant #** on the Plant Layout chart and either homozygous or heterozygous.
21. Go to the Plant Growth Center, put the wooden sticks next to the identified T-DNA tagged plants.
22. Observe T-DNA tagged plants for abnormal phenotypes.

EXPERIMENT 7 – GENE EXPRESSION STUDY IN *ARABIDOPSIS THALIANA* (GENE TWO)

Purpose: To determine mRNA accumulation patterns of genes encoding transcription factors in Arabidopsis leaves and siliques/seeds.

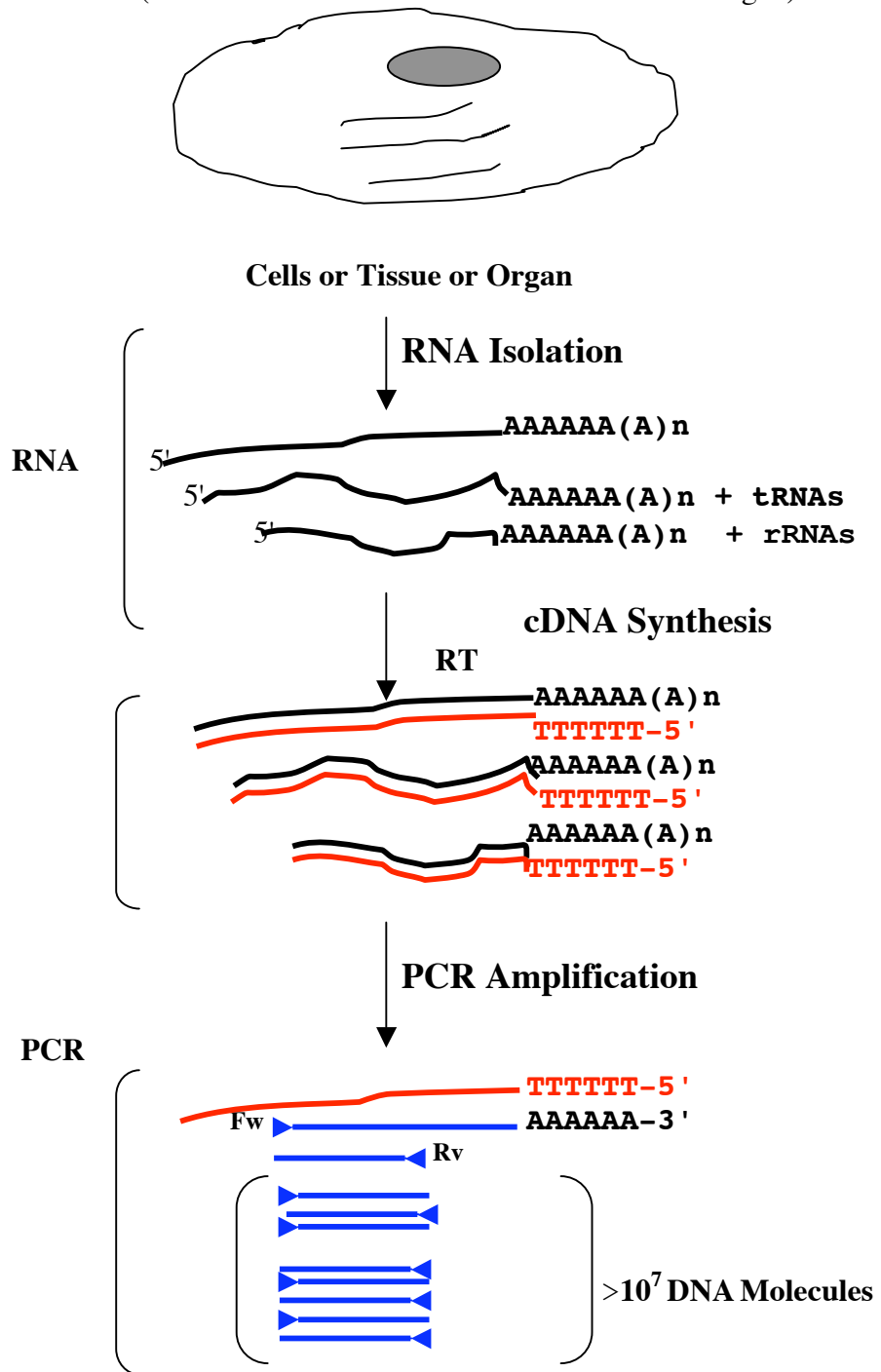
Attention: *In the previous Experiment 3, you had isolated total RNA from leaves and siliques of wild type Arabidopsis plants and synthesized cDNA templates using Reverse transcriptase for these RNA samples. The cDNA (or RT) solutions have been stored in the –20°C freezer and still good for the PCR amplification of the SECOND gene. In this experiment, you, therefore, do NOT need to carry out steps I and II, but perform step III.*

OVERVIEW OF RT-PCR

STRATEGY

- I. ISOLATION OF TOTAL RNA USING QIAGEN RNEASY PLANT MINI KIT (SEE EXPERIMENT 3)**
- II. SYNTHESIZING FIRST STRAND cDNA USING REVERSE TRANSCRIPTASE (REVERSE TRANSCRIPTION or RT) (SEE EXPERIMENT 3)**
- III. CARRYING OUT PCR AMPLIFICATION ANALYSIS**

OVERVIEW OF RT-PCR (Based on RT-PCR Technical Note from Invitrogen)



- I. ISOLATION OF TOTAL RNA USING QIAGEN RNEASY PLANT MINI KIT (SEE EXPERIMENT 3)
- II. SYNTHESIZING FIRST STRAND cDNA USING REVERSE TRANSCRIPTASE (REVERSE TRANSCRIPTION or RT) (SEE EXPERIMENT 3)
- III. CARRYING OUT PCR AMPLIFICATION ANALYSIS

Purpose: To amplify DNA template corresponding to mRNA that is either absent or present at different levels in different plant organs throughout plant development.

Materials and Reagents Needed:

- Reverse transcription (+RT & -RT) reactions
- 10X Ex-Taq buffer (Takara Mirus Bio)
- dNTP mix (Takara Mirus Bio)
- **12 μM** Gene-specific **RT-PCR Fw** primers
- **12 μM** Gene-specific **RT-PCR Rv** primers
- **12 μM Tubulin Fw** primers
- **12 μM Tubulin Rv** primers
- Sterile water
- Ex-Taq DNA polymerase (Takara Mirus Bio, 5 U/μL)
- Agarose
- 1X TAE buffer
- 1 KB DNA ladder (Invitrogen)
- 6X Loading buffer
- 0.2 mL PCR tubes
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube racks
- PCR Machine (Bio-Rad MyCycler or PE 9700)
- Gel apparatus
- Power supplies

PROCEDURE

1. Get ice from the icemaker in room 2911 or 3906.
2. Determine **how many RT reactions**, including **+RT's** and **-RT's**, will be amplified.
3. Make a **Table** with information such as **tube #**, **plant organ(s)**, and **+RT's/-RT's** (see the **example** Table below)

Tube #	1	2	3	4	5 (Positive)	6 (Negative)
Organ & RT	Leaf +RT	Leaf -RT	Silique +RT	Silique -RT	Genomic DNA	Sterile Water

4. Label on the **lids** and **sides of FIVE 0.2 mL PCR tubes** with **Number** and **your initial**.
5. Put the labeled tubes on a PCR rack sitting **on ice**.
6. Prepare a **master mix** in a **1.5-mL microcentrifuge tube** for the **number of PCR solutions** being carried out **plus 1 extra solution volume** as followings: (**How many reactions are carried out?**)

	Mmix for 1 Reaction	Mmix for 7 Reactions
10X Ex-Taq buffer	5.0 μ L	35.0 μ L
dNTP mix	4.0 μ L	28.0 μ L
12 μM RT-PCR Gene-specific Fw primer	1.0 μ L	7.0 μ L
12 μM RT-PCR Gene-specific Rv primer	1.0 μ L	7.0 μ L
*12 μM Control (Tubulin) Fw primer	1.0 μ L	7.0 μ L
*12 μM Control (Tubulin) Rv primer	1.0 μ L	7.0 μ L
Sterile water	34.5 μ L	241.5 μ L
Ex-Taq DNA Polymerase (5 U/μL)	0.5 μ L	3.5 μ L
Total Volume	48.0 μL	336.0 μL

* *Control primers are used to ensure that the absence of Gene-specific PCR product in +RT samples is NOT due to technical mistakes. The expected size of the Tubulin PCR product is ~0.45 kb.*

16. Pipet **48 μL** of the **master mix** to the labeled tubes and **2 μL** of **appropriate RT** to each of the tubes shown on the table below. Mix the contents by pipetting **gently** up and down for five times.

