A Gene Discovery Lab Manual For Undergraduates:

Searching For Genes Required
To Make A Seed

Honors Collegium 70AL Laboratory
Spring 2009

By
Ahnthu Q. Bui
Brandon Le
Bob Goldberg

Department of Molecular, Cell, &
Developmental Biology
UCLA

Sponsored by NSF

HC70AL Protocols
Spring 2009
Professor Bob Goldberg

Table of Contents

Experiments

Appendixes:

EXPERIMENT 1 – INTRODUCTION TO GENERAL MOLECULAR BIOLOGY TECHNIQUES

STRATEGY

I. **MICROPIPETTING EXERCISE**
II. **SERIAL DILUTION EXPERIMENT**
III. **POLYMERASE CHAIN REACTION (PCR)**
IV. **PURIFICATION OF PCR PRODUCTS**
V. **SEQUENCING REACTION WITH BIG DYE V. 3**
VI. **RETRIEVING AND ANALYZING DNA SEQUENCES**

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 1-kb 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.

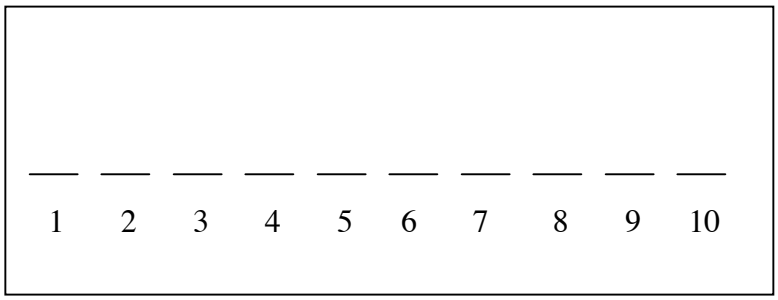
B. Determination of Pipetting Accuracy by Gel Electrophoresis

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:

from	**Dil. #1**	**Dil. #2**	**Dil. #3**
to	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 **cathode** (positively charged) of the gel box. (The **cathode** 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 cathode to anode. (Opposite direction from DNA migration). Remember that DNA is negatively charged; so, it migrates to the positive end of the gel. (DNA migrates from anode to cathode).
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.

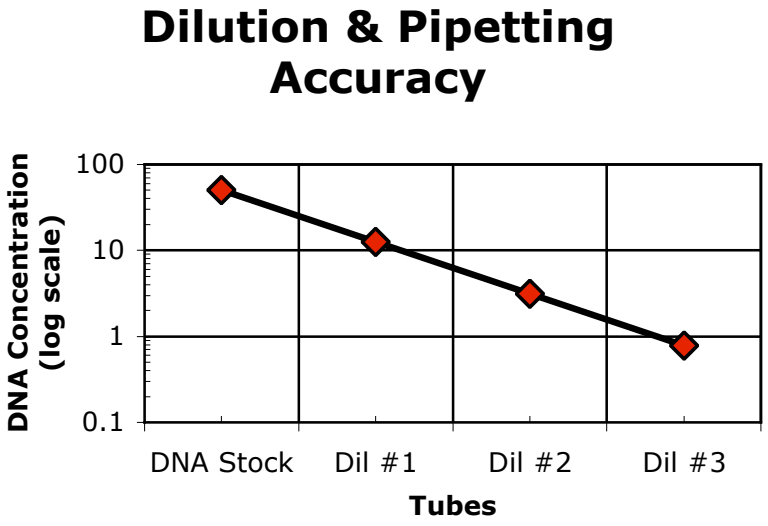
C. Determination of Pipetting Accuracy Using a Spectrophotometer

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/μL**) 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 μM Gene-specific **Forward** primer
- 12 μM Gene-specific **Reverse** primer
- 0.2 ng/μL *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 μL, 1-20 μL and 1-200 μL) for PCR
- Rack for 1.5-mL microcentrifuge tubes
- Rack for 0.2-mL PCR tubes
- Gloves
- Black Ultra-fine sharpie pen
- Ice bucket or plastic container
- Microcentrifuge
- PCR machine (MyCycler, BioRad)
- Agarose
- Diluted 100-bp DNA ladder solution
- Regular pipet tips
- 1X TAE buffer for gel electropheris

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**, **dNTP mix, 12 μM Forward** and **Reverse primer solutions** on a microcentrifuge-tube rack at **room temperature** for about 5 minutes.
3. Flick or vortex the tubes to ensure the solutions are completely thawed out. Spin the tubes for 5 seconds in a microcentrifuge, and put them on ice until needed.
4. Obtain **THREE** 0.2-mL sterile PCR tubes and set them on a PCR-tube rack.
5. Write on the **lids** of the tubes the number **1**, **2**, **3** and on the **side** of the tubes the number **1, 2, 3**, **your Initial** and **Date** as follows: (*your TA will show you how to write on the tubes*)

   Tube #1: **Genomic DNA** isolated from Arabidopsis leaf

   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.
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 a 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 µL	146.4 µL
10x Ex-Taq buffer	5.0 µL	20.0 µL
dNTP mix	4.0 µL	16.0 µL
12 µM Gene-specific **Forward** primer	1.0 µL	4.0 µL
12 µM Gene-specific **Reverse** primer	1.0 µL	4.0 µL
Ex-Taq DNA polymerase (**5 Units/µL**)	0.4 µL	1.6 µL
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
Arabidopsis genomic DNA (Columbia-0 ecotype)	2 µL	0 µL	0 µL
Control template DNA (Positive control)	0 µL	1 µL	0 µL
Sterile water (negative control)	0 µL	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, ∞

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.

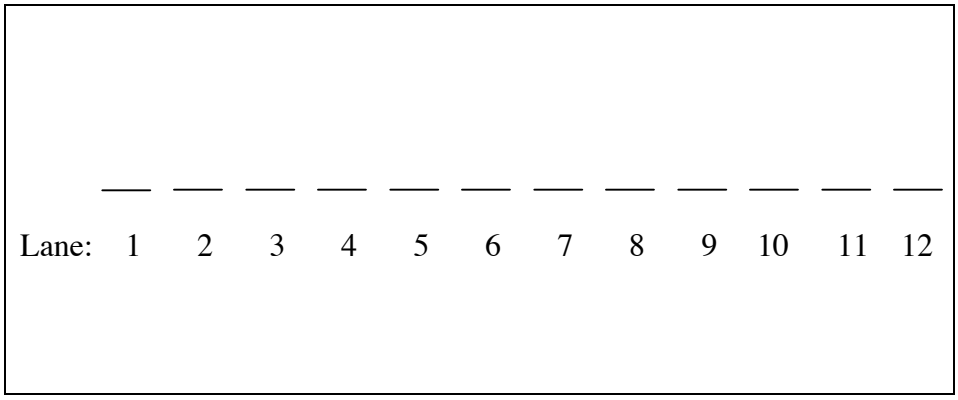
GEL ELECTROPHORESIS ANALYSIS OF PCR PRODUCT

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 μL	2 μL	2 μL
PCR Solution #1	10 μL	0 μL	0 μL
PCR Solution #2	0 μL	10 μL	0 μL
PCR Solution #3	0 μL	0 μL	10 μL

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

4. Load the samples along with diluted 100-bp DNA ladder on the 1% agarose gel.
5. Record the loading pattern of samples.



6. Run the gel at ~**120 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 µL** 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 µL** 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 µL** 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 µL** 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 μM 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-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.
- 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 ng/4.5 ng/µL = 2.2 µL***

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** 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.

Components	**Gene-specific <u>Forward</u> primer**	**Gene-specific <u>Reverse</u> primer**
Mmix	19 μL	19 μL
3 μM **Gene-specific Forward** primer	1 μL	0 μL
3 μM **Gene-specific Reverse** primer	0 μL	1 μL
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, ∞

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

6. Take the purified sequencing reaction to UCLA Sequencing Facility located on the 5th floor in Gonda Building. *<u>Note:</u> Make sure to copy down the **assigned file number** (example, # 5678); that is, automatically given by the Facility, after you enter the samples into the Facility computer.*

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 – SHOTGUN SEQUENCING OF SCARLET RUNNER BEAN (SRB) GENOME USING MASSIVELY PARALLEL PYROSEQUENCING (OR 454 SEQUENCING)

Purpose: To introduce the concepts of assembly and annotating genomic DNA sequences generated by shotgun sequencing using the next-generation sequencing technique.

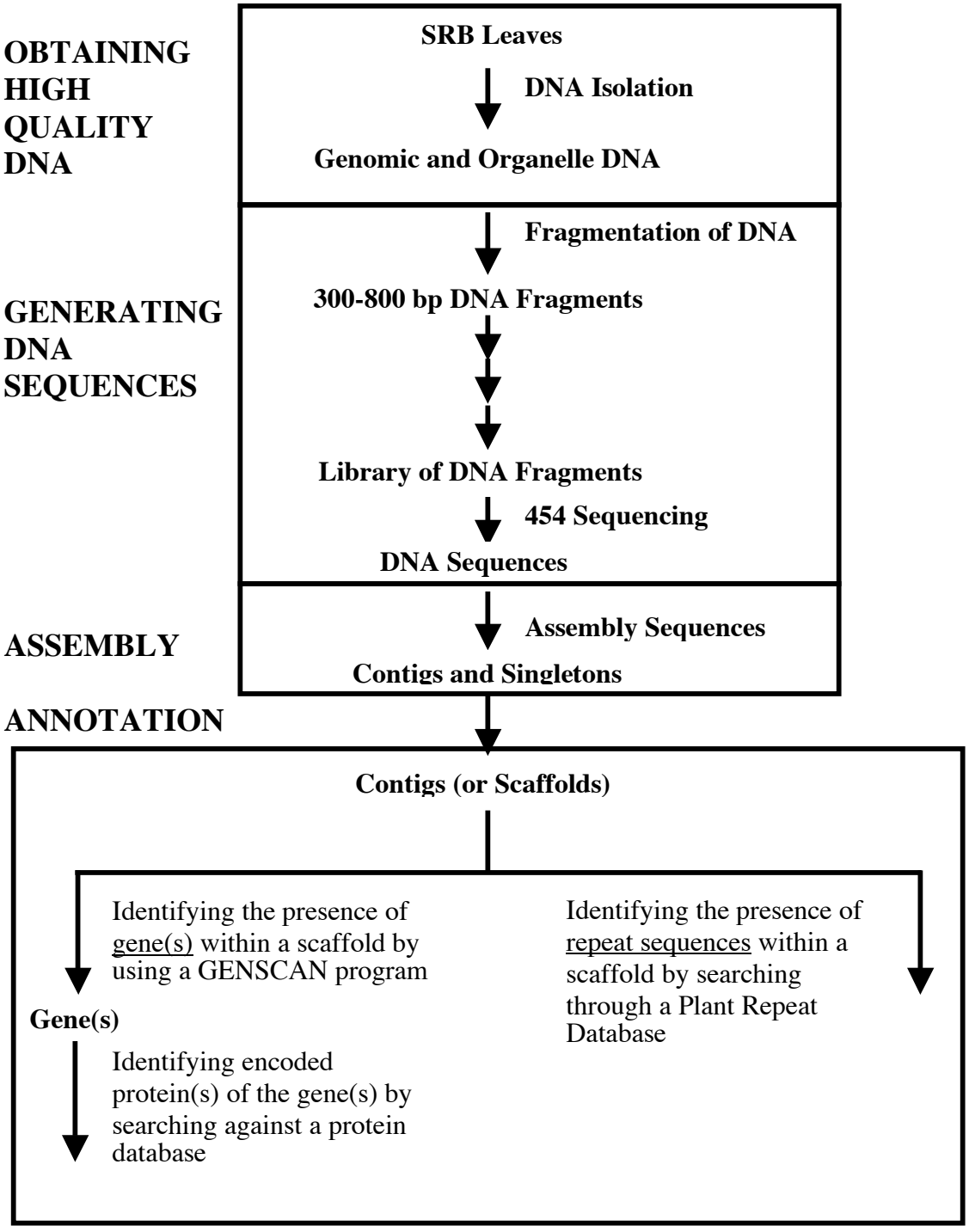
STRATEGY

I. **ISOLATION OF GENOMIC DNA FROM SRB LEAVES USING QIAGEN PLANT DNEASY MINI KIT**

II. **GENERATING DNA SEQUENCES USING 454 SEQUENCING TECHNOLOGY**

III. **ASSEMBLY AND ANNOTATING A CONTIG (OR SCAFFOLD)**

Strategy:


OBTAINING HIGH QUALITY DNA
SRB Leaves
DNA Isolation
Genomic and Organelle DNA
GENERATING DNA SEQUENCES
Fragmentation of DNA
300-800 bp DNA Fragments
Library of DNA Fragments
454 Sequencing
DNA Sequences
ASSEMBLY
Assembly Sequences
Contigs and Singletons
ANNOTATION
Contigs (or Scaffolds)
Identifying the presence of gene(s) within a scaffold by using a GENSCAN program
Gene(s)
Identifying encoded protein(s) of the gene(s) by searching against a protein database
Identifying the presence of repeat sequences within a scaffold by searching through a Plant Repeat Database

ISOLATION OF GENOMIC DNA USING DNEASY PLANT MINI KIT

References:

DNeasy Plant Mini Kit Manual (Qiagen)
454 Sequencing Technology (www.454.com)

Materials and Reagents Needed:

- Liquid nitrogen. *Caution: It is very cold (at least -210°C. Avoid getting frostbite.*
- Styrofoam container for liquid nitrogen
- Aliquots of 14-mL RNase-free tubes containing ~100 mg of frozen powder of SRB leaves stored in a -70°C freezer. *Note: Prepared by Teaching assistant(s).*
- DNeasy Plant Mini kit (Qiagen, Cat. #69104 for 50 extractions) containing AP1 extraction buffer, RNase A, AP2 buffer, AP3/E buffer
- Black ultra-fine sharpies
- 1.5-mL microcentrifuge tubes
- Racks for microcentrifuge tubes
- Pipetman set of P-10, P-20, P-200, P-1000
- Aerosol-barrier PCR tips
- Vortex mixer
- 65°C waterbath
- Gloves (small, medium, large, or extra-large)
- Microcentrifuges
- Kimwipes
- UV Spectrophotometer (Nanodrop)
- Diluted 1-kb DNA Ladder (50 ng/ μL, Invitrogen)
- Agarose
- Microwave
- 1X TAE buffer
- Gel Electrophoresis system
- Power supply
- 10 mg/mL ethidium bromide (EtBr) solution
- Gel Documentation system

PROCEDURE

1. Keep a 14-mL tube containing ~**100 mg** of frozen leaf powder in liquid nitrogen for a few minutes.
2. Set the P-1000 pipetman to **400 µL** (or 4 volumes of the sample weight).
3. Remove the tube from the liquid nitrogen container.
4. Immediately, tap the tube five times against the base of the vortex mixer to loosen the frozen powder. <u>*Caution:*</u> *Do NOT tap the tube too forcefully nor too softly!*
5. Quickly, pipet **400 µL** of buffer **AP1** and dispense it into the tube.
6. Mix the contents by vortexing the tube until the powder completely dissolved in the buffer. *It may take one minute or so to dissolve the powder. The mixture is now call the homogenate.*
7. Transfer the homogenate to a 1.5-mL microcentrifuge tube using the P-1000 pipetman and a pipet tip.
8. Add **4 µL** of **RNase A** (supplied with the kit) to the homogenate. Mix the content by flicking or inverting the tube several times.
9. Incubate the tube in the **65°C waterbath** for **10 minutes**.
10. Spin the tube briefly (5 seconds) to bring the water condensation down.
11. Add **130 µL** (or **1.3 volumes** of the sample weight) of buffer AP2 in the tube. Mix by shaking or inverting the tube. Spin the tube for 5 seconds.
12. Incubate the tube **on ice** for **5 minutes**.
13. Spin the tube at **13,000 rpm** for **5 minutes**.
14. Transfer the lysate into a **QIAshredder** (**light purple**) mini spin column sitting in a 2-mL collection tube. <u>*Note:*</u> *While pipetting the lysate, AVOID touching the pellet at the bottom of the tube.*
15. Spin the QIAshredder column and its collection tube at **13, 000 rpm** for **2 minutes**. *A liquid passed through the column is called a* ***flow-through****.*
16. Meanwhile, write on a lid of a new 1.5-mL microcentrifuge tube your initial and set the tube on a rack.
17. Transfer the flow-through from the collection tube to a newly labeled 1.5-mL tube.

18. Determine a volume of the flow-through using a P-1000 pipetman set at **530 µL**. *Note: Adjust the dial up and down to get the approximate volume.*
19. Record the volume: _____ **µL.** Multiply this volume by **1.5** for step 20 below.
20. Add **1.5 volumes** of buffer **AP3/E** to the flow-through. Mix the contents by pipetting the mixture up and down for 10 times.
21. Transfer ~**650 µL** of the mixture to a **DNeasy** (**clear**) mini spin column in a 2-mL collection tube.
22. Spin the column and its tube at **8,000 rpm** for **1 minute**. Pour off the flow-through solution in a beaker labeled "Waste".
23. Repeat steps 21 & 22 for the remaining volume of the mixture at step 20.
24. Transfer the **DNeasy** mini spin column into a NEW 2-mL collection tube.
25. Pipet **500 µL** of buffer **AW** to the DNeasy mini spin column.
26. Spin at **8,000 rpm** for **1 minute** to wash the membrane in the column.
27. Pour off the flow-through in the beaker labeled "Waste".
28. Wash the column with another **500 µL** of buffer **AW**. Spin at **13,000 rpm** for **2 minutes**. *Inspect the membrane of the column to ensure that the membrane is dry.*
29. Meanwhile, label a new 1.5-mL microcentrifuge tube according to the sample gDNA, your initial, and date.
30. Transfer the DNeasy spin column to the newly labeled tube.
31. Pipet **50 µL** of buffer **AE** or **sterile water** to the center of the membrane.
32. Incubate the column for **5 minutes** at **room temperature**.
33. Spin the tube and column at **8,000 rpm** for **1 minute**. *Caution: Do NOT discard the flow-through solution, which is the genomic DNA solution.*
34. Repeat steps 31-33.
35. Discard the spin column and its collection tube.
36. Close the lid of the microcentrifuge tube containing the isolated genomic DNA solution and put the tube on ice.
37. Determine the total volume of the genomic DNA solution using a P-200 pipetman and an aerosol-barrier PCR P-200 pipet tip. *Note: there is approximately 98* ***µL*** *of the genomic DNA solution. So, you can set the P-200 pipetman to 98.*

38. Determine the concentration of DNA solution using a Nanodrop UV spectrophotometer. Your TA will help you to use the spectrophotometer. Determine the total amount of isolated genomic DNA.
39. Determine the quality of the isolated genomic DNA on a 0.7% agarose/ethidium bromide gel along with the 1-kb DNA ladder. <u>*Note:*</u> *(1) Your TAs will prepare the agarose gel for you.* (2) *Two students will share one agarose gel.* (3) *As little as 60 ng of genomic DNA can be observed on the agarose/EtBr gel.*
    a. Label a new 1.5-mL microcentrifuge tube as "**SRB gDNA**", **your initial, date**.
    b. Pipet 10 μL of the genomic DNA solution into the new labeled tube.
    c. Add 1.5 μL of 6x Loading dye to the DNA solution. Discard the pipet tip. Flick the tube to mix its contents. (Option) Spin the tube for 5 seconds to bring all liquid droplets to the bottom of the tube.
    d. Pipet 10 μL of a ready-to-use 1-kb DNA ladder (50 ng/μL) into a well of the 0.7% agarose gel.
    e. Pipet 11.5 μL of the DNA/loading dye mixture to the next well adjacent to the 1-kb ladder well.
    f. Record the loading pattern of your sample on the agarose gel below:

    g. Pipet 10 μL of 10 mg/mL Ethidium bromide stock solution to the running TAE buffer near the bottom of the gel.
    h. Cover the gel box with its lid.
    i. Connect the electrodes according to their colors to the power supply's plug-ins (example, RED to RED and BLACK to BLACK)
    j. Switch on the power supply and adjust the voltage to ~120 volts.
    k. Run the gel for 1-1.5 hrs.
    l. Record the Starting time: _____ and the Ending time:_____ .
    m. After electrophoresis, turn off the power supply, slide the lid off the gel box, transfer the gel and the gel cast into a plastic container, carry the container to

the gel documentation for picture taken. Your TAs will help you taking the picture of your gel.

n. Place the gel picture in a glassine envelope that is pasted on this page.

o. Analyze the quality of the isolated genomic DNA sample.
   Is the DNA intact or fragmented?
   What is the estimate size of DNA?

40. Store the tube of genomic DNA solution in your assigned white box in the refrigerator.

EXPERIMENT 3 – SCREENING SALK T-DNA MUTAGENESIS LINES (GENE ONE)

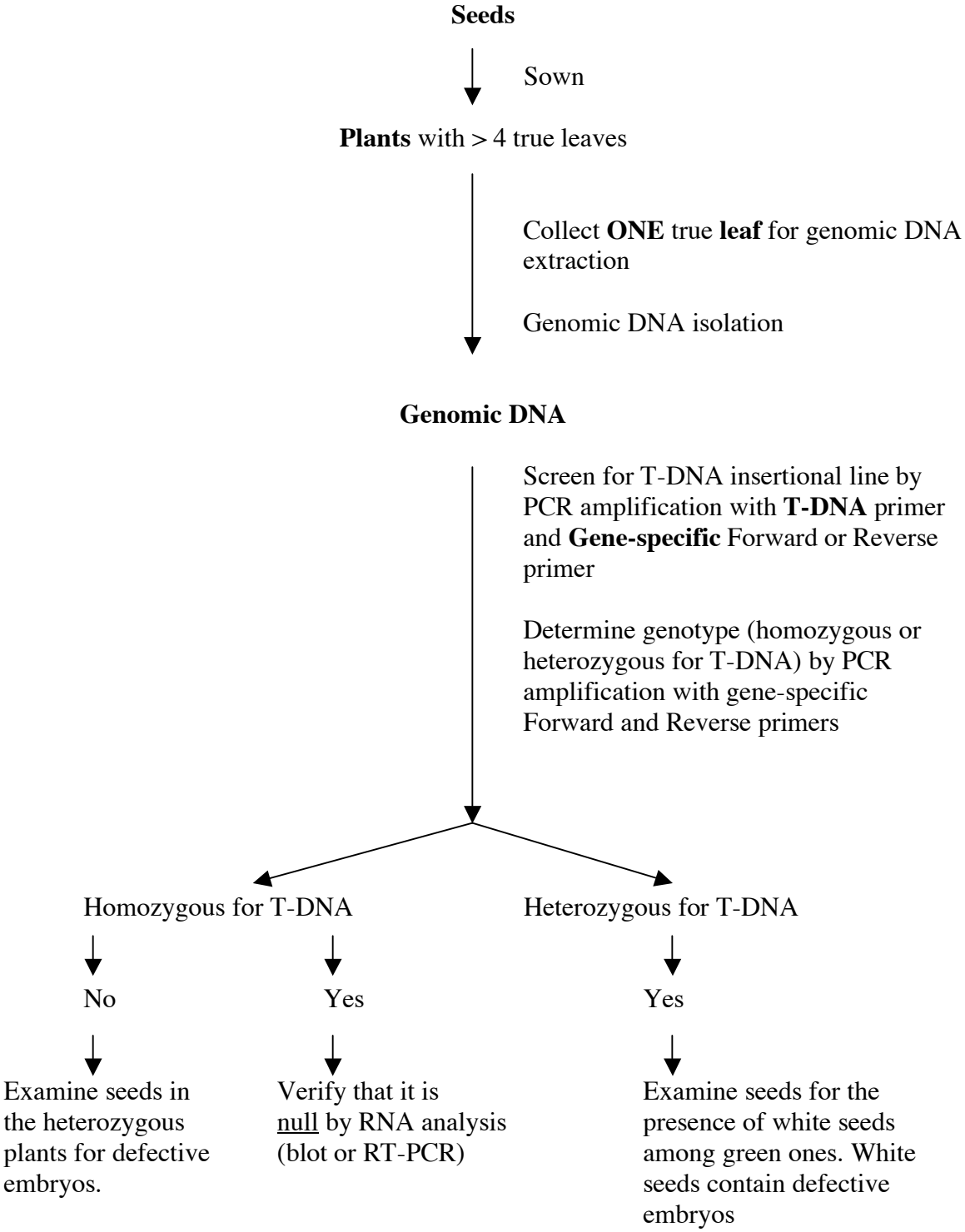
Purpose: To identify a knockout line for the gene of interest and characterize phenotype of mutant plant(s).