Tube #	1	2	3	4	5 (Positive)	6 (Negative)
Mmix	48 μL	48 μL	48 μL	48 μL	48 μL	48 μL
Leaf +RT	2 μL	---- μL	---- μL	---- μL	---- μL	---- μL
Leaf -RT	---- μL	2 μL	---- μL	---- μL	---- μL	---- μL
Silique +RT	---- μL	---- μL	2 μL	---- μL	---- μL	---- μL
Silique -RT	---- μL	---- μL	---- μL	2 μL	---- μL	---- μL
0.2 ng/μL Genomic DNA	---- μL	---- μL	---- μL	---- μL	2 μL	---- μL
Water	---- μL	---- μL	---- μL	---- μL	---- μL	2 μL
Total Volume	50 μL	50 μL	50 μL	50 μL	50 μL	50 μL

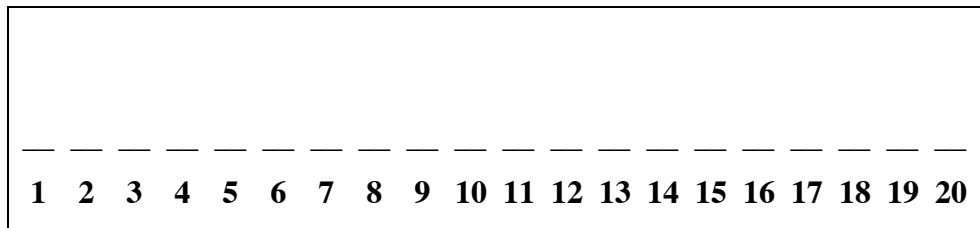
17. Carry out PCR reactions with the **RT-PCR program** containing the following profile: 1 cycle of 96°C, 3 min. → 40 cycles of 94°C, 10 sec./60°C, 30 sec./72°C, 45 sec. → 1 cycle of 72°C, 4 min. → 4°C, ∞.

18. Prepare **100 mL** of **1.5% agarose gel** in **1X TAE** buffer as usual (Use a **20-tooth** comb).

Note: The percentage of agarose gels depends on the difference in size of two PCR products. If there is at least 100-bp difference between two PCR products, then use a 1% agarose gel. However, if there is 50-100 bp difference between two PCR products, then use 1.5-2% agarose gel. For example, the size of PCR products is 0.6 kb and 0.55 kb for the control and gene A, respectively. The 2.0% agarose gel resolves these two PCR products as two discreet DNA bands whereas the 1.0% agarose gel shows these two PCR products as a single DNA band.

19. Label 1.5 mL microcentrifuge tubes according to the PCR solutions being performed.

20. Add to the labeled 1.5 mL microcentrifuge tubes **20 μ L** of **PCR solution** and **3 μ L** of **6X loading dye**. *Note: 20 μ L of PCR solution was loaded so that you can see the PCR products clearly.*
21. Load samples on a 1.5% agarose gel along with **20 μ L** of **50 ng/ μ L 1-Kb ladder** solution. Record RNA loading pattern.



22. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) is about two-thirds of the gel.

Starting time:

Ending time:

23. Take a picture of the gel.
24. Analyze the data.

How many DNA fragments do you see on the gel?

Is there different brightness between the fragments from one organ to the other?

What are the sizes of DNA fragments?

Note: The expected size of the Tubulin PCR product is ~0.45 kb.

What is the size of the PCR fragment corresponding to mRNA of the gene of interest?

What is a conclusion on gene expression of the gene of interest for the tested plant organs, leaves and siliques?

Are the RT-PCR results correlated to the GeneChip data?

EXPERIMENT 8 – CLONING THE PROMOTER REGION OF THE GENE OF INTEREST (GENE TWO)

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.

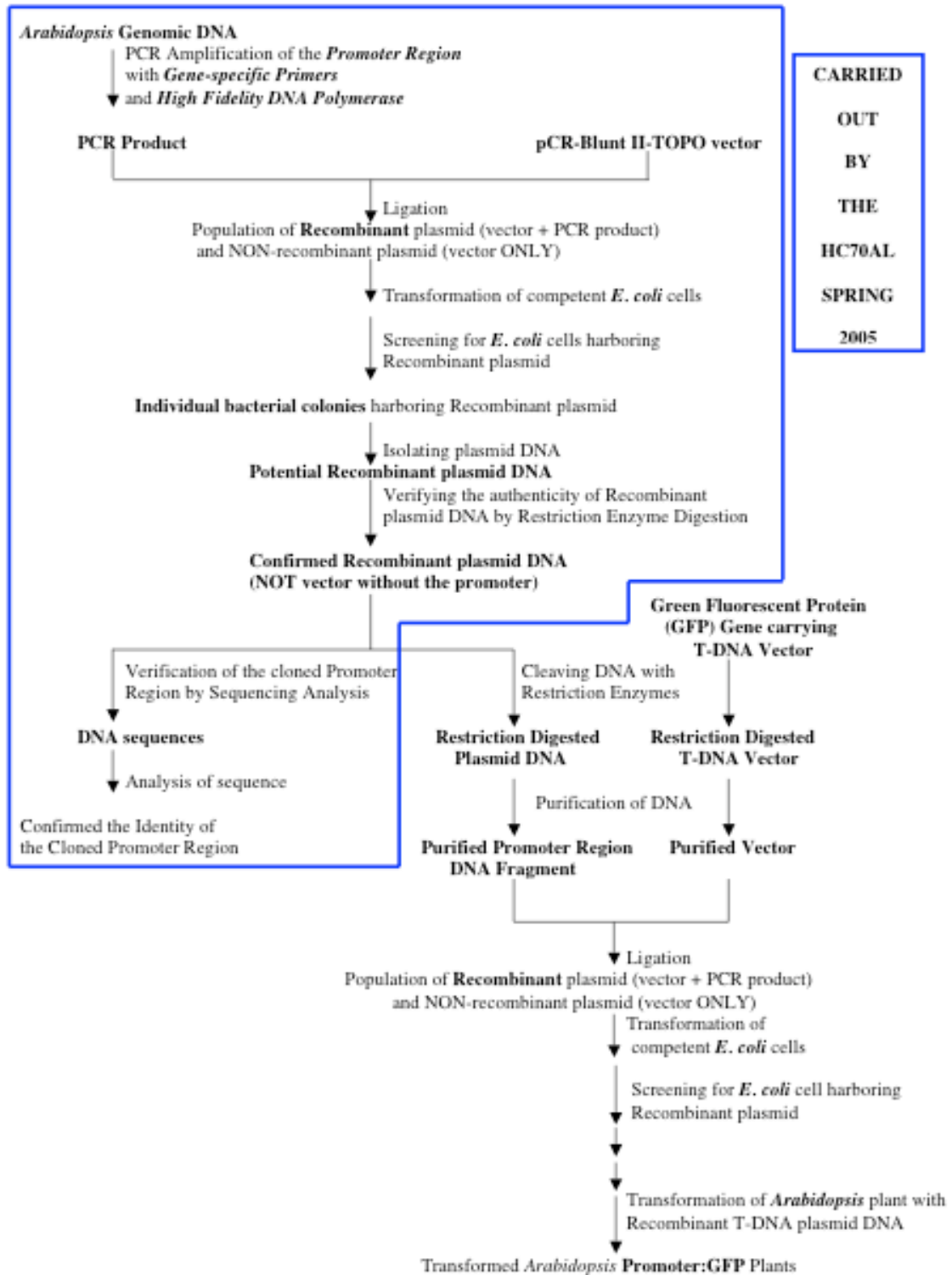
Reference:

1. KOD HiFi DNA Polymerase Technical Note (Novagen) (see Attachment 1)
2. TOPO Cloning Instruction Manual (Invitrogen) (see Attachment 2)
3. QIAprep Miniprep Handbook (see Attachment 3)

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



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
KOD Hot Start DNA Polymerase kit (Cat.# 71086-3, Novagen)
Sterile water
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 **10x Buffer for KOD Hot Start DNA Polymerase, 25 mM MgSO₄ and dNTPs (2 mM each)** 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 **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)** as follows:

Master Mix (*Note: Amplification of targets greater than 3 kbp may require more DNA polymerase*)

	Mmix for 1 Reaction	Mmix for 4 Reactions	Final Concentration
Sterile water	27.0 μ L	108.0 μ L	
10x Buffer for KOD Hot Start DNA polymerase	5.0 μ L	20.0 μ L	1x
dNTP mix (2 mM each dNTP)	5.0 μ L	20.0 μ L	0.2 mM
25 mM MgSO₄	2.0 μ L	8.0 μ L	1.0 mM
12 μM Gene-specific Forward primer	1.0 μ L	4.0 μ L	0.24 μ M
12 μM Gene-specific Reverse primer	1.0 μ L	4.0 μ L	0.24 μ M
Hot Start DNA polymerase (1.0 Units/μL)	1.0 μ L	4.0 μ L	0.2 Unit
Total volume	42.0 μL	168.0 μL	

- Pipet into the tube the reagents with order from top down (*example: water, 10x Buffer, dNTP mix, etc.*).
- 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	Tube #3
Mmix	42 μL	42 μL	42 μL
~100 ng <i>Arabidopsis</i> genomic DNA (12 ng/μL)	8 μL	0 μL	0 μL
Positive control DNA template (PCR product using Ex-Taq 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 and holding the "**Standby**" button for **TWO** seconds.
 - 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 "**KOD HiFi**" protocol by pressing yellow arrowheads surrounding the "**ENTER**" button. Once it is selected, the "**KOD HiFi**" protocol is highlighted. Press the "**ENTER**" button.

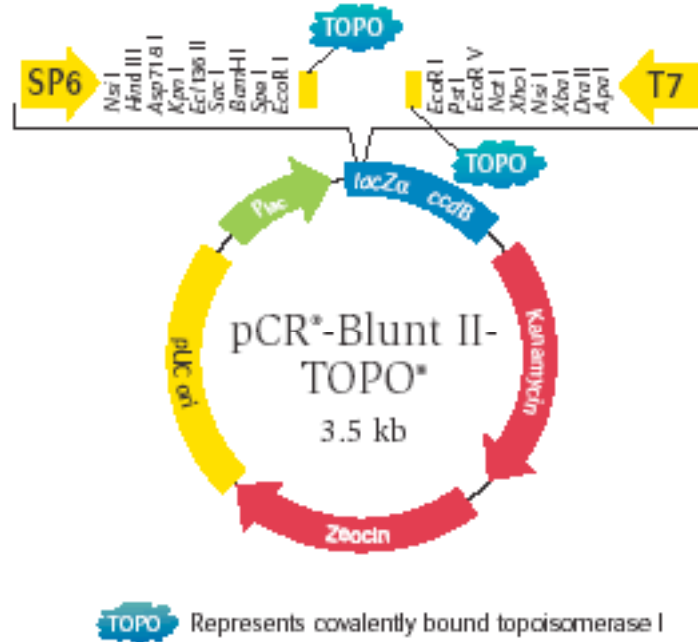
The **PCR profile for Genomic DNA templates.**

Cycling parameters	Up to 3 kbp of PCR product
Activation Enzyme step	94°C for 2 minutes
Number of cycle	1
Denaturation step	94°C for 15 seconds
Annealling step	55°C (or $T_m - 5^\circ\text{C}$) for 30 seconds
Extension step	72°C for 75 seconds
Number of cycles	30

- 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 μ L** of the reaction products on a **1% agarose** gel containing **0.5 μ g/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. Pipet the following reagents into the labeled tube as listed in the **Table** below:

Reagent	
Freshly prepared PCR product	2.0 μL
Salt Solution	1.0 μL
Sterile water	2.5 μL
pCR-Blunt II – TOPO	0.5 μL
Total Volume	6.0 μL

- a. Mix reaction **GENTLY** by **flicking** the tube. Do **NOT** vortex the tube!

- b. 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

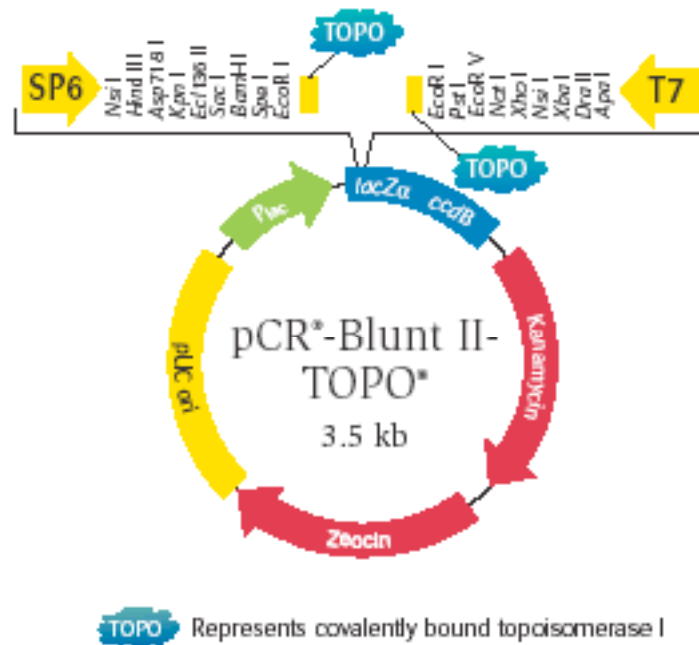
1. Label on the lids of **1.5-mL microcentrifuge tubes** with **your Initial** and the **number (1-6)**. Set labeled tubes on the microcentrifuge rack.
2. Arrange the culture tubes and labeled microcentrifuge tubes in their corresponding order. For example, 1 to 1, 2 to 2,... , 6 to 6.
3. **Carefully**, 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) Spin tubes in the microcentrifuge at **FULL** speed for **10 minutes**.

14. 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.
15. 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 26**).
16. After 10 minutes of spinning, transfer 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.*
17. Spin the columns in their collection tubes at full speed for 30 seconds.
18. Lift the column off the collection tube and discard the flow-through liquid into a glass **BEAKER**.
19. Put the column back in its collection tube.
20. Pipet **500 µL** of **Buffer PB** to each column. Spin the columns at **FULL** speed in the microcentrifuge for 30 seconds.
21. Lift the column off the collection tube and discard the flow-through liquid into a glass **BEAKER**.
22. 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.*
23. Lift the column off the collection tube and discard the flow-through liquid into a glass **BEAKER**.
24. 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. Also, ethanol will inhibit enzyme activity in later steps.*
25. Transfer the QIAprep columns in **NEWLY** labeled tubes. Discard the collection tubes. *Note: make sure the numbers on the columns and microcentrifuge tubes matched.*

26. Pipet **50 μ L** of **Buffer EB (10 mM Tris-HCl, pH 8.5)** to the center of each QIAprep column.
27. Let the columns stand for **1 minute**. Note: it is okay to incubate **longer than 1 minute**.
28. Spin the tubes with columns at **FULL** speed for **1 minute**. Steps 28 and 29 are for eluting plasmid DNA off the column.
29. After spinning, discard the columns. **Save the eluted plasmid DNA** in the microcentrifuge tubes.
30. Determine **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 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/mL)
- 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

Components	Standard Reaction	Final Concentration	<u>Example</u> 500 ng DNA (@200 ng/μL)
DNA	x μL	-----	2.5 μL
Sterile water	y μL	---	15.0 μL
*10x React buffer #2 (Invitrogen)	2.0 μL	1x	2.0 μL
<i>EcoRI</i> (10 U/μL, Invitrogen)	0.5 μL	0.25 U/μL	0.5 μL
Total Volume	20.0 μL	---	20.0 μL

Explanation of volumes

- ✓ $x \mu\text{L}$ = Volume of Plasmid DNA depends on the **amount** (in ng) of DNA to be digested and the **concentration** of plasmid DNA (in ng/μL)
- ✓ Volume of 10x 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 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!

Components	Sample A (300 ng/μL)	Sample B (200 ng/μL)	Sample C (100 ng/μL)	Final Concentration
500 ng of DNA	1.7 μL	2.5 μL	5.0 μL	25 ng/μL
Sterile water	8.3 μL	7.5 μL	5.0 μL	---
2x Enzyme mix	10.0 μL	10.0 μL	10.0 μL	1x
Total volume	20.0 μL	20.0 μL	20.0 μL	

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?

Components	Enz Mix for 1 Reaction	Enz Mix for #__Reactions
10x React buffer #3	2.0 μL	____ μL
Sterile water	7.5 μL	____ μL
<i>EcoRI</i> (10 U/μL)	0.5 μL	____ μL
Total Volume	10.0 μL	____ μL

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.

Components	Sample 1 (ng/ μL)	Sample 2 (ng/ μL)	Sample 3 (ng/ μL)	Sample 4 (ng/ μL)
500 ng of DNA	____ μL	____ μL	____ μL	____ μL
Sterile water	____ μL	____ μL	____ μL	____ μL
2x Enzyme mix	10.0 μL	10.0 μL	10.0 μL	10.0 μL
Total volume	20.0 μL	20.0 μL	20.0 μL	20.0 μL

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 1 hour.

2. Meantime, prepare a 1% agarose gel in 1X TAE buffer with 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.
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.

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.
9. 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 μ M T7 primer
- 3 μ M 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:

	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 μ L	x (x 3) μ L
Sterile water	y μ L	y (x 3) μ L
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 **gene-specific primer** into TWO labeled 0.2-mL PCR tubes.

either or

Components	T7 primer	SP6 primer
Mmix	19 µL	19 µL
3 µM T7 primer	1 µL	-----
3 µM SP6 primer	-----	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, ∞

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/>
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.

EXPERIMENT 9 - IDENTIFYING FEATURES OF MUTANT EMBRYO USING NOMARSKI MICROSCOPY (GENE TWO)

Purpose: To introduce **Differential Interference Contrast (DIC)** or **Nomarski Interference Contrast (NIC)** microscopy technique as a tool to identify features of defective embryos as illustrated in a T-DNA knockout mutant *dicer-like*, *lec1*, *raspberry3*, and *titan*.

Reference: The protocol was written by Dr. Miguel Aguilar in Professor Robert L. Fischer laboratory at University of California, Berkeley.