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


Seeds
Sown
Plants with > 4 true leaves
Collect ONE true leaf for genomic DNA extraction
Genomic DNA isolation
Genomic DNA
Screen for T-DNA insertional line by PCR amplification with T-DNA primer and Gene-specific Forward or Reverse primer
Determine genotype (homozygous or heterozygous for T-DNA) by PCR amplification with gene-specific Forward and Reverse primers
Homozygous for T-DNA
Heterozygous for T-DNA
No
Yes
Yes
Examine seeds in the heterozygous plants for defective embryos.
Verify that it is null by RNA analysis (blot or RT-PCR)
Examine seeds for the presence of white seeds among green ones. White seeds contain defective embryos

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 plactic 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 wild type Seeds (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. *<u>Note:</u> 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 mount 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 Seeds to slide down into the soil. The tweezers are a useful tool to guide one seeds off of the paper to a precise location in the pot without dumping all of the seeds from the paper.
11. Sow 4 seeds per pot for 11 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 seeds 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).

    <u>CAUTION:</u> *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.

    <u>NOTE:</u> *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.

    <u>NOTE:</u> *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 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, then put "fertilizer-supplemented" water in.

    <u>NOTE:</u> *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.

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^{o}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^{o}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 **12** sterile 1.5-mL microcentrifuge tubes on a microcentrifuge-tube rack.
2. Label number 1-6 on lids of the tubes.

   Tube #**1 - 11**: seedlings/plants #1 - 11 of **Knockout** lines

   Tube #**12**: **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 would 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 5 seconds.
17. Repeat steps 14-17 for **other tubes**.
18. Centrifuge tubes of homogenates at **room temperature** for **5 minutes** at **FULL speed**.
19. Meanwhile, label **a set** of **microcentrifuge tubes** with **Gene Name** and **tube #.**
20. 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.
21. After centrifugation, transfer tubes from the microcentrifuge onto a microcentrifuge-tube rack.
22. Organize tubes such that the **numbers** on the **lids** of **NEW tubes match** with **numbers** on **the lids** of **tubes containing homogenates**.
23. 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.
24. Mix the isopropanol and homogenate by inverting the tube **5-10 times**.
25. Incubate the mixture at **room temperature** for **5 minutes** to precipitate **nucleic acids** *(both genomic DNA and total RNA).*
26. Centrifuge tubes at **room temperature** for **10 minutes** at **FULL** speed.

27. 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.*
28. 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.*
29. Centrifuge the tubes at **room temperature** for **5 minutes**.
30. 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.*
31. Put the tubes on a microcentrifuge-tube rack with their lids opened allowing ethanol to be evaporated.
32. 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 up to 60 minutes**.
33. *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.
34. Spin tubes in a microcentrifuge for **1 minute** to bring down liquid and any contaminants to the bottom of the tubes.
35. Store DNA solutions at **4ºC** (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.

36. 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 **120 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?

37. 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	
Plant #7	
Plant #8	
Plant #9	
Plant #10	
Plant #11	
Plant WT	

Question: Why do you use the Fluorometer instead of the Nanodrop spectrophometer 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 detemined 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 Excitation 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.

38. Dilute **5 µL** of **original DNA solutions** to a **final concentration** of **1 ng/µL** with **TE** buffer. Label on the lids and sides of microcentrifuge tubes with the following information: **1 ng/µL**, **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,

$\mathbf{V_i \cdot C_i = V_f \cdot C_f}$
where,

$\mathbf{V_i}$ = **inital volume** (the volume of original DNA solution is **5 µL**)

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

$\mathbf{V_f}$ = **final volume** (depends on the initial concentration)

$\mathbf{C_f}$ = **final concentration** (**1 ng/µL**)
then,

$\mathbf{V_f = (V_i \cdot C_i)/ C_f}$ = (**5 µL** x **8 ng/µL**)/(**1 ng/µL**) = **40 µL** of **total volume**
What is the volume of TE to be used in dilution?

$\mathbf{V_{TE} = V_f - V_i}$ = **40 µL - 5 µL = 35 µL** 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			
Plant #7			
Plant #8			
Plant #9			
Plant #10			
Plant #11			
Plant WT			

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 μM Gene-specific Forward primer
- 12 μM Gene-specific Reverse primer
- 12 μM **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 12** plants to be characterized and **2 controls** (genomic DNA isolated by TA + No DNA template), prepare a **master mix** for **14 + 1 extra = 15 reactions**.*

1. Label on the lids and sides **14 PCR tubes** and put them on a PCR rack sitting on ice.
2. Prepare a **master mix** for **15 PCR reactions** in a 1.5 mL microcentrifuge tube labeled as "**Mmix**" sitting on ice.

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

	Mmix for ONE reaction	**Mmix for 15** reactions
Sterile water	16.8 µL	252.0µL
10x Ex-Taq buffer	2.5 µL	37.5 µL
dNTP mix	2.0 µL	30.0 µL
12 µM **Gene-specific Forward** primer	0.5 µL	7.5 µL
12 µM **Gene-specific Reverse** primer	0.5 µL	7.5 µL
12 µM **LBb1** primer (for SALK lines)	0.5 µL	7.5 µL
Ex-Taq DNA polymerase (5 U/µL)	0.2 µL	3.0 µL
Total Volume	**23.0 µL**	**345.0 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 µL** of the **Mmix** into each of **13 PCR tubes**.
5. Pipet **2 µL** of **1 ng/µL** genomic DNA extracted from each of **12** 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 µL** of **1 ng/µL genomic DNA** extracted (by TAs) from **wild type** (Col-0) seedlings into each of tubes **#13**. Pipet up and down for five times to mix the contents.
7. Pipet **2 µL** 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 96ºC for 3 minutes
    36 cycles of 94 ºC, 15 seconds -> 60ºC, 30 seconds -> 72ºC, 2 minutes
    1 cycle of 72ºC, 4 minutes
    4ºC, ∞
11. Prepare a **1.5% 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 µL** of **loading dye** to each tube.
14. Pipet **10 µL** of **PCR solutions** to each tube.
15. Load samples on the **1% agarose gel** along with **10 µL** 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 **120 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* ***11 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 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
- 50ºC water bath
- 1.5-mL microcentrifuge tubes
- Microcentrifuge
- Scale
- Metal waste container for sharp objects

PROCEDURE

1. Prepare a **1.5% agarose** gel with a **20-tooth comb**.
2. Add **4 µL** of **6X loading dye** to each tube of ~**25-µL** 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 **120 volts** for 1 - 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 **10 seconds**.
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 µL**.*
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 µL**. Therefore, add **450 µL** of **buffer QG** to the tube.*
18. Incubate tube at **50 ºC** 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 µL** of the mixture into the column. If the total volume is **more than 800 µL**, 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** into a beaker labled as "Waste". 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 µL** 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** of **µL 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** to 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 µL** 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/µL**

    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 μL 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 μL** 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 μL** 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 μL** 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 µL** 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**

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 μM LBb1 primer (for T-DNA)
- 3 μM Gene-specific Forward primer
- 3 μM 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-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.
- 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 ng/4.5 ng/µL = 2.2 µL***

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** 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	**LBb1 primer**	**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**	0 µL	0 µL
3 µM Gene-specific Forward primer	0 µL	**1 µL**	0 µL
3 µM Gene-specific Reverse primer	0 µL	0 µL	**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, ∞

    <u>or</u> **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. <u>*Note:*</u> *Make sure to copy down the* ***assigned file number*** *(example, # 5678); that is, automatically given by the Facility, after you enter the samples into the Facility computer.*

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 4 – GENE EXPRESSION STUDY IN *ARABIDOPSIS THALIANA* (GENE ONE)

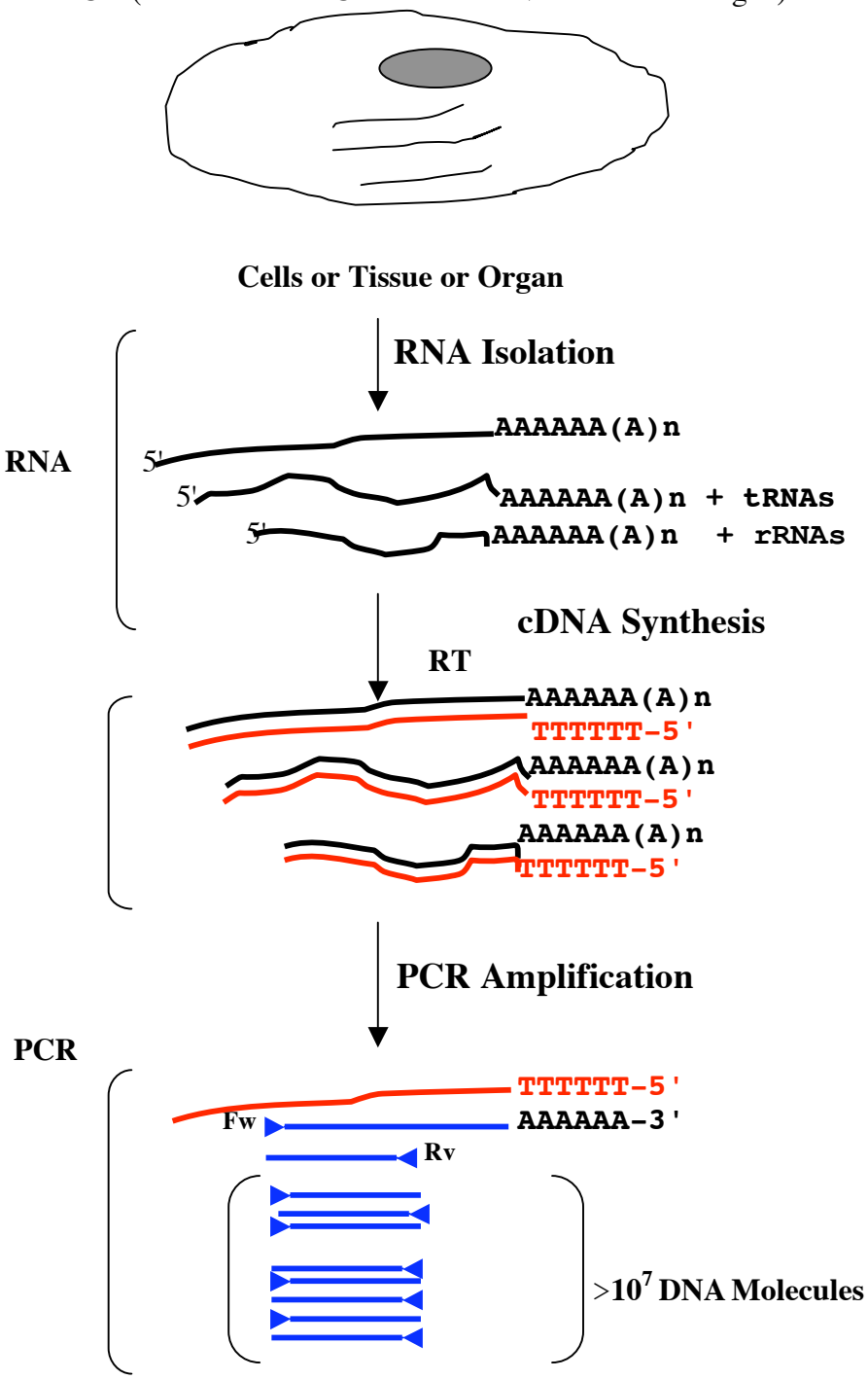
Purpose: To determine mRNA accumulation patterns of genes encoding transcription factors in *Arabidopsis* leaves and siliques.

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 USING CAPILLARY GEL ELECTROPHORESIS

Materials and Reagents Needed:

- Frozen powder of leaves or siliques from *Arabidopsis* Columbia 0 ecotype (prepared by Teaching Assistants). *Note: Leaves are collected from plants with 8 true leaves. Siliques contain SEEDs with embryos ranging from globular to torpedo stages.*
- Liquid Nitrogen. *Caution: It is very cold (at least -210˚C). Avoid getting frost bite.*
- Porcelain mortars and pestles for grinding leaves or siliques to fine powder
- Aliquots of 14-mL RNase-free tubes containing ~100 mg of frozen powder of leaf/silique stored in a -70ºC freezer
- Qiagen RNeasy Plant Mini Kit: (Cat. #74904 for 50 extractions) containing extraction buffer, RPE 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.*
- **500 mL** of freshly prepared 0.05% **DEPC**-treated water (**non-autoclaved**) for cleaning up pipetman, microcentrifuge rotor and chamber, racks for microcentrifuge tubes, vortex mixer.
- β-mercaptoethanol. *Caution: work in the fume hood because this chemical has very bad odor.*
- Ambion DNase I kit (stored at -20ºC)
- **Autoclaved** DEPC-treated (**DEPC'd**) water
- 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
- Pipetman set of P-10, P-20, P-200, P-1000
- RNase-free filtered PCR tips for P-20, P-200, P-1000
- White Revco storage boxes
- Gloves (small, medium, large, or extra-large)
- Microcentrifuges
- Kimwipes
- Aluminum foil
- UV Spectrophotometer (Nanodrop)
- Capillary Gel Electrophoresis system Experion (Bio-Rad)
- Experion StdSens (Nanogram) RNA kit (Bio-Rad)
- RNA Ladder for Experion (Bio-Rad)
- Vortex mixers
- Ice bucket or styrofoam containers
- 85 ºC Heat block
- 65 ºC Heat block
- 42 ºC Heat block
- 37 ºC 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., Siliques).
- Buffer **RLC** (Guanidine Hydrochloride) is used for Siliques 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, test-tube racks, pens and sharpies) to be used in isolating RNA.*

A. RNA ISOLATION

Note: Steps 1-6 are done a few days ahead of the classs time by Teaching Assistants (TAs).