Materials Needed:

- Siliques containing seeds with a wide range of embryo stages (globular to mature green) from Arabidopsis
 - a. Wild type
 - b. T-DNA knockout mutant *raspberry3* or *titan* or *dicer-like* or *lec1*
 - c. homozygote or heterozygote mutant
- Absolute ethanol
- Acetic acid
- Chloral Hydrate (C-8383, Sigma-Aldrich)
- Glycerol (Gibco/Invitrogen)
- Fine point forceps
- 30-gauge hypodermic needles
- Microcentrifuge tubes (1.5 mL)
- Microcentrifuge tube rack
- Disposable transfer pipets or Pasteur pipets
- Double-distilled water
- Pipetman P-200 and P-1000
- Pipette tips
- Rulers with METRIC scale (cm)
- Fine-point scissors or razor blades
- Coverslips
- Microscope Slides

Equipment Needed:

- Dissecting microscopes (we got 10 extra dissecting microscopes from our Biology department storage)
- A microscope equipped with Normarski optical parameter (Leica CTR5000)

PROCEDURE

Each student collects the followings from Wild type, one of the known mutants, and his/her homozygous/heterozygous mutant:

(a) 5 siliques containing white seeds with embryo stages of globular to heart or torpedo. Note: silique length is in a range of 0.5 cm to 1.5 cm.

(b) all seeds from One silique containing mature green embryos.

A. Fixing Plant Materials

14. Prepare 5 mL of fixative solution of ethanol: acetic acid (9:1, v/v) in a 14-mL sterile Falcon tube using disposable 5-mL pipets.

Absolute ethanol	4.5 mL
<u>Acetic acid</u>	<u>0.5 mL</u>
Total volume	5.0 mL

- Close the tube with its cap.
- Invert the tube to mix the content.

15. Pipet **0.75 mL** of the **fixative solution** into **SIX** 1.5 mL microcentrifuge tubes sitting on the microcentrifuge rack at room temperature.

16. Label on the lid and side of each tube in step 2 with your initial, number (1-6), and information (mutant/wild type, siliques/seeds).

17. Label on the lids and sides of **THREE** microcentrifuge tubes with information (Wild type, known mutant, or your homozygote/heterozygote mutant). *Note: siliques with different lengths from wild type, known mutant, and homozygote/heterozygote mutant plants will be collected into each of these three tubes.*

18. Bring the **tubes in step 4** and the **Plant Chart** with information about plant number and the genotype of those plants to the Plant Growth Center (or greenhouse).

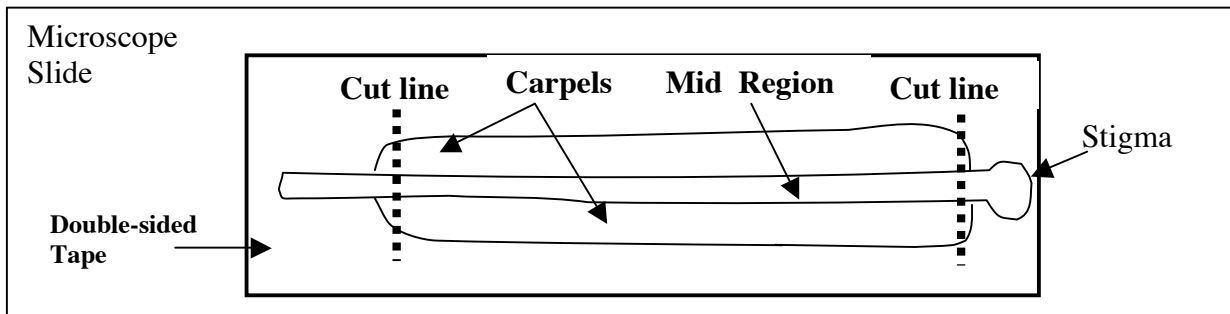
19. Measure and collect **3 siliques** for **each** of **two different lengths** (one short and one long) in a **range of 0.5-1.5 cm** from the wild type, a known T-DNA knockout (*raspberry 3*, *dicer-like*, *lec1*, or *titan*), and homozygous/heterozygous mutant.

Note: collect the same length of siliques for wild type, known mutant, and homozygous/heterozygous mutant.

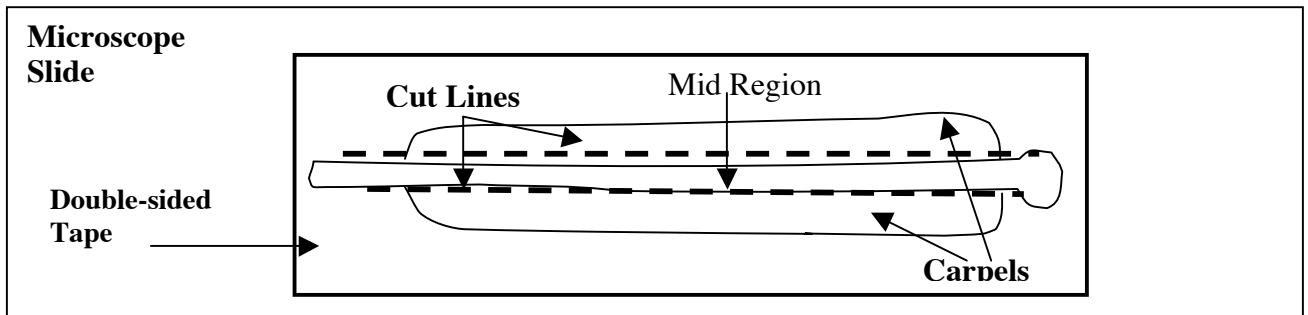
What are the genotype and phenotype of the mutant plants?

20. Collect **siliques** and **seeds** and put them in the fixative solution as follows:

- a. For **siliques with length < 0.7 cm**, Place a **piece of double-sided tape** on a **microscope slide** --> Carefully, use a **fine-point forceps** to place a silique on the tape --> **Excise the top and bottom of the silique** at its **ends of carpels** to allow solutions to penetrate the seeds and embryos during fixation, washing, and clearing steps (see a diagram below) --> **Immediately**, use the fine-point forceps to transfer the cut silique into the Fixative solution. Repeat the cutting of siliques for other siliques.



- b. For **siliques with length > 0.7 cm**, including those containing seeds with mature green embryos, **dissect** the seeds out of the siliques as illustrated below (NOT drawn to scale): Place a **piece of double-sided tape** on a **microscope slide** --> Carefully, use a **fine-point forceps** to place a **silique** on the tape and **rearrange** the silique such that the **mid region** is facing you --> Use a **28G or 30G hypodermic needle attached to a 1cc syringe** to **slice the carpels** along the **mid region** as shown in the diagram, then Use the fine-point forceps to **tear off the carpels** to expose the seeds --> Use either the forceps or the needle to **collect and transfer seeds** into the **Fixative solution**.



21. Fix seeds and siliques in the fixative solution for 2 hours to overnight.

*Note: It is recommended to fix the seeds from siliques with length > 0.7 cm **overnight** to ensure that fixative solution penetrates the seeds and their embryos.*

22. Next day, CAREFULLY pipet off **650 μ L** of the fixative solution using a **P-1000** pipetman and then the **remaining volume** with a **P-200** pipetman. *Note: Do not let the seeds and siliques dried out.*

23. **Immediately**, pipet 0.5 mL of 90% ethanol solution into the tube using a P-1000 pipetman. *The 90% ethanol solution will remove chlorophyll from the embryos.*

90% ETHANOL SOLUTION

Absolute ethanol	4.5 mL
<u>Double-distilled water</u>	<u>0.5 mL</u>
Total volume	5.0 mL

24. Incubate seeds and siliques in the ethanol solution for 0.5 - 1 hour.

Note: It is safe to leave the materials in the ethanol solution longer than 1 hour (or indefinite).

25. Replace the 90% ethanol solution with 70% ethanol similar to steps 9 & 10.

70% ETHANOL SOLUTION

Absolute ethanol	3.5 mL
<u>Double-distilled water</u>	<u>1.5 mL</u>
Total volume	5.0 mL

26. Incubate seeds and siliques in the ethanol solution for 0.5 - 1 hour.

Note: it is safe to leave the materials in the ethanol solution longer than 1 hour (or indefinite).

B. Observation of Seeds and Embryos

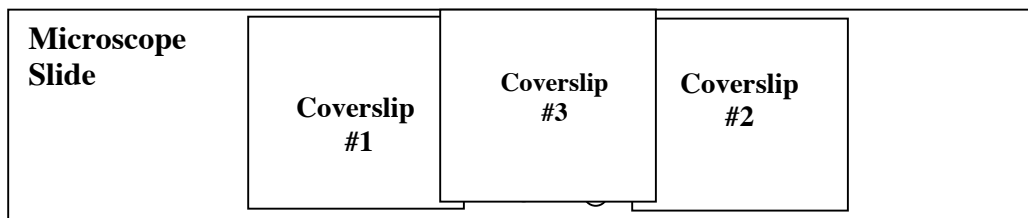
Note: At least **ONE** hour before observation of the seeds and their embryos. Seeds must be submerged in the clearing solution. (a) The older the silique, the longer it takes to clear and sink. For young seeds, clearing is usually fast, i.e., seeds can be observed under a microscope within 30 minutes. (b) Tissues **CANNOT** be stored in the **CLEARING** solution for more than **TWO** days because they will lose their structures quickly.

1. Prepare a **fresh** CLEARING solution of chloral hydrate/glycerol/water (8:1:2, w/v/v) in a 14-mL Falcon tube (**Note:** The TA will prepare this solution before the lab class begins)

CLEARING SOLUTION

Chloral hydrate	8 g
Glycerol	1 mL
Water	<u>2 mL</u>
Total volume	3 mL

2. Replace the 70% ethanol solution with the CLEARING solution. Wait for 30-60 minutes or until the seeds and siliques **SINK** to the bottom of the tubes.
3. Set a new glass microscope slide on the bench.
4. Gently, pipet the clearing solution up and down for 5 times to mix up the seeds using a P-200 pipetman.
5. Dispense **100-150 μ L** of the clearing solution with seeds in the center of the microscope slide.
6. Carefully, place two squared coverslips, one on each side of the solution. Then, place a third coverslip over the clearing solution (see below)



7. Observe the seeds under Nomarski optic using Leica CTR5000 microscope.
8. Take pictures of the embryos.

Note: For siliques, you need to dissect seeds out of the silique on a microscope slide under a dissecting microscope. Then, proceed steps 5-8.

APPENDIXES

Appendix 1A

Preparation of a 1% Agarose Gel for Gel Electrophoresis

2. For a **1% agarose gel**, weigh out **1 gram** of agarose (powder) on a weighing scale.
Note: percentage of the gel reflects the amount of agarose in gram in 100 mL of 1X TAE buffer, depending on the final percentage of agarose in the gel.
Example: If you want to make a 0.7% agarose gel (0.7 g/100 mL, w/v), weigh out 0.7 g of agarose for 100 mL of agarose solution
3. Carefully, put the agarose in a 250-mL Erlenmeyer flask.
4. Measure out 100 mL of 1X TAE buffer using a plastic or glass graduated cylinder.
5. Add 100 mL of 1X TAE buffer into the flask in step 2.
6. Cover the flask with a piece of plastic wrap. Poke 3-4 holes on the plastic wrap using a pointed end of a pencil or pen (*Note: the holes allow the steam to escape during microwaving in step 6 below*). Swirl the solution to break up any lumps of agarose granules.
7. Microwave the solution for about 2 minutes or until the agarose granules have completely melted.
 - *Be careful with the flask. The solution gets very hot.*
 - *Constantly watch over the solution because when it starts boiling, it might overflow.*
 - *Swirl gently the solution several times while microwaving to help melt agarose evenly.*
 - *Once the agarose has melted completely, the solution is clear.*
8. Cool down the agarose solution for at least 30 min in a 55°C water bath.
9. While the agarose solution is cooling, prepare the gel cast with the appropriate comb.
 - *The comb depends on the number of samples to be loaded on the gel. For example, if there are less than 18 samples, then use a 20-tooth comb; but, if there are 21 samples, then use a 30-tooth comb.*

- *Remember to add two more wells to the number of wells needed for the samples. These two wells will be for loading 1kb DNA ladder in the first and the last wells (or left and right sides of loaded samples).*

10. After the agarose solution has been cooled down, add 5 μ L of Ethidium Bromide (EtBr) into the solution and swirl the flask GENTLY to mix. Note: Do NOT swirl vigorously to generate many bubbles.

11. Pour the agarose/EtBr solution into the gel cast. Wait for 30 min for the agarose solution to solidify.

Note: IMMEDIATELY after pouring the agarose solution, inspect the agarose solution's surface for the present of bubbles. If there are many bubbles floating on the surface of the gel solution, use a pipette tip to pop them before the gel is completely solidified.

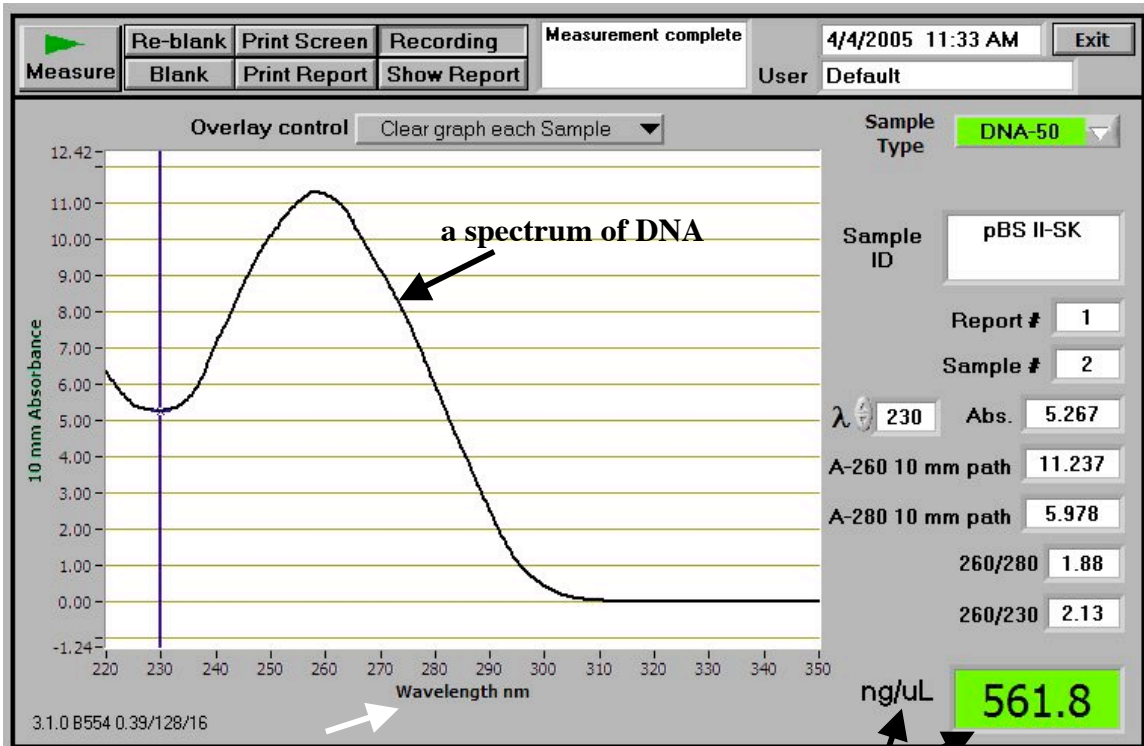
12. Pour ~600 mL of 1X TAE buffer into the gel box.

After the agarose has solidified into a gel, take out the comb gently by pulling it straight up out of the gel and put the gel in the gel box containing the 1XTAE running buffer.

Appendix 1B

What is a **spectrophotometer**?

It is an **instrument** that **measures** the **amount** of **molecules** absorbing at a **given wavelength of energy**. In this exercise, we measure the amount of DNA molecules in a given volume in the **ultraviolet wavelengths** of **200 – 280 nm** (nm stands for **nanometer**, which is **1 billionth** of a **meter**). The bases of DNA have the absorbance at the wavelength of 254 nm. The absorbance of DNA molecules over the wavelength range of 220 - 350 nm is represented as a **spectrum** with a **peak** at **near 260 nm** as illustrated by the **Nanodrop spectrophotometer readout** below:

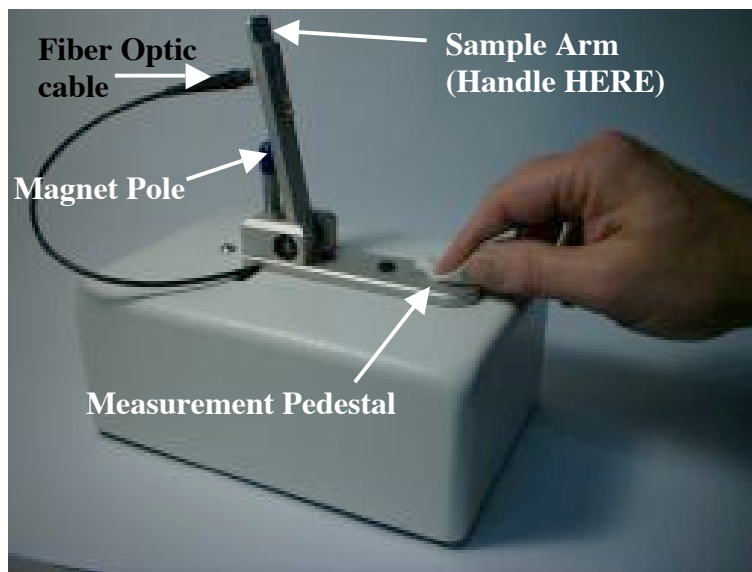


DNA concentration

OPERATION OF NANODROP SPECTROPHOTOMETER ND-1000

Note: The Nanodrop is powered by the computer via the USB port.

1. **Turn ON** the **Computer (laptop)** that connects to the Nanodrop.
2. **Turn ON** the **Nanodrop** by clicking an "ND-1000 v3.1.0" icon on the computer desktop. Wait for a few seconds for the Nanodrop to be up. You see the Nanodrop 3.1.0 Diagnose panel with **User field** as **Default**.
3. **Click** on "**Nucleic Acid**" button on the top left column for reading concentration of DNA and RNA solutions.
 - ❖ You see a following message:
"Ensure Sample Pedestals are clean and then load a water sample. After loading water sample, click OK to initialize instrument"
4. a. **Clean the sample pedestals as followings:**
 - i. **Raise the Sample Arm up** by holding at its end as shown on the picture below.
Caution: NEVER hold the Optical Cord when lifting and lowering the Sample Arm because the cord is fragile; and it is very expensive to replace it.
 - ii. **Wipe both the Measurement Pedestal** and the **Sample Arm** with a piece of Kimwipes slightly wetted with distilled water.



b. Pipet **1.5 - 2 μL** of water on the **Measurement pedestal**.