1. Label on the WHITE area on the side of TWO RNase-free **14-mL disposable centrifuge tubes "Leaf" or Silique"**. 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 box).
2. Chill **RNase-free** spatulas in a Dewar flask containing liquid nitrogen.
3. Remove bottles/tubes containing **frozen ground organs** from a **–70 °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, Siliques) to ensure that the frozen powder is not partially thawed out.
5. Use a **chilled spatula** to transfer a 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.
6. Store tubes of aliquots in the **–70 °C freezer** until the RNA extraction step.
7. Determine the **total volume** (**= # of organs x 500 µL**) of **lysis buffer** needed for RNA isolation. *Note: if the total volume is greater than 1.5 mL, it is best to use a **14-mL centrifuge tube** for preparing the **lysis buffer** with **β-mercaptoethanol**. (TAs will prepare lysis buffer and β-mercaptoethanol).*
8. Add **10 µL** of **β-mercaptoethanol** to every **1 mL** of **lysis buffer** in a fume hood. Mix the contents in the tube 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. The newly prepared lysis buffer with β-mercaptoethanol is stable for a few hours at room temperature.*

Volume of lysis buffer (**mL**)	Volume of β-mercaptoethanol (**μL**)
_____ RLT	_____
_____ RLC	_____

9. Remove the **chilled tube** containing **~100 mg** of **ground organ powder** from the styrofoam container and set on the rack at room temperature. Quickly, **tap** the **tube** on the **bench** or a base of the vortex mixer for 3-5 times to loosen frozen powder.
10. **Immediately**, pipet **450 μL** of **lysis buffer containing β-mercaptoethanol** into the 14-mL tube containing **~100 mg** of starting organ. **Cap** the **tube**. **Immediately**, **vortex** the tube **vigorously** for at least **1 minute**. 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 rich in starch, such as siliques or old leaves,.*
11. **Repeat steps 9-10** for all organs.
12. Label on the lid of each of two **QIAshredder** (**purple**) **spin columns** already in **2-mL collection tubes "your initial"** and **"Leaf"** or **"Silique"**.
13. Pipet the **entire volume** of **lysate** from the 14-mL tube into the appropriate labeled QIAshredder spin column. *Example: Leaf → Leaf; Silique → Silique.*
14. Centrifuge the spin columns in the collection tubes at **FULL speed** (13,200 rpm) for **2 minutes**. *Attention: Do **NOT discard** the **flow through** (or **supernatant)** because it contains total RNA and genomic DNA.*
15. Meanwhile, label on the lids of TWO **1.5-mL RNase-free microcentrifuge tubes** and TWO **RNeasy** (**pink**) **mini columns** already placed in the **2-mL collection tubes** "**Leaf**" or "**Silique**" and **your initial**. Set the labeled tubes on a microcentrifuge-tube rack at room temperature.
16. Transfer ~ **450 μL** 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.

17. Add **0.5 volume** (or **225 µL**) of **room temperature 96-100% ethanol** to the **flow-through solution**. **Immediately**, mix the mixture by pipetting **up and down** for **10 times**.
18. Pipet the **entire volume** (**~675 µL)** of the **"Leaf" mixture** (including any precipitate) in step 17 to the "**Leaf**" labeled **RNeasy** (**pink**) **mini column** already placed in a **2-mL collection tube**. Close the lid of the tube **gently**.
19. Repeat steps 16-18 for the "**Silique**" mixture.
20. Centrifuge the spin columns already placed in a **2-mL collection tubes** for **15 seconds** at **10,000 rpm** (or FULL speed).
21. Carefully, 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** into a **"waste" beaker**. Put the column back on the collection tube.
    Note: if the sample volume is >700 µL, pipet the remaining volume of the mixture onto the RNeasy column and centrifuge as before.
22. Pipet **700 µL** of **buffer RW1** to the RNeasy column. Close the lid of the tube.
23. Centrifuge for **15 seconds** at **10,000 rpm** to wash the column.
24. Transfer the column(s) to **NEW 2-mL collection tube**(s). *Attention: at this point, total RNA and small amount of genomic DNA are bound to the silica membrane of the pink RNeasy spin column.*
25. Discard the flow-through solution and collection tubes.
26. Pipet **500 µL** of **buffer RPE** onto each RNeasy column.
27. Centrifuge for **15 seconds** at **10,000 rpm** to wash the column.
28. Discard the flow-through solution as done earlier in the step 20.
29. Pipet another **500 µL** of **buffer RPE** to the RNeasy column.
30. Centrifuge for **1 minute** at **10,000 rpm** to wash the RNeasy silica-gel membrane again.
31. Discard the flow-through solution as done earlier in the step 20.
32. 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 34-37. If this is the case, RNA solution will float up when it is loaded on an agarose gel.*

33. While spinning at step 30, label on the **lid** and **side** of 1.5-mL RNase-free microcentrifuge tubes "**Leaf** or **Silique RNA**", "**your initial**", and "**date**".
34. Transfer the **spin columns** to these **NEW labeled tubes**.
35. Pipet **30 µL** 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.
36. Wait for **1 minute** to allow water to evenly absorb in the membrane.
37. Centrifuge for **1 minute** at **10,000 rpm** to elute RNA off the membrane.
38. Repeat **steps 35-37** with **20 µL** of **RNase-free water**. *Note: the total volume of RNA solution is about **50 µL.***
39. Mix the content in the tubes with gentle flicking. Put tubes **on ice**. *Note: From this step on, **KEEP** RNA solution **ON ICE** to prevent **RNA degradation**.*
40. Determine the **total volume** of **RNA solution** using a P-200 pipetman. The volume should be **~48 µL**.
41. Determine **RNA concentration** and **total amount** using a UV **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** UV 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:

[RNA] = (OD_{A260} reading) (Dilution factor) (40 µg/mL.OD) = **X µg/mL** or

= **X µg/µL**

Total amount of RNA = (X µg/µL) (Volume of RNA solution in µL) = **Y µg**

Records of organs and their RNA concentration and total amount

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

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^{o}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 2 μg of DNA*** *from RNA in a* ***25-100 μL*** *reaction volume.*

PROCEDURE

1. Label new RNase-free tubes with "RNA sample + DNase" and initials.
2. 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 **37ºC** (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			

3. Mix the solutions gently by flicking the tubes. Spin briefly (**5-10 seconds**).
4. Incubate at **37 ºC** in a **heat block** for **20-30 minutes**.
5. After incubation, spin tubes for **10 seconds** in a microcentrifuge to bring down water condensation to the bottom of the tubes.
6. To inactivate DNase I, pipet **0.1 volume** (or **3.0 μL**) of the **DNase inactivation reagent** (**WHITE slurry)** to the sample using a P-20 pipet tip. Mix well by **flicking the tube**.

   Note: Make sure the slurry is WHITE. If the DNase inactivation reagent is CLEAR, vortex the mixture for a few seconds.
7. Incubate the tube at **room temperature** for **2 minutes**. Flick the tube **once more during the incubation** to re-disperse the **DNase inactivation reagent**.

8. At the meantime, label on the **lids** and **sides** of **NEW RNase-free microcentrifuge tubes** "**Purified Leaf RNA**" or "**Purified Silique RNA**", "**your initial**", and "**date**".
9. Spin the tube at ~**10,000 rpm** for **1 minute** to pellet the **DNase inactivation reagent**.
10. **Carefully**, Pipet ~**28-30 µL** of the **RNA solution** (*AVOID pipetting the PELLET!*) and transfer it into NEW labeled tubes. *Note: It is **okay** if a **tiny amount** of the pellet **is carried over** in the RNA solution.*
11. Keep RNA tubes **on ice**.
12. Determine RNA concentration using a Nanodrop or Beckman UV spectrophotometer

    Calculations (if Using Beckman spectrophotometer):

    [RNA] = (OD_{A260} reading) (Dilution factor) (40 µg/mL.OD) = X µg/mL

    Total amount = (X µg/mL) (volume of RNA solution) = Y µg

    *Note: **1 µg = 1,000 ng**; therefore, you need to **convert ng/µL** to **µg/µL***

Samples	**[RNA] (µg/µL)**	**Volume (µL)**	**Estimate Total Amount (µg)**
Purified Leaf RNA			
Purified Silique RNA			

13. Store the RNA solution at **-20°C for up to 1 week** or **-70°C for up to 6 months**.

(Option) 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 <u>BEFORE</u> AND <u>AFTER</u> DNase I-TREATMENT USING A CAPILLARY GEL ELECTROPHORESIS SYSTEM (EXPERION, BIO-RAD)

<u>Note:</u> A single StdSens RNA chip can hold 12 RNA samples.

1. Equilibrate Experion RNA StdSens reagents (filtered RNA gel solution, RNA loading buffer (yellow cap), a previously prepared "gel-stain" solution in an amber tube) stored in a refrigerator to room temperature for at least 15 minutes.
2. Make sure to have a heat block at 65-70ºC.
3. At the meantime, clean the **Electrodes** in the **platform** of the **electrophoresis station** with **DEPC'd water**
   a. Fill a **cleaning chip** (**clear**) with 800 µL of DEPC'd water
   b. Open the **lid** of the **electrophoresis station** and place the chip on the **platform**.
   c. Close the **lid** and **leave** the **chip in the instrument** for **1 minute**.
   d. Open the **lid** and **remove** the **cleaning chip**. Discard the water in the chip, but save the chip for the future usage.
   e. Leave the lid open and wait for 30-60 seconds for any water remaining on the electrodes to evaporate.
   f. Close the lid of the electrophoresis system to prevent dust gets on the electrodes.
4. Get ice from an ice maker.
5. Label FOUR 1.5-mL RNase-free microcentrifuge tubes according to the RNA samples (Leaf RNA before DNase, Purified Leaf RNA, Silique RNA before DNase, Purified Silique RNA). Set tubes on ice.
6. Take out tubes of purified RNA samples from a -70ºC freezer and spin them for 10 seconds in a microcentrifuge. Thaw RNA solution on ice.
7. Transfer 1.0 µL of each RNA solution from the original RNA samples tube to the appropriate newly labeled tubes. Keep tubes on ice.
8. Immediately, put the tubes of original RNA samples back to their box in the -70ºC freezer.

9. Remove a tube of **1-µL RNA ladder aliquot** (**160 ng/µL**) from the -70ºC freezer. Spin the tube in a microcentrifuge for **10 seconds** to bring down any water condensation, and keep it on ice.
10. Heat **FOUR tubes** of RNA solutions and the **tube** of **RNA ladder solution** on a **65-70ºC heat block** for **2-3 minutes**. *Note: it is okay to heat the samples for up to 5 minutes*.
11. Quench tubes **on ice** for **at least two minutes**.
12. Spin tubes in a microcentrifuge for 30 seconds. Keep tubes on ice.
13. Pipet **5 µL** of **loading buffer (yellow cap)** to each RNA solution. Mix the contents by flicking the tube several times. Keep the tube on a microcentrifuge-tube rack at room temperature. After adding loading buffer to all RNA solutions, spin tubes for 10 seconds.
14. Remove an RNA StdSens chip from its plastic wrap, pipet **9 µL of gel-stain solution** into the well labeled **GS** with an **orange highlight**.
15. Put the chip on the priming station. Make sure the setting as **B1**.
16. Press the **START** button on the priming station. Wait for ~30 seconds.
17. Open the priming station.
18. Pipet another **9 µL** of **gel-stain solution** to the other well labeled **GS**.
19. Pipet **9 µL** of **filtered gel solution** into the well labeled **G**.
20. Pipet **6 µL** of RNA mixtures prepared in step 13 into each well sample (1-4) and into the ladder well (labeled L). If there are only 4 samples, then pipet **6 µL** of loading buffer into the remaining wells (5-12). *Caution: Do NOT leave any sample well empty*.
21. Tap the **chip** on the **bench 3-5 times** to remove any **bubbles** present in the sample wells. Inspect the wells for the presence of bubbles. *Note: run the chip within 5 minutes of loading samples.*
22. Place the sample-loaeded chip on the platform of the electrophoresis station and close the lid.
23. Launch the **Experion software**, select **New Run** and then **RNA StdSens**.
24. Select **Eukaryotic total RNA assay**.