Note: Even though the Nanodrop Inc. claims that the Nanodrop can read as low as **1 μL** , the concentration reading is NOT consistent at this volume. Therefore, the **minimal** volume for the concentration reading is **1.5 μL**

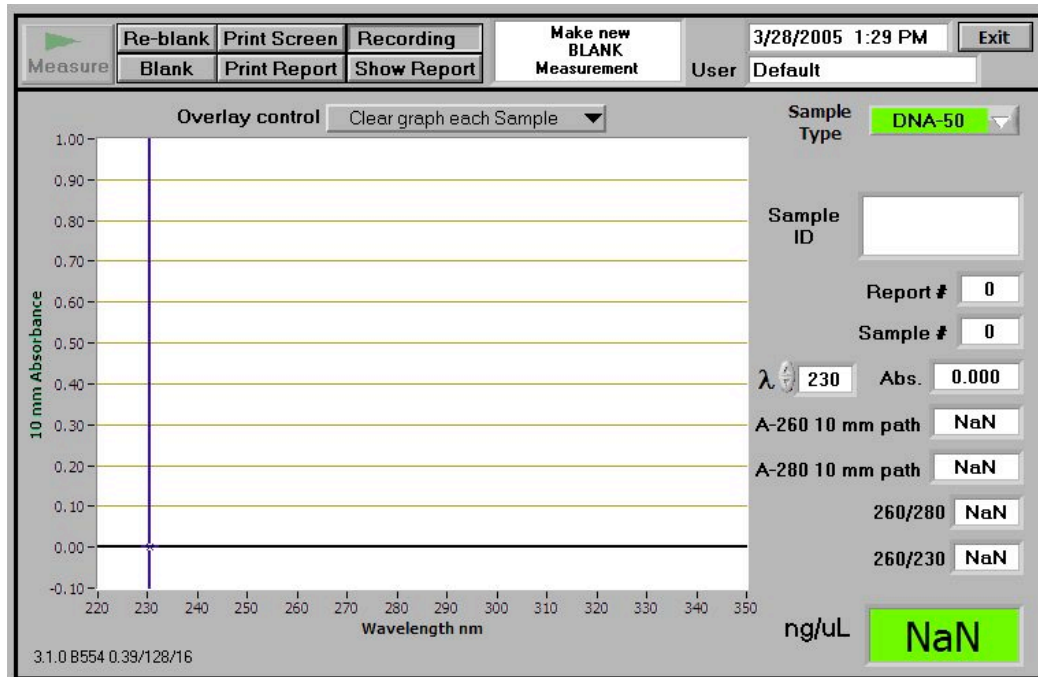


c. **Slowly Lower** the SAMPLE Arm to its horizontal position.

Caution: NEVER let the arm fall freely.

d. Click the **OK** button. The Nanodrop is **INITIALIZED**.

You see a **Dialog panel** as shown below



What do you need to do, NEXT?

- a. **Change SAMPLE TYPE** (if necessary) from **DNA-50** (by Default) to **RNA-40** or **Other** (for Oligonucleotides), depending on your sample.
 - b. **Type in** the **SAMPLE ID** field the Information of your sample.
 - c. **Make** a **NEW BLANK measurement**
 - d. (Option) **Change** the **OVERLAY CONTROL** field from the Default setting of “CLEAR GRAPH EACH SAMPLE” to “CLEAR GRAPH ON NEW REPORT” or “ACCUMULATE UNTIL CLEAR” or “CLEAR GRAPH NOW”
-
5. **Wipe off** the **liquid** on **BOTH** the **ARM** and **Measurement pedestals** with a piece of Kimwipes.
 6. **Make** a **Blank measurement** by pipetting 1.5 - 2.0 mL of either double-distilled water or TE (depending on whether your sample solution is in double-distilled water or TE) on the Measurement Pedestal. Then lower the Sample Arm to its horizontal position.
 7. **Click** the **BLANK** button. The blank was made.
 8. After the reading is done, **bring** the Sample Arm **up** to the vertical position and **Wipe off** the **liquid** on **BOTH** the **ARM** and **Measurement pedestals** with a piece of Kimwipes.
 9. **Pipet** 1.5 - 2.0 mL of **SAMPLE** on the Measurement Pedestal and lower the Sample Arm.
 10. **Type in** the **SAMPLE ID** field **Information** of a sample solution.
 11. **Click** the **MEASURE** button to determine concentration of your sample.
 12. After the reading is done, a sample concentration (in **ng/mL**) and a spectrum of the sample along other information are shown. You can either
 - a. **Save** the **window** of measured sample by clicking on **FILE** → choose **SAVE WINDOW** → Select an existing folder or Create a **NEW** folder (give a name for the **NEW** folder) → Type in a Name file in the **FILE NAME** field → Click the **SAVE** button to save the file **or**
 - b. **Print** the **window** by **clicking** the **PRINT SCREEN** button.

Note: To print the current spectrum of the sample, you **MUST** print it before reading the next sample. Otherwise, you need to repeat reading the sample.

13. **Repeat** steps 8-12 for other samples.
14. After reading the **last sample**, **click** the **PRINT BATCH** button to print concentrations of all read samples.
15. If done with the Nanodrop, **click** the **EXIT** buttons.
16. **Clean** the **Measurement Pedestal** and the **Sample Arm** with a piece of Kimwipes slightly wetted with distilled water.

Appendix 1C

1-kb DNA Ladder (Taken from Invitrogen website)



1 Kb DNA Ladder

Cat. No. 15615-016

Size: 250 µg

Conc.: 1.0 µg/µl

Store at -20°C.

Description:

The 1 Kb DNA Ladder (U.S. Patent No. 4,403,036) is suitable for sizing linear double-stranded DNA fragments from 500 bp to 12 kb. The bands of the ladder each contain from 1 to 12 repeats of a 1018-bp DNA fragment. In addition to these 12 bands, the ladder contains vector DNA fragments that range from 75 to 1636 bp. The 1636-bp band contains 10% of the mass applied to the gel. The ladder may be radioactively labeled by one of the following methods: (i) Partial exonucleolytic degradation and resynthesis with T4 DNA polymerase. This method is preferred because higher specific activity is achieved with less ³²P input; (ii) Labeling the 5' ends with T4 polynucleotide kinase; (iii) Filling in the 3' recessed ends with *E. coli* DNA polymerase I or the large fragment of DNA polymerase I.

Storage Buffer:

10 mM Tris-HCl (pH 7.5)

50 mM NaCl

0.1 mM EDTA

Recommended Procedure:

Invitrogen recommends the use of 10X BlueJuice[®] Gel Loading Buffer (10816-015) at a concentration of 2X [for electrophoresis of this ladder on agarose gels]. Alternatively, the DNA ladder can be diluted in a buffer such that the final concentration of NaCl is 20 mM. Apply approximately 0.1 µg of standard per mm lane width. **DO NOT HEAT!**

Quality Control:

Agarose gel analysis shows that all bands larger than 500 bp are distinguishable.

Doc. Rev.: 011602

This product is distributed for laboratory research only. CAUTION: Not for diagnostic use. The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

For technical questions about this product, call the Invitrogen Tech-LineSM U.S.A. 800 955 6288

APPENDIX 2 - BIOINFORMATICS (PARTS I AND II)

HC70AL Spring 2004

An Introduction to Bioinformatics -- Part I

By

Brandon Le

April 6, 2004

What is a Gene?

An ordered sequence of nucleotides

What are the 4 Nucleotides in DNA?

**A - Adenine
T - Thymine
C - Cytosine
G - Guanine**

What are the Characteristics of a Gene?

- An ordered sequence of nucleotides
- A unique position/location in the genome
- Polarity (5' to 3')
- Exons and Introns

What are the Anatomical Features of Genes?

- Discrete beginning and discrete end
- Two strands of DNA
- Double helical
- Strand one (5' to 3')
- Strand two (3' to 5')
- Sense strand (5' to 3')
 - specifies the trait
- Nonsense strand (3' to 5')
 - template for transcription

Sense Strand

5' - ACGTCAGTCGATGCATGCTAGCTAGC - 3'
3' - TGCAGTCAGCTACGTACGATCGATCG - 5'

Nonsense Strand

Genes Have a Unique Position in the Genome!

Task: Where is your gene located in the genome?

Tools: The Arabidopsis Information Resources (TAIR)
(<http://www.arabidopsis.org>)

Procedure:

1. Select Seqviewer
2. Enter gene number (ex. AT1G18260)
3. Submit

01 AT2G22800
02 AT2G23290
03 AT2G37120
04 AT3G09735
05 AT3G12840
06 AT3G50060
07 AT3G53370
08 AT4G37260
09 AT4G37790
10 AT5G03220
11 AT5G03500
12 AT5G19490
13 AT5G67300

Results/Question:

1. What chromosome is your gene in?
2. What other genes/markers are next to your gene?
3. What is the exact position of your gene in the genome?

Genes Have a Unique Order of Nucleotides!

Task: What is the order of nucleotides for your gene?

Tools: The Arabidopsis Information Resources (TAIR)
(<http://www.arabidopsis.org>)

Procedure: (Continue from previous slide)

1. Click on Location

Results/Question:

1. What are your neighbor genes?
2. What is the orientation of your gene?
3. How big is your gene?

Genes Have Exons and Introns!

Task: How many exons and introns does your gene have?

Tools: The Arabidopsis Information Resources (TAIR)
(<http://www.arabidopsis.org>)

Procedure: (Continue from previous slide)

1. Click on gene information on the right

Results/Question:

1. How many exons/introns in your gene?
2. What are exons?
3. What are introns?

Gene Encodes a Protein

Task: Determine the protein encoded by gene?

Tools: The Arabidopsis Information Resources (TAIR)
(<http://www.arabidopsis.org>)

Results/Question:

1. How large is your protein?
2. What are the anatomy of a protein?

N-terminal



C-terminal

What is the identity of your gene?

Task: What does your gene code for?

Tools: NCBI BLAST Tools
(<http://www.ncbi.nlm.nih.gov/BLAST>)

What is BLAST?

Basic Local Alignment Search Tool (BLAST)

What does BLAST do?

A family of programs that allows you to input a query sequence and compare it to DNA or protein sequences in db.

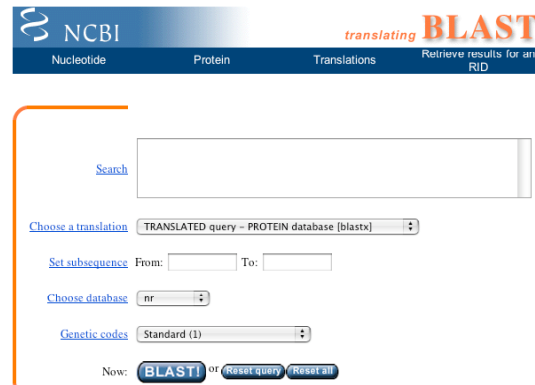
What are the steps to performing BLAST search?

Paste sequence of interest into BLAST input box

Select BLAST program

Select db

Select Optional Parameters



What are the different BLAST Programs?

Fastest

blastp - protein query vs protein db

blastn - DNA query vs DNA db

blastx - translated DNA query vs protein db

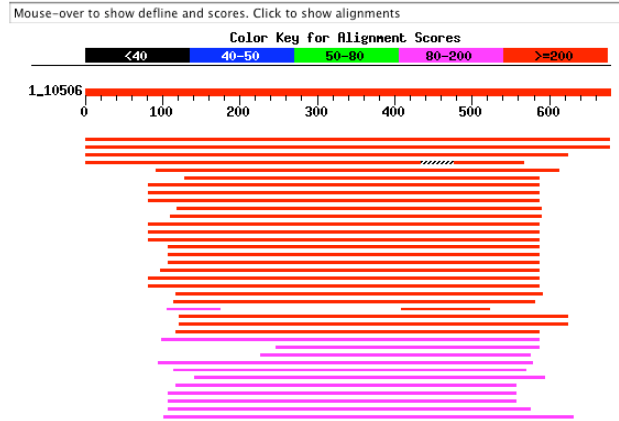
tblastx - protein query vs translated DNA db

Slowest

tblastn - translated DNA query vs translated DNA db

Anatomy of a BLAST Result -- Part I

Distribution of 339 Blast Hits on the Query Sequence



Anatomy of a BLAST Result -- Part II

Sequences producing significant alignments:	Score	E	Value
	(bits)		
gi 14532716 gb AAK64159.1 unknown protein [Arabidopsis tha...	1206	0.0	
gi 18394588 ref NP_564049.1 suppressor of lin-12-like prot...	1209	0.0	
gi 15219499 ref NP_177498.1 suppressor of lin-12-like prot...	877	0.0	
gi 11120786 gb AAG30966.1 hypothetical protein, 3' partial...	426	e-118	
gi 41151276 ref XP_046437.5 chromosome 20 open reading fra...	291	3e-77	L
gi 13559241 emb CAB65792.2 dJ842G6.2 (novel protein imilar...	282	2e-74	L
gi 19923669 ref NP_005056.3 sel-1 suppressor of lin-12-lik...	268	4e-70	L
gi 6851089 gb AAF29413.1 SEL1L [Homo sapiens] >gi 17646138...	268	4e-70	L
gi 9967440 dbj BAB12403.1 SEL1L [Mesocricetus auratus]	264	4e-69	
gi 31203035 ref XP_310466.1 ENSANGP00000019196 [Anopheles ...	263	1e-68	
gi 21355295 ref NP_651179.1 CG10221-PA [Drosophila melanog...	263	1e-68	L
gi 20857527 ref XP_127076.1 Sell (suppressor of lin-12) 1 ...	261	4e-68	L
gi 4159995 gb AAD05210.1 SEL1L [Mus musculus] >gi 20073079...	259	1e-67	L
gi 29336095 ref NP_808794.1 Sell (suppressor of lin-12) 1 ...	259	2e-67	L
gi 29612522 gb AAH49959.1 Sellh protein [Mus musculus]	258	4e-67	L
gi 17563256 ref NP_506144.1 Suppressor/Enhancer of Lin-12 ...	247	9e-64	L
gi 1255199 gb AAC47112.1 sel-1 gene product	247	9e-64	

Anatomy of a BLAST Result -- Part III

```
>gi|14532716|gb|AAK64159.1| unknown protein [Arabidopsis thaliana]
Length = 678

Score = 1206 bits (3120), Expect = 0.0
Identities = 614/678 (90%), Positives = 614/678 (90%)

Query: 1 MRILSYGIVILSLLVFSFIEFGVHARPVVLVXXXXXXXXXXXXXXXXXXXXXXXXXXXX 60
MRILSYGIVILSLLVFSFIEFGVHARPVVLV V
Sbjct: 1 MRILSYGIVILSLLVFSFIEFGVHARPVVLVLSNDDLNSGGDDNGVGESSDFDFGESEP 60

Query: 61 XXXXXLDPGSWRSIFEPDDSTVQAASPQYYSGLKKILSAASEGNFRLMEEAVDEIEAASS 120
LDPGSWRSIFEPDDSTVQAASPQYYSGLKKILSAASEGNFRLMEEAVDEIEAASS
Sbjct: 61 KSEEEIDPGSWRSIFEPDDSTVQAASPQYYSGLKKILSAASEGNFRLMEEAVDEIEAASS 120

Query: 121 AGDPHAQSIMGFVYIGIMMREKSKSKSFLHHNFAAAGNMQSKMALAFTYLRQDMHDKAV 180
AGDPHAQSIMGFVYIGIMMREKSKSKSFLHHNFAAAGNMQSKMALAFTYLRQDMHDKAV
Sbjct: 121 AGDPHAQSIMGFVYIGIMMREKSKSKSFLHHNFAAAGNMQSKMALAFTYLRQDMHDKAV 180

Query: 181 QLYAELAETA AVNSFLISKDSPVVEPTRIHSGTEENKALRKS RGEEDDFQILEYQAQKG 240
QLYAELAETA AVNSFLISKDSPVVEPTRIHSGTEENKALRKS RGEEDDFQILEYQAQKG
Sbjct: 181 QLYAELAETA AVNSFLISKDSPVVEPTRIHSGTEENKALRKS RGEEDDFQILEYQAQKG 240

Query: 241 NANAMYKIGLFYFGLRGLRRDHTKALHWFLKAVDKGEPRSMELLGEIYARGAGVERNYT 300
NANAMYK GLFYFGLRGLRRDHTKALHWFLKAVDKGEPRSMELLGEIYARGAGVERNYT
Sbjct: 241 NANAMYKNGLFYFGLRGLRRDHTKALHWFLKAVDKGEPRSMELLGEIYARGAGVERNYT 300
```

PubMed - Endless Resources

The screenshot shows the PubMed search interface. At the top, there are logos for NCBI, PubMed, and the National Library of Medicine (NLM). Below the logos is a search bar with the text "PubMed" and "for" followed by a search input field. There are "Go" and "Clear" buttons next to the search input. Below the search bar are navigation tabs: "Limits", "Preview/Index", "History", "Clipboard", and "Details". On the left side, there is a sidebar with links for "About Entrez", "Text Version", "Entrez PubMed Overview", "Help | FAQ", "Tutorial", "New/Noteworthy", "E-Utilities", "PubMed Services", "Journals Database", "MeSH Database", and "Single Citation". In the main content area, there are three bullet points providing search tips:

- Enter one or more search terms, or click [Preview/Index](#) for advanced searching.
- Enter [author names](#) as smith jc. Initials are optional.
- Enter [journal titles](#) in full or as MEDLINE abbreviations. Use the [Journals Database](#) to find journal titles.

Below the bullet points, there is a paragraph of text: "PubMed, a service of the National Library of Medicine, includes over 14 million citations for biomedical articles back to the 1950's. These citations are from MEDLINE and additional life science journals. PubMed includes links to many sites providing full text articles and other related resources."