25. Click the Start button in the software to begin the run. The run would take up to 30 minutes for all 12 samples.
26. While the electrophoresis is running, enter the samples information in the "**data info**".
27. After the run is complete, remove the chip from the platform and discard the used chip.
28. Immediately, place a **cleaning chip** containing **800 μL of DEPC'd water** on the platform. Close the lid of the electrophoresis system for **1 minute** to clean the electrodes.
29. Open the lid for **30 seconds** to allow water to evaporate.
30. Remove the **cleaning chip**. **Dispose** the **water** and **store** the **cleaning chip** for the future usage.
31. If there is no further run, turn off the electrophoresis system and quit the Experion software.
32. Export data (electropherograms and gel-like images) to the desktop.
33. Copy the **exported data** on an **USB 2.0 flash drive** and **upload** them on the **HC70AL server**.

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 **-20ºC RNA** Freezer, room 2918)
- Nuclease-free water (came with the iScript RT, Bio-Rad, stored in a **-20ºC RNA Freezer**, room 2918)
- iScript Reverse transcriptase (iScript RT, Bio-Rad, stored in a **-20ºC RNA Freezer**, room 2918)
- Total RNA samples (stored in a **-20ºC RNA Freezer**, room 2918)
- DEPC'd water
- 42ºC and 85ºC dry baths (or heating blocks)
- 1.5 mL RNase-free 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 **μg/μL**.*

	Leaves	**Siliques**
RNA concentration	_____ μg/μL	_____ μg/μL

.

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 μg/μL, then the volume of **1 μg RNA** will be (1 μg) / (0.5 μg/μL) = **2 μL***

	Leaves	**Siliques**
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	**0.0** μL
- RT Mix	**0.0** 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**, spin tubes in a microcentrifuge for **10 seconds**, and 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 + Siliques + 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	0.0 µL	0.0 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	0.0 µL	0.0 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 **25ºC** (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 **42ºC** for **30 minutes** on a **dry bath** (or heating block). *This step is to synthesize first strand cDNAs.*
12. **After 30 minutes at 42ºC**, inactivate **reverse transcriptase**, *which is known to interfere with Taq DNA polymerase in the PCR amplification step*, by heating the mixture at **85ºC** 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 **-20ºC 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 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
- 100-bp DNA ladder (Invitrogen)
- 6X Loading buffer containing ONLY xylene cyanol
- 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 sush 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 SIX 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 ONE 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	34.6 μL	242.2 μL
Ex-Taq DNA Polymerase (5 U/μL)	0.4 μL	2.8 μL
Total Volume	**48.0** μL	**336.0 μL**

* ***Control primers*** *are used to ensure that the absence of Gene-specific PCR product in* ***the +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**	0 µL	0 µL	0 µL	0 µL	0 µL
Leaf -RT	0 µL	**2 µL**	0 µL	0 µL	0 µL	0 µL
Silique +RT	0 µL	0 µL	**2 µL**	0 µL	0 µL	0 µL
Silique -RT	0 µL	0 µL	0 µL	**2 µL**	0 µL	0 µL
0.2 ng/µL Genomic DNA	0 µL	0 µL	0 µL	0 µL	**2 µL**	0 µL
Water	0 µL	0 µL	0 µL	0 µL	0 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 ºC, 3 min. → 40 cycles of 94 ºC, 10 sec./60 ºC, 30 sec./72 ºC, 45 sec. → 1 cycle of 72 ºC, 4 min. → 4 ºC, ∞.
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 µL** of **PCR solution** and **3 µL** of **6X loading dye**. *Note: 20 µL 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 µL** of **50 ng/µL 100-bp ladder** solution. Record RNA loading pattern.

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

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

BIO-RAD

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 µL
iScript Reverse Transcriptase	1 µL
Nuclease-free water	x µL
RNA template (100fg to 1µg Total RNA)*	x µL
Total Volume	20 µL

Reaction Protocol

Incubate complete reaction mix:
- 5 minutes at 25ºC
- 30 minutes at 42ºC
- 5 minutes at 85ºC
- Hold at 4ºC (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 2µL.
*When using larger amounts of input RNA (>1µg) the reaction should be scaled up e.g. 40 µL reaction for 2µg, 100 µL reaction for 5 µg 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 5 – CLONING THE UPSTREAM REGION OF THE GENE OF INTEREST

Purpose:

To determine the activity of the promoter of the gene of interest at the cellular and tissue levels in *Arabidopsis* plants via the expression of the β-glucuronidase (GUS) or Green Fluorescent Protein (GFP) gene fused to the promoter.

Reference:

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

STRATEGY

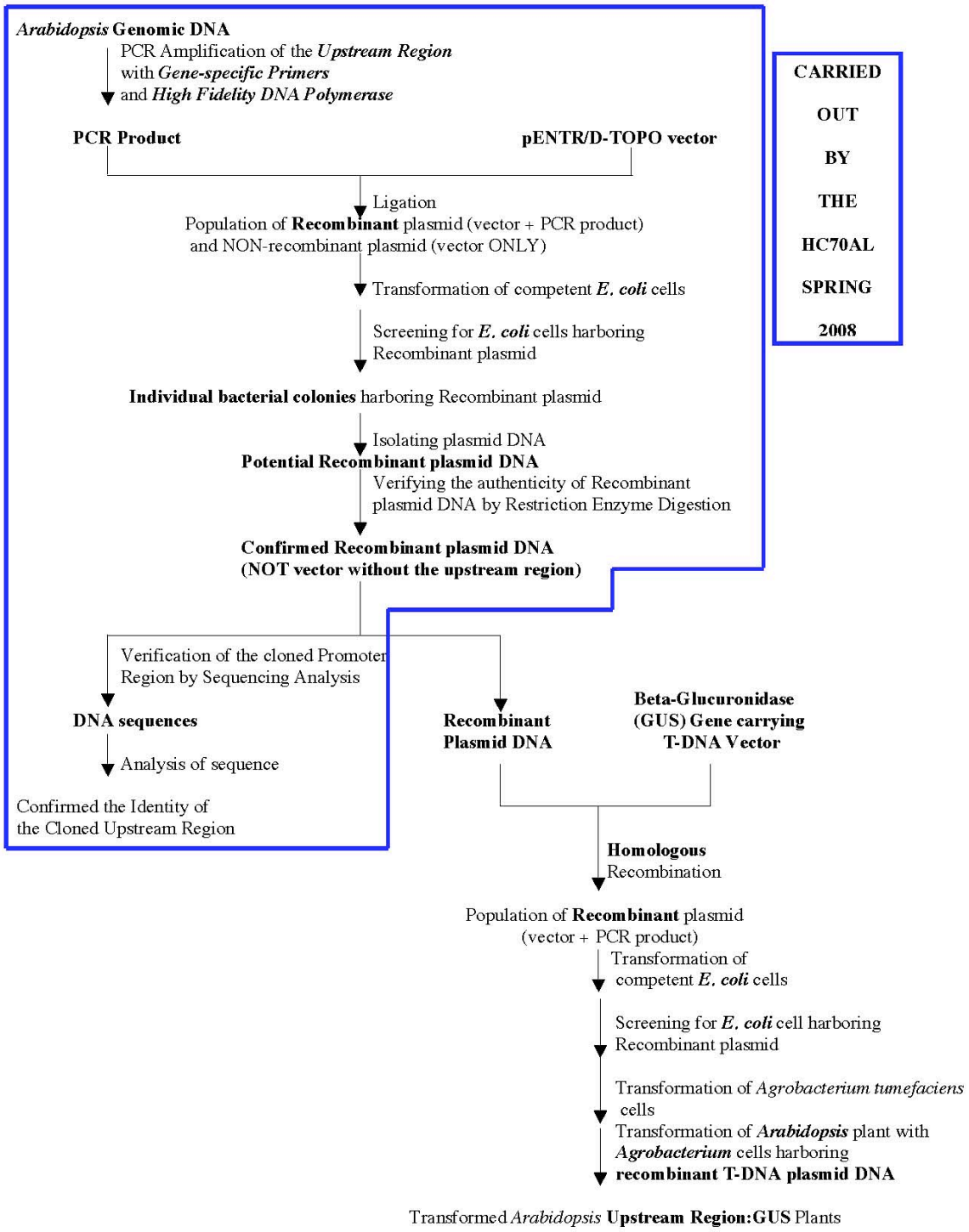
I. AMPLIFICATION OF THE UPSTREAM REGION OF THE GENE OF INTEREST USING HIGH FIDELITY (HIFI) DNA POLYMERASE

II. CLONING THE AMPLIFIED PROMOTER REGION INTO A PLASMID VECTOR

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

III. STRATEGY OF PROMOTER ACTIVITY ANALYSIS

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



Materials Needed:

High quality *Arabidopsis* genomic DNA (12 ng/μL)
PCR product of the promoter region tested with iProof DNA Polymerase
Filter tips for PCR
iProof High Fidelity DNA polymerase kit (Cat.# 172-5301, Bio-Rad)
Sterile water
dNTP mix (2.5 mM each dNTP, from Ex-Taq DNA polymerase package, Takara)
12 μM Promoter Gene-specific Forward primer
12 μM Promoter Gene-specific Reverse primer
Pipetman sets of P-10, P-20, P-200
PCR tubes or strip of tubes
1.5-mL Microcentrifuge tubes
Microcentrifuge-tube rack
Ice bucket
pENTR/D-TOPO Cloning kit (Cat.# K2400-20, Invitrogen)
One Shot TOP10 Competent cells (Included in the TOPO Cloning kit)
pUC19 plasmid (10 pg/μL) (Included in the TOPO Cloning kit)
S.O.C. medium
Sterile toothpicks
Terrific Broth (TB) medium
50 mg/mL Kanamycin
LB + Kanamycin plates
LB + Ampicillin plates for pUC19 plasmid (control for transformation efficiency)
37^{o}C Air incubator
Orbital shaker in the 37^{o}C air incubator
42^{o}C Water bath
Bacterial cell spreader
A glass jar containing 95% ethanol solution
Regular Pipet tips for P-10, P-20, P-200, P-1000
QIAgen Plasmid Miniprep kit
Nanodrop spectrophotometer
TE buffer

PROCEDURE

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

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

1. Get ice from an icemaker in room 2911 or 3906.
2. Thaw out tubes of **5x iProof HF Buffer with 7.5 mM $MgCl_2$** and **dNTP mix (2.5 mM each dNTP)** on a microcentrifuge rack for 1.5-mL microcentrifuge tubes at **room temperature** for few minutes. Once the solutions are thawed out → **vortex** for **5 seconds** to mix the contents → **spin** tubes for **10 seconds** → put the tubes **on ice** until needed.
3. Thaw out **Promoter Forward** and **Reverse primer** solutions corresponding to a gene of interest as in step 2.
4. Obtain **THREE 0.2-mL sterile PCR tubes** and set them on a **PCR rack**.
5. Label **Name** and **Date** on the **lids** and **sides** of the tubes as follows: (your TA will show you how to write on the tubes)

   Tube #1: "**Name of a gene**" (**same name** as **primer's gene**)

   Tube #2: **Pos.** (**Positive** control for the gene of interest = PCR product using Ex-Taq DNA polymerase)

   Tube #3: **Neg.** (**Negative** control for the gene of interest containing same components as in tube #1, but **NO** genomic DNA)
6. Obtain **ONE 1.5-mL microcentrifuge** tube and set them on a microcentrifuge-tube rack.
7. Write on the **lid** of the tube with black ultra-fine sharpie as "**Mmix**" (for Master mix). Keep the tube on ice.

8. Prepare the **Master Mix (Mmix)** solution for **4 reactions** (**3 samples + 1 extra**) (see table below)

   Caution: Keep tube on ice at all the time.

   Note: Amplification of targets greater than 3 kbp may require more DNA polymerase, but NOT to exceed 2 units of enzyme per 50 µL reaction.