HC70AL Spring 2004

An Introduction to Bioinformatics -- Part II

By

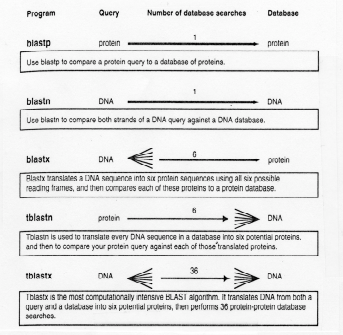
Brandon Le

April 8, 2004

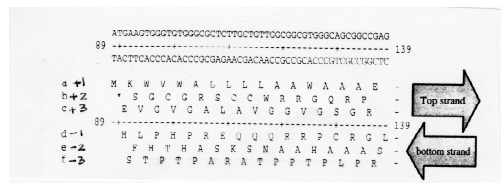
Review of BLAST Search

1. **What is the purpose of running BLAST Search?**
2. **What are the steps to performing BLAST search?**
3. **What does the e-value from a blast result tell you?**
4. **How many BLAST programs can you perform?**
5. **What BLAST program(s) takes the least computational time?**
6. **What BLAST program(s) takes the most computational time? Why?**

What are the Five BLAST Search Programs?



•How many proteins can a short DNA sequence potentially encode?



Question:

You have DNA Sequence. You want to know which protein in the main protein database is most similar to some protein encoded by your DNA.

Which BLAST program should you use?

Suppose you have a protein sequence.
Which BLAST program should you use?

HOW to interpret BLAST results?

Expect = 8×10^{-74}
 probability of finding this alignment in a database by chance and chance alone.

Identifier of protein in database
 d384206.2 (novel protein similar to SEDL (sw1-1)) (Homo sapiens)

origin of protein sequence

Score = 297 bits (760), Expect = 8×10^{-74}
 Identical = 177/468 (37%), Positives = 269/468 (57%), Gaps = 9/468 (1%)
 Frame = +1

What does frame mean?

Query: 388 MGFV--YGIHMKREKSEKSLHNFPAAGNMQSKHALAFTYLRQ----DHDKAVQLY 549
 +GF+ YGIH+ E +K+ +*+ *+* *+* *AGNM *R+ L+ ID + +A+ 3
 Sbjct: 2 MGLFQVYQIM--EYDQASALIVYVDSGSGHMHGHILOVYVLOGIIVLQNEVALQVY 59

Query: 540 ARLAFTAVNEPLTKDSEVVEPRTRISGTEENKGLRKSERGEEDFQILEYQAGKGNAN 729
 +*+ +*+ +*+ S+ E+ R+ TE + E + +G +*+ R+G+
 Sbjct: 60 KKVADYIADTFESESQVY-EVRL---TEPENLSSSEILMDIYQVYFLAERGQVQ 115

Query: 730 AMYKIGLFYFGLRGLRDHTKALNFKAVDKGPRSMELGELIYAG-AGVERNVA 708
 +G+ +G +G +G+ KALVFKKA G +R +R+*+ G A V +R A 708
 Sbjct: 116 IQVSLQLLHIGHGLEDQVYKAVYFLRAAKAGAHAFIHSYLSGHAAVFQHNK 175
 exact match at amino acid

Query: 907 LENVLAAKEGLYAFNGIGYLVKGVGVKKNYKAREYFEKAVDNEPDSCHYHLGVLY 1086
 +*+AA +G +*+G LV G GV NY +A +*+KA + + + LG +3
 Sbjct: 176 FKTFHAKRSCHGILGRLGILLFPGKGYL--NIAELITFQFAEKGNPDQGLGFNY 234

Query: 1087 LKIGVNRDVRQATYFFVAANAGQPFAYQLAKMFTGVLKELNEMATSYKLVAEKG 1240
 G G+ +D + A RIF+*+ *GDP A Y LAKM+ TG G+ +* A YK V E G
 Sbjct: 235 YSRSHMDEVFLAPYVLAAGQSGALVYLAHNVATQGVVNSCHYAVELVREGVSLG 294

Query: 1247 PWEELEWALHAYKGVGKALIVSRHAEKGYEVAQSNAMILDKYGRSMCHGVSGFC 1446
 W+ A AY GD+ +L+ YK +AENGYEVAQSN+*+IL+ + +----- 546
 Sbjct: 295 HWAKEFLTATFAYKGGDSEELVQALLRNCYVAQSNAILSEKKNIL----- 546
 what is this?

Query: 1447 TDKER-HERASLWRAEQGNEHAALLIGDAYYGRGTERDFVRAAEAY-NHAKSQSNA 1620
 +*E+ + A LW RA+ QUN A + IGL +YIG G*+D+ AA Y + A HA
 Sbjct: 347 -EERDHYFALSLWRAEQGNEHAALVYVYDDESLOSDAARLPTLALAI 1764

Query: 1471 QAMFELVYHREHGLFQDLHLKAVYVDESLOSDAARLPTLALAI 1764
 QAMFELVYHREHGLFQDLHLKAVYVDESLOSDAARLPTLALAI 1764
 Sbjct: 406 QAMFELVYHREHGLFQDLHLKAVYVDESLOSDAARLPTLALAI 1764

what does these number corresponds to?

what is query?
 what is subjct?
 positively charged amino acids

what does the "+" mean?
 K = Lysine
 R = Arginine

4

Review of gene transcription

1. What product is made after transcription?
2. How is the product similar/different from the gene?
3. What is cDNA?
4. What important information does a cDNA tell you about a gene?
5. What are ESTs?
6. What important information does ESTs tell you about a gene?

Annotation of your gene

1. What chromosome is your gene in?
2. How “big” is your gene?
3. How many exons and introns in your gene?
4. What orientation is your gene in the genome?
5. What is the specific position of your gene in the genome?
6. What gene is “upstream” of your gene?
7. What gene is “downstream” of your gene?
8. How far are the other genes (6 & 7) from your gene?
9. What is the “structure” of your gene?
10. What is the size of the protein in your gene encodes?
11. What protein does your gene encode
12. Is your gene structure predicted by a program?

Webbook - A Virtual Lab Notebook

Webbook is a web lab notebook

Purpose/goal: To have access to experiments carried out by
Lab members, etc... from anywhere
Also serves as a repository for protocols, stocks/reagents

Created by: Harry Hahn
Brandon Le
Bob Goldberg

<http://estdb.biology.ucla.edu/webbook>

Using the Webbook

- 1. Username: email username**
Password: 9 digit student id
- 2. Check message board for important news/updates**
- 3. An overview of the different sections**
 - Projects** - list of experiments
 - Stocks** - catalog of stocks/reagent in the lab
 - Protocols** - procedures carried out in the lab (pdf format)
 - Calendar** - calendar to plant your experiments
 - Browse** - search and look at other members experiments
 - Contact** - email for help
 - Logout** - will logout if idle for 30 min

Webbook Login Page

Help Login

webBOOK Login

Username: ble Password: Login

Last modified August 03 2003 21:16:09.
Copyrighted by the University of California (2003)
Created by Harry Hahn and Brandon Le, Laboratory of Bob Goldberg, UCLA

Creating Projects / Experiments

1. Title of project
2. Questions/Purpose of project
3. Summary of project (ideas)

Entering Gene Information

Genes

Create gene

Fields marked with a red asterisk (*) are **REQUIRED**

Gene Name:*	<input type="text"/>
Species:	<input type="text"/>
Sequence:	<input type="text"/>
Sequence Type:*	-- Select --
Amino Acid Sequence:	<input type="text"/>
Chromosome:	<input type="text"/>
EST Data:	<input type="text"/>
Functional Category:	<input type="text"/>
Promoter:	<input type="text"/>
Domains:	<input type="text"/>
Hits:	<input type="text"/>
Attach a file:	Title: <input type="text"/> File: <input type="button" value="Choose File"/> no file selected Description: <input type="text"/>

Entering Experiments Information Part 1

Experiments	
Fields marked with a red asterisk (*) are REQUIRED	
Title: *	<input type="text"/>
Goal: *	<input type="text"/>
Background Info: *	<input type="text"/>
Approach: *	<input type="text"/>
Controls: *	<input type="text"/>
Discussion:	<input type="text"/>
Next:	<input type="text"/>

Entering Experiment Information Part II

Materials	Primer * <input type="text" value="AT2G22800-FW
AT2G22800-RV
AT2G23290-FW
AT2G23290-RV
AT2G37120-FW
AT2G37120-RV
AT3G09735-FW
AT3G09735-RV"/>
Protocols:	Protocols * <input type="text" value="*Sequencing Using SPPCR
Alkali Lysis Plasmid Isolation
Arabidopsis Tissue Harvest for GeneChip Experiment
Bacteria Chromosome Mini-Prep
Bacteriophage
Chromatin Immunoprecipitation with Leaves from Arabidopsis"/>
Attach a file:	Title: <input type="text"/> File: <input type="button" value="Choose File"/> no file selected Description: <input type="text"/> <small>All files must have a file name extension. Images must end in .jpg, .png, or .gif. Additional files can be attached by later editing this record.</small>

Entering References Relating to your Gene

References

Create reference record

Fields marked with a red asterisk (*) are **REQUIRED**

Author(s):*	<input type="text"/>
Title:*	<input type="text"/>
Journal:*	<input type="text"/>
Year*	<input type="text"/>
PDF File	<input type="button" value="Choose File"/> no file selected