   Master Mix

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

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

   b. After pipetting all reagents into the master mix tube, close its lid. Mix the contents by vortexing at **setting of slow speed** for **2 seconds**. *Caution: Do **NOT** vortex the mixture with the enzyme, such as DNA polymerase, **vigorously** as well as for **> 5 seconds** because these two factors will **break down enzyme**, resulting **LOW** or **NO yield** of PCR product.* Spin the tube in a microcentrifuge at full speed (13,200 rpm) for **10 seconds**. Put the tube back **on ice**.

9. Prepare PCR-reaction tubes by
   a. pipetting **42 µL** of the Mmix solution into PCR tubes (see table below)
   b. pipetting **8 µL** of genomic DNA or water to tubes #1-3.
   c. Immediately, mix the contents by pipetting up and down **five times**

PCR reactions:

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

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

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

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

e. Under the "**Choose Operation**" window, "**Run Protocol**" is highlighted. Press the "**ENTER**" button to run the protocol.

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

11. Analyze **10 µL** of the reaction products on a **1% agarose** gel containing **0.5 µg/mL ethidium bromide** and visualize the DNA bands under UV illumination.

II. CLONING THE AMPLIFIED PROMOTER REGION INTO A PLASMID VECTOR

A. LIGATING THE PCR PRODUCT AND A pENTR/D-TOPO VECTOR

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

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

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

c. Incubate the reaction for **5 minutes** at **room temperature** (22-24^{o}C).

Note: For most applications, 5 minutes will yield many colonies for analysis. The length of the TOPO Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. ***For large PCR products (>1 kb), increase the reaction time will yield more colonies*** *(Taken from TOPO Cloning Manual, Invitrogen).*

4. After the ligation reaction is done, place the ligation mixture tube on ice.

Note: If you ***don't have time****, you* ***can store*** *the* ***ligation mixture*** *at* ***–20^{o}C overnight****.*

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

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

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

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

	Vial #1	**Vial #2**	**Vial #3**
pENTR + Gene	**2 µL**	**0 µL**	**0 µL**
pENTR Only	**0 µL**	**2 µL**	**0 µL**
pUC19	**0 µL**	**0 µL**	**1 µL**

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

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

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

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

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

    Plate #2: **50 µL** of transformation mixture

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

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

INOCULATION OF A LIQUID MEDIUM WITH BACTERIAL COLONIES

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

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

ISOLATING PLASMID DNA

1. Label on the lids of **1.5-mL microcentrifuge tubes** with **your Initial** and the **number (1-6)**. Set labeled tubes on the microcentrifuge rack.
2. Arrange the culture tubes and labeled microcentrifuge tubes in their corresponding order. *For example, 1 to 1, 2 to 2, … , 6 to 6.*

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

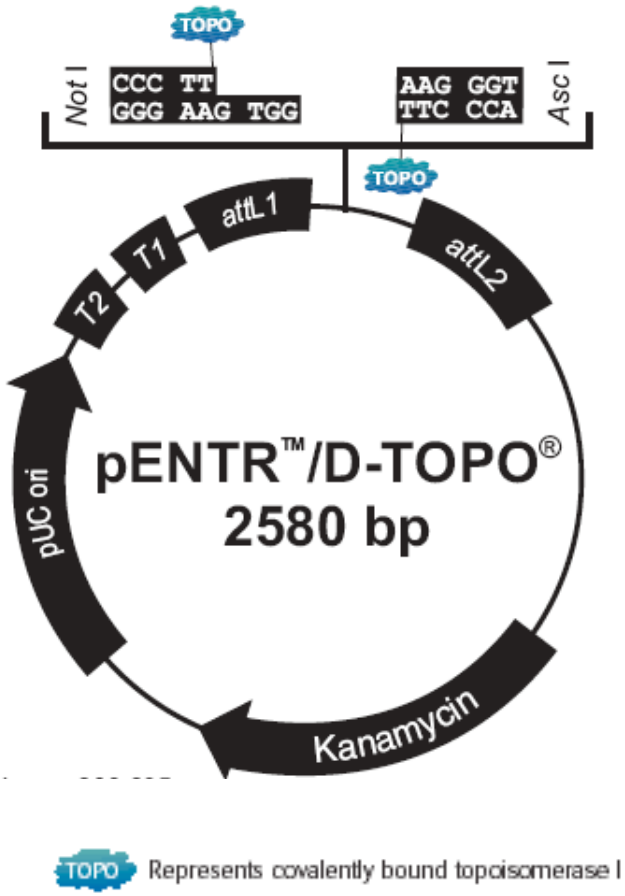
3. **Carefully,** pour the liquid culture into the microcentrifuge tube. Close the lids of the tubes.
4. Spin tubes in a microcentrifuge at **FULL** speed for **2 minutes**.
5. Pour off the supernatant into a glass Erlenmyer flask labeled as "CULTURE WASTE". Dab off the extra liquid on a piece of paper towel.
6. Place the tubes back on the microcentrifuge rack.
7. Pipet **250 µL** of **Buffer P1** (**Resuspension buffer + RNase A**) to each tube. Close the lid **tightly**.
8. Resuspend pelleted bacterial cells by either raking the tube on a microcentrifuge rack for 10 times or vortexing the tube for a few minutes until NO cell lumps are observed.
9. Place the tube back on the microcentrifuge rack.
10. Add **250 µL** of **Buffer P2** (**Lysis buffer**) to each tube. Close the lid.
11. Invert tubes for **10 times** or **until** the mixture is clear. This step is for breaking open bacterial cells to release their contents (chromosomal DNA, plasmid DNA, proteins, carbohydrates) into the solution. *Note: **Do NOT vortex** the contents to prevent shearing bacterial chromosome DNA into many tiny pieces that have the same size as the plasmid DNA.*
12. Add **350 µL** of **Buffer N3** (**Neutralization buffer**) to each tube. Close the lid. Immediately, invert the tube to mix the solution as in **step 11**. The solution appears cloudy. *Note: **Do NOT vortex** the mixture!*
13. Repeat step 12 for other tubes (one by one).
14. Spin tubes in the microcentrifuge at **FULL** speed for **10 minutes**.

15. Meanwhile, label on the **SIDE** of the QIAprep columns (**Light blue**) with your **Initial** and the **number**. Set these columns in their collection tubes on the microcentrifuge rack.
16. Also, label on the lids and side of a new set of tubes with the following information: **pENTR-**"**gene name**"; **Number**, **your initial**, **date**. **(Tubes will be used at step 27).**
17. After 10 minutes of spinning, transfer **800 µL** of the **supernatant** from step 14 to the QIAprep column by pipetting. *Caution: Make sure that the **numbers** on the **lids** of tubes and the **SIDE** of QIAprep columns are corresponding*.
18. Spin the columns in their collection tubes at **FULL** speed for **30 seconds**.
19. Lift the column off the collection tube and discard the flow-through liquid into a glass BEAKER.
20. Put the column back in its collection tube.
21. Pipet **500 µL** of **Buffer PB** to each column. Spin the columns at **FULL** speed in the microcentrifuge for **30 seconds**.
22. Lift the column off the collection tube and discard the flow-through liquid into a glass BEAKER.
23. Pipet **750 µL** of **Buffer PE** to each column. Spin the columns at **FULL** speed in the microcentrifuge for **30 seconds**. This step is to wash off residual salt and proteins from the membrane on the column. *Note: Make sure that ethanol is added to the PE buffer before use*.
24. Lift the column off the collection tube and discard the flow-through liquid into a glass **BEAKER**.
25. Spin the columns at **FULL** speed for **1 minute** to remove residual wash buffer. *Note: if the residual wash buffer is NOT completely removed, DNA solution will float up when the sample is loaded into the well of the agarose gel due to the presence of ethanol in the DNA solution. Also, ethanol will inhibit enzyme activity in later steps.*
26. Transfer the QIAprep columns in **NEWLY labeled tubes (prepared in step 16)**. Discard the **collection tubes** and ethanol residue. *Note: make sure the numbers on the columns and microcentrifuge tubes matched.*

27. Pipet **50 µL** of **Buffer EB (10 mM Tris-HCl, pH 8.5)** to the center of each QIAprep column.
28. Let the columns stand for **1 minute**. *Note: it is okay to incubate* ***longer than 1 minute***.
29. Spin the tubes with columns at **FULL** speed for **1 minute**. Steps 28 and 29 are for eluting plasmid DNA off the column.
30. After spinning, discard the columns. **Save** the **eluted plasmid DNA** in the microcentrrifuge tubes.
31. Determine **DNA concentration** and its **purity** using the Nanodrop UV spectrophotometer. Record DNA concentration,

D. CONFIRMING THE AUTHENTICITY OF RECOMBINANT PLASMID DNA VIA RESTRICTION ENZYME DIGESTION

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



Reagents and Materials Needed:

1.5-mL Microcentrifuge tubes
Microcentrifuge-tube rack
Sterile water
AscI restriction enzyme (New England Biolabs or NEB, 10 units/μL)
10 NEB buffer #4 (supplied with *AscI*)
37°C water bath
Agarose
Gel Apparatus
Gel Document system

PROCEDURE

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

(**Why *Asc I*?** Check the presence of the ***Asc I***site in the **Multiple Cloning Site** of the pENTR/D-TOPO vector diagram in the previous page). *Note: It is best to set up reactions with a master mix to minimize the chance of leaving out one or several components in the reactions.*

***Table 1. Setting up** the **standard** restriction enzyme reaction*

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

Explanation of volumes

- ✓ ***x µL** = Volume of Plasmid DNA depends on the **amount** (in **ng**) of DNA to be digested and the **concentration** of plasmid DNA (in **ng/µL**)*
- ✓ ***Volume** of **10x React buffer** is **1/10th** the **total volume** of the reaction so that the **final concentration** of the React buffer in the reaction is **1x**.*
- ✓ *The **volume** of **restriction enzyme** depends on the **amount of DNA** to be digested. Usually, **ONE Unit** of **Restriction endonuclease** (Enzyme) is defined as the amount of enzyme required to digest **1 µg** (or **1,000 ng**) of **DNA** completely in **ONE hour** under the **conditions specified for that enzyme** (**most enzymes** have **optimal temperature at 37°C**). To ensure that DNA is completely digested after 1 hour, we use **2-10 units** of enzyme **per microgram** of DNA.*

✓ ***y µL*** *= The volume of sterile water is the remaining volume added to the reaction for bringing up the total volume.*

<u>Note:</u> the volume of NEB buffer + AscI for each reaction is fixed as 2.5 µL while the volume of the plasmid DNA + water is 17.5 µL. Therefore, it is best to make an Enzyme Mix containing the buffer and Asc I enzyme (see the Table below) for seven reactions (6 samples + 1 extra reaction).

a. Label on the lid of a microcentrifuge tube as "**Enz Mix"** → Prepare the **Enzyme Mix** for the **number of plasmid DNA samples + 1 Extra reaction** to be analyzed (use the **Table 2** below) → **Keep the Enzyme Mix tube on ice**. → Mix the contents by flicking the tube gently. Spin the tube for 10 seconds (if necessary). Keep the tube on ice.

How many DNA samples will be digested?

Table 2. Preparation of the Enzyme Mix

Components	**Enz Mix** for **1 Reaction**	**Enz Mix** for **#____Reactions**
10x NEB buffer #4	2.0 µL	µL
***Asc* I (10 U/µL)**	0.5 µL	µL
Total Volume	**2.5 µL**	**µL**

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

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

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

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

f. Incubate the reactions in the **37°C water bath** for about **1 hour**.

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

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

6. Run the gel at **~105 volts** for **1-2 hours**.
   Starting time:
   Ending time:
7. Take a **picture of the gel** and paste it below.
8. Analyze the results.
   How many DNA fragments do you see from each plasmid DNA sample?
   Which fragment corresponds to the vector?
   What is the size of the vector?
   Which fragment corresponds to the PCR-amplified promoter region?
   What is the size of the PCR-amplified promoter region?

How many samples have the PCR-amplified promoter region?

Which plasmid DNA or clone will be used for sequencing analysis?

F. VERIFYING THE SEQUENCE OF THE PROMOTER REGION BY SEQUENCING ANALYSIS

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

SEQUENCING REACTION WITH BIG DYE V. 3

Reference: Perkin Elmer/Applied Biosystems

Solutions Needed:

- Applied Biosystems Big Dye version. 3 (Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- Dye Dilution Mix (Sigma, Cat. # S3938; also, obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- 3 μM M13 Forward primer
- 3 μM M13 Reverse primer
- Sterile water

Materials Needed:

- Applied Biosystems GeneAmp 9700 or BioRad MyCycler
- 0.2 mL PCR tubes or Strips of 8 tubes/strip
- PCR Rack
- Aerosol-barrier (or PCR) Pipet Tips
- Sequencing Reaction Purification Columns (Edge Biosystem) (can be bought directly from Edge Biosystem or Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)

Overview:

Generally, **20-µL** reactions are set up with the following components in **0.2 mL PCR** tubes for a **single DNA template** (see table below). *Note: If you set up several sequencing reactions that share all, except one component, such as DNA template or primer, you should use the format of Master Mix (Mmix) solution to minimize number of pipettings and mistakes of not adding some components into the individual reaction tubes resulting in negative.*

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

General Components of One Reaction:

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

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

y µL = the **remaining volume** to bring the **total volume** to **20 µL**

*** Amount of DNA template** depends on **type of DNA**:

- For **plasmid** DNA, use **250-500 ng**. We found that 500 ng of plasmid DNA gives the best read.

What is the concentration of plasmid DNA? _____ **ng/µL**

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

What is the volume of plasmid DNA solution to be used? _____ **µL**

PROCEDURE

1. Get ice from the icemaker in room 2911 or 3906.
2. Label on the **side** of **TWO 0.2-mL PCR tubes** with **your initial** and **primer name**. Set the tube on a PCR rack sitting on ice.
3. Label on the **lid** and **side** of a **1.5-mL microcentrifuge tube** as "**Mmix**" and **your initial**. Set the tube on ice.
4. Prepare a **master mix (Mmix)** for **3 reactions** (2 reactions + 1 extra) by pipetting the following components into the **Mmix tube** as shown in the table below. *Note: use information on the previous page to fill in the volume of DNA solution to be added and calculate the volume of water to be added to the Mmix tube for 3 reactions.*

Master Mixes (Mmix) of Sequencing Reactions:

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

- Mix the content by flicking the tube five times or vortexing at the mixer setting of 2-3 for **5 seconds**.
- Spin the tube for **10 seconds** to bring all the contents to the bottom of the tube.
- Set the tube back on ice.

5. Pipet **Mmix** and **specific primer** into TWO labeled 0.2-mL PCR tubes.

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

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

<u>or</u> **BioRad MyCycler** with a **Big Dye** protocol with the same profile as above.

7. After the cycling reaction is finished, clean up sequencing reactions using Edge Biosystems spin columns (stored in the cold room) as following:
   a. Spin the pre-packed columns in a microcentrifuge at 3,000 rpm for 2 minutes at room temperature.
   b. Meanwhile, label a new set of 1.5 mL microcentrifuge tubes according to your reactions.
   c. Transfer the columns to new tubes.
   d. Pipet 20 µL of sequencing reaction to appropriate columns.
   e. Spin the columns as in step a.
   f. Discard the columns.
8. Take the purified sequencing reaction to UCLA Sequencing Facility located on the 5th floor in Gonda Building. <u>*Note:*</u> *Make sure to copy down the* ***assigned file number*** *(example, # 5678); that is, automatically given by the Facility, after you enter the samples into the Facility computer.*
9. After one to two days, retrieve your sequences from the Sequencing Facility web page.

RETRIEVING AND ANALYZING DNA SEQUENCES

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

1. From any computers in the lab, Log in to the UCLA Sequencing Retrieval System via http://www.genetics.ucla.edu/webseq/
2. Enter in the USER NAME field: **goldberg_r**
3. Enter in the PASSWORD field: **embryo**
4. Find your sequence files by looking up the ***assigned file number*** and the name of the gene you are working on.

   Example: the ***assigned file number*** is **5677**, and the gene of interest is **At5g09250**. You would see the following files:

   5677 GOLDR_At5g09250Fw_080.ab1

   5677 GOLDR_At5g09250Rv_081.ab1

   What are the annotations?

   5677 = assigned file number; **GOLDR** = user name; **At5g09250Fw** = sequence name obtained with the Forward sequencing primer, **080** = capillary position used in loading sequencing sample in the Sequencer ABI 7700 (Perkin-Elmer/Applied Biosystems); abi = ABI file format. Select "PROCESS INDIVIDUAL SEQUENCES" instead of "PROCESS COMPLETE SET OF 96 SEQUENCES".
5. Select sequences to be downloaded, and click "DOWNLOAD SELECTED" or click on "SEQUENCE FILE TO DOWNLOAD".
6. Select "SAVE TO DISK" and choose "THE DESKTOP".
7. Open the saved file using a SEQUENCE VIEWER PROGRAM (CHROMAS on Windows or EDITVIEW on Mac).
8. Copy DNA sequences to a Microsoft Word file. Note: Name the files according to the name of gene of interest (for example, At5g09250).

9. Process the DNA sequences by "BLASTN" and "BLASTX" searches, respectively. Note: Blast search may take a few minutes or longer to complete depending on how busy is the NCBI server in Washington D.C (i.e. how many sequences have been processed by the NCBI server at the fraction of time).
10. Determine if the DNA sequence corresponds to the gene of interest.
11. Print out the Blast results as hard-copy records.
12. Save the Blast results in the **pdf** format so that you can upload them in your webbook.

EXPERIMENT 6 - IDENTIFYING FEATURES OF MUTANT EMBRYO USING NOMARSKI MICROSCOPY

Purpose: To introduce **D**ifferential **I**nterference **C**ontrast (**DIC**) or **N**omarski **I**nterference **C**ontrast (**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 (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
Acetic acid	0.5 mL
Total volume	5.0 mL

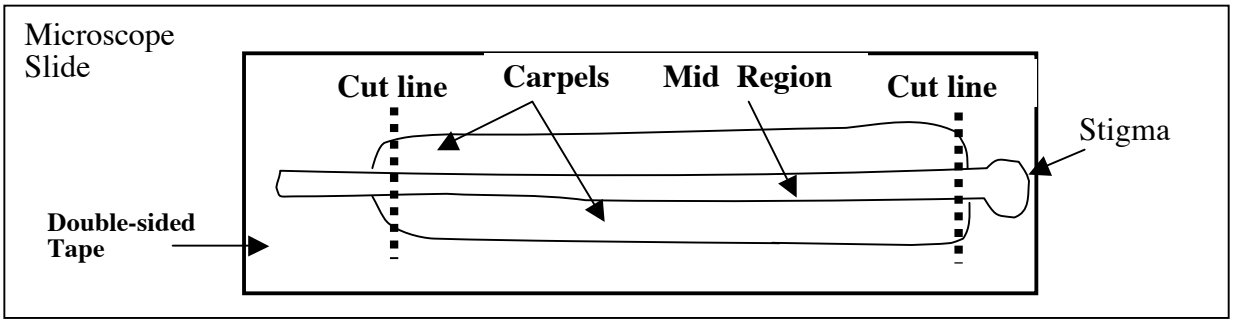
- Close the tube with its cap.
- Invert the tube to mix the content.

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/Siliques).
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).

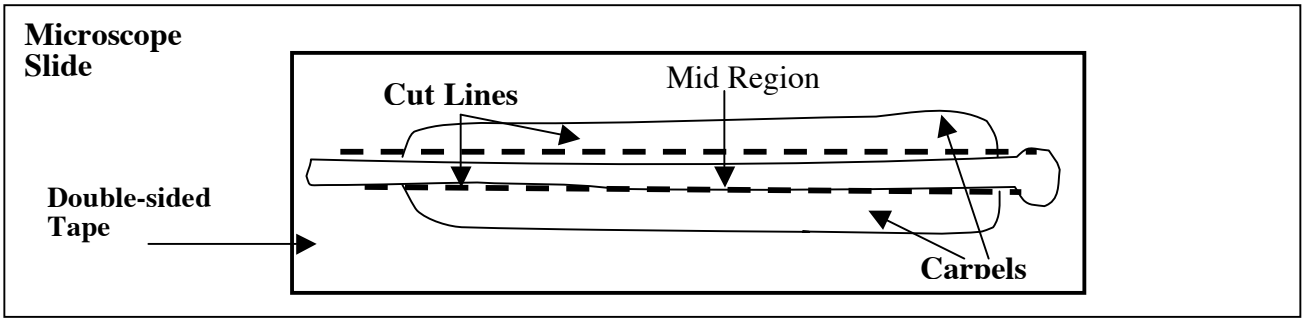
6. Measure and collect **3 siliques** for **each** of **two different lengths** (one short and one long) in a **range of 0.5-1.5 cm** from the wild type, a known T-DNA knockout (*raspberry 3*, *dicer-like, lec1*, or *titan*), and homozygous/heterozygous mutant. *Note: collect the **same length** of siliques 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:
   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 Siliques 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 Siliques with mature green embryos, **dissect** the Siliques 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 Siliques --> 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 Siliques and their embryos.*
9. Next day, CAREFULLY pipet off **650 µL** of the fixative solution using a **P-1000** pipetman and then the **remaining volume** with a **P-200** pipetman. *Note: Do not let the Siliques 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
Double-distilled water	0.5 mL
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
Double-distilled water	1.5 mL
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

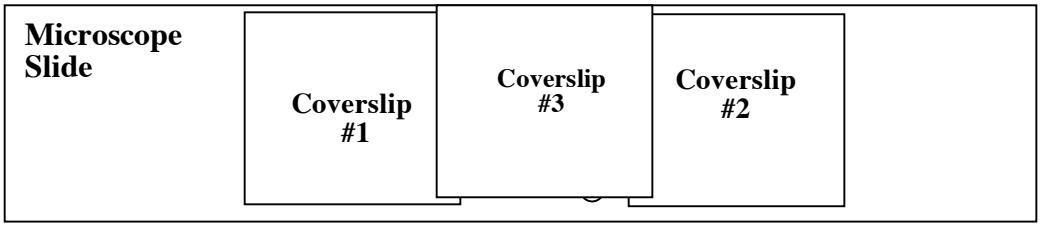
Note: ***At least ONE hour before observation of the seeds and their embryos. Seeds must be submerged in the clearing solution. (a) The older the seed, the longer it takes to clear and sink. For young seeds, clearing is usually fast, i.e., seeds can be observed under a microscope within 30 minutes. (b) 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	2 mL
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 μL** 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.

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

EXPERIMENT 7 – 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

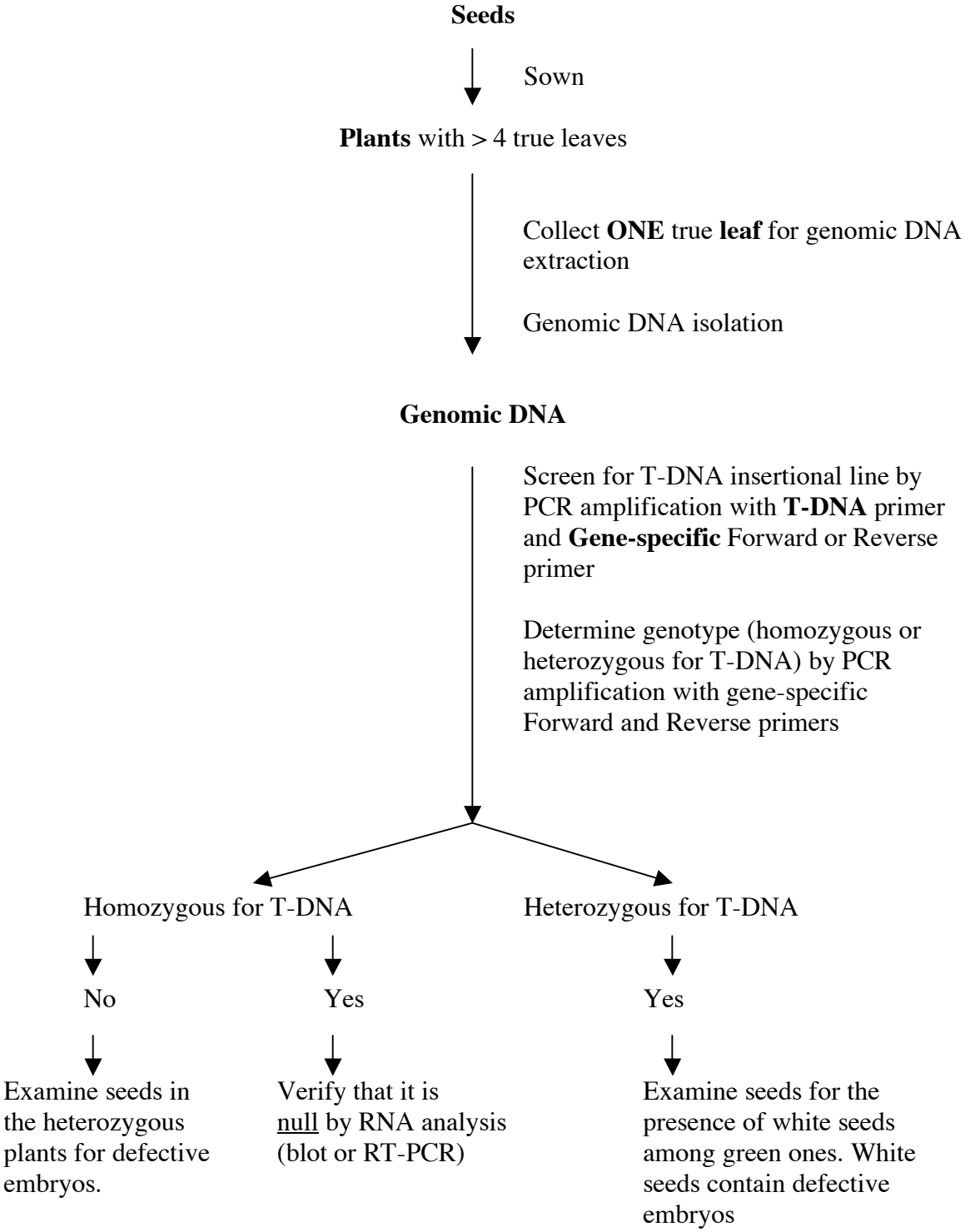
STRATEGY

IV. SOWING SEEDS AND GROWING PLANTS

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

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

STRATEGY


Seeds
Sown
Plants with > 4 true leaves
Collect ONE true leaf for genomic DNA extraction
Genomic DNA isolation
Genomic DNA
Screen for T-DNA insertional line by PCR amplification with T-DNA primer and Gene-specific Forward or Reverse primer
Determine genotype (homozygous or heterozygous for T-DNA) by PCR amplification with gene-specific Forward and Reverse primers
Homozygous for T-DNA
Heterozygous for T-DNA
No
Yes
Yes
Examine seeds in the heterozygous plants for defective embryos.
Verify that it is null by RNA analysis (blot or RT-PCR)
Examine seeds for the presence of white seeds among green ones. White seeds contain defective embryos

IV. SOWING SEEDS AND GROWING PLANTS

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

Caution: Be extremely CAREFUL with Siliques. Do NOT mix up labeled tags and actual Silique 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 plactic 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 (Columbia for Salk lines).*
2. If **plastic tags** are available in the lab, label them with a black sharpie.

   For **knockout line**: **Gene name**
   SALK line #
   Date
   Pot # 1-10 (for 1 flat with 11 pots)

   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 mount 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 Siliques 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 Silique 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 4 seeds per pot for 11 pots.
12. Put the labeled tags for the **knockout line** into **each** of the **10 pots** containing knockout Siliques.
13. Put the Siliques that were not used back into the **appropriate knockout seed** microcentrifuge **tube**.

14. For pot **#11**, pour out wild-type Siliques 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 Siliquelings with 2 cotyledons to the glasshouse #3
24. Put the flats of Siliquelings on a table.

    NOTE: Choose a table that has no mature Arabidopsis plants bearing ripened Siliques because these Siliques could accidentally get in the soil of the Siliqueling 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 Siliquelings nor will the surface of the soil be too warm which is favorable for molds to grow.
26. Wait until most of Siliquelings 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 Siliquelings** 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, then put "fertilizer-supplemented" water in.

<u>NOTE:</u> 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 #**

V. 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.

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^{o}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^{o}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 **12** sterile 1.5-mL microcentrifuge tubes on a microcentrifuge-tube rack.
2. Label number 1-6 on lids of the tubes.

   Tube #**1 - 11**: seedlings/plants #1 - 11 of **Knockout** line

   Tube #**12**: **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)
   The Nikon 5400 digital camera
   A pen
   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 would 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 10 seconds.
17. Repeat steps 14-17 for **other tubes**.
18. Centrifuge tubes of homogenates at **room temperature** for **5 minutes** at **FULL speed**.
19. Meanwhile, label **a set** of **microcentrifuge tubes** with **Gene Name** and **tube #.**
20. 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.*
21. After centrifugation, transfer tubes from the microcentrifuge onto a microcentrifuge-tube rack.
22. Organize tubes such that the **numbers** on the **lids** of **NEW tubes match** with **numbers** on **the lids** of **tubes containing homogenates**.
23. 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.*
24. Mix the isopropanol and homogenate by inverting the tube **5-10 times**.

25. Incubate the mixture at **room temperature** for **5 minutes** to precipitate **nucleic acids** *(both genomic DNA and total RNA)*.
26. Centrifuge tubes at **room temperature** for **10 minutes** at **FULL** speed.
27. 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.*
28. 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.*
29. Centrifuge the tubes at **room temperature** for **5 minutes**.
30. 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.*
31. Put the tubes on a microcentrifuge-tube rack with their lids opened allowing ethanol to be evaporated.
32. 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**.
33. *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.
34. Spin tubes in a microcentrifuge for **1 minute** to bring down liquid and any contaminants to the bottom of the tubes.
35. Store DNA solutions at **4ºC** (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.

36. 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?

37. 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 spectrophometer 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 detemined 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 Excitation 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.

38. Dilute **5 µL** of **original DNA solutions** to a **final concentration** of **0.2 ng/µL** with **TE** buffer. Label on the lids and sides of microcentrifuge tubes with the following information: **0.2 ng/µL**, **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,

$\mathbf{V_i \cdot C_i = V_f \cdot C_f}$
where,

$\mathbf{V_i}$ = **inital volume** (the volume of original DNA solution is **5 µL**)

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

$\mathbf{V_f}$ = **final volume** (depends on the initial concentration)

$\mathbf{C_f}$ = **final concentration** (**0.2 ng/µL**)
then,

$\mathbf{V_f = (V_i \cdot C_i)/ C_f}$ = (**5 µL** x **8 ng/µL**)/(**0.2 ng/µL**) = **200 µL** of **total volume**
What is the volume of TE to be used in dilution?

$\mathbf{V_{TE} = V_f - V_i = 200\ \mu L - 5\ \mu L = 195\ \mu L}$ 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			
Plant #7			
Plant #8			
Plant #9			
Plant #10			
Plant #11			
Plant WT			

VI. 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 μM Gene-specific Forward primer
- 12 μM Gene-specific Reverse primer
- 12 μM **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 12** plants to be characterized and **2 controls** (genomic DNA isolated by TA + No DNA template), prepare a **master mix** for **14 + 1 extra = 9 reactions**.*

23. Label on the lids and sides **14 PCR tubes** and put them on a PCR rack sitting on ice.
24. Prepare a **master mix** for **15 PCR reactions** in a 1.5 mL microcentrifuge tube labeled as "**Mmix**" sitting on ice.

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

	Mmix for ONE reaction	**Mmix for 15** reactions
Sterile water	16.8 µL	252.0µL
10x Ex-Taq buffer	2.5 µL	37.5 µL
dNTP mix	2.0 µL	30.0 µL
12 µM **Gene-specific Forward** primer	0.5 µL	7.5 µL
12 µM **Gene-specific Reverse** primer	0.5 µL	7.5 µL
12 µM **LBb1** primer (for SALK lines)	0.5 µL	7.5 µL
Ex-Taq DNA polymerase (5 U/µL)	0.2 µL	3.0 µL
Total Volume	**23.0 µL**	**345.0 µL**

25. 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**.
26. Pipet **23 µL** of the **Mmix** into each of **14 PCR tubes**.
27. Pipet **2 µL** of **1 ng/µL** genomic DNA extracted from each of **12** seedlings/plants into PCR tubes #1-12. Pipet up and down for five times to mix the contents. Put the first tube back on ice and work on the remaining tubes.
28. Pipet **2 µL** of **1 ng/µL genomic DNA** extracted (by TAs) from **wild type** (Col-0) Siliquelings into each of tubes **#13**. Pipet up and down for five times to mix the contents.
29. Pipet **2 µL** of **sterile water** to tube **#14** (**negative control** without DNA template). Pipet up and down for five times to mix the contents.

30. Spin PCR tubes in the microcentrifuge for PCR tubes for **5 seconds** to bring the liquid to the bottom of the tubes.
31. Put the tubes on the **wells** of the PCR machine.
32. Perform PCR with the "**KNOCKOUT**" program with the following profile:

    1 cycle of Hot start or 96ºC for 3 minutes
    36 cycles of 94ºC, 15 seconds -> 60ºC, 30 seconds -> 72ºC, 2 minutes
    1 cycle of 72ºC, 4 minutes
    4ºC, ∞
33. Prepare a **1.5% agarose gel** in 1X TAE buffer with a **20-tooth** comb.
34. Label **14 1.5-mL microcentrifuge** tubes and set them on a rack.
35. Add **2 µL** of **loading dye** to each tube.
36. Pipet **10 µL** of **PCR solutions** to each tube.
37. Load samples on the **1.5% agarose gel** along with **10 µL** 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**

38. Run the gel at **120 volts** for **1-2 hours** or until the front dye (bromophenol blue) is two-thirds of the gel.
39. Stop the gel electrophoresis.
40. Take a picture of the gel.
41. 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?

42. 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.
43. Go to the Plant Growth Center, put the wooden sticks next to the identified T-DNA tagged plants.
44. 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* ***11 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 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
- 50ºC water bath
- 1.5-mL microcentrifuge tubes
- Microcentrifuge
- Scale
- Metal waste container for sharp objects

PROCEDURE

32. Prepare a **1.5% agarose** gel with a **20-tooth comb**.
33. Add **4 µL** of **6X loading dye** to each tube of ~**25-µL** PCR solutions.
34. Load the samples on the gel.
35. Record loading pattern below:

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

36. Run the gel at **120 volts** for 1.5 - 2 hours.
37. Take a picture of the gel.
38. Verify the presence of **expected size PCR product**.

39. Label on the **lids** of **TWO** microcentrifuge tubes "**T-DNA**", "**WT**", and **your initial**.
40. Place a NEW piece of plastic wrap on an UltraViolet (UV) box, then place your gel on the plastic wrap.
41. Put on a UV shield to protect your eyes and face.
42. Turn **on** the **UV box**. *Note: Turn **off** the UV box as soon as you are done with excising DNA band(s).*
43. 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).*
44. Place the agarose slice in the **appropriate 1.5-mL microfuge tube**. Repeat this step for more than one DNA fragments.
45. Take a picture of the gel **after removing excised agarose slice(s)**. *This step serves as a record of DNA fragment(s) being collected.*
46. Centrifuge the gel fragment for **10 seconds**.
47. 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 µL**.*
48. 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 µL**. Therefore, add **450 µL** of **buffer QG** to the tube.*
49. Incubate tube at **50 ºC** 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.*
50. 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.*
51. 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.**
52. Pipet the **mixture** from **step 19** to the appropriate **spin columns** (**purple**). *Do NOT pipet **more than 800 µL** of the mixture into the column. If the total volume is **more than 800 µL**, repeat **steps 21-23**.*
53. Centrifuge the tube for **1 minute**.

54. Separate the **spin column** from the **collection tube** and then **pour off** the **flow-through solution** in a beaker labeled as "Waste". 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.*
55. Add **500 µL** of **buffer QG** to the **spin column** and centrifuge for **1 minute**. Discard the flow-through solution. *This step removes all traces of agarose.*
56. Add **750** of **µL buffer PE** and let the tube stand for **2-5 minutes**. Centrifuge the tube for **1 minute**. *This step washes the column.*
57. Discard the **flow-through solution** and centrifuge **1 minute** to remove all the **ethanol** from the column.
58. 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**.
59. After spinning, transfer the **spin columns** to the **appropriate labeled microcentrifuge tubes**. *Note: Make sure that the labels on the spin columns corresponding to those on the microcentrifuge tubes.*
60. Add **30 µL** 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**.*
61. Discard the collection tube.
62. 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/µL**
    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 μL of PCR products on the gel and identify homozygote for T-DNA or wild type.

14. Write on the **lids** and **sides** of **1.5-mL microcentrifuge** tubes "**T-DNA**" or "**WT**", and **your initial**.
15. Pipet **15 μL** 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.
16. Add **75 μL** 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.
17. Place a **QIAquick spin column** in a provided **2-mL collection tube**.
18. 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*
19. Discard the **flow-through solution** in the collection tube. Put the QIAquick column back into the same collection tube.
20. Add **750 μL** of **Buffer PE** to the QIAquick spin column and spin at **FULL speed** for **1 minute**.
21. Discard the **flow-through solution** in the collection tube. Put the **QIAquick column back** into the same collection tube.
22. 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.

23. 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**.
24. Transfer the appropriate **QIAquick columns** in the **NEWLY labeled microcentrifuge tubes**. Discard the flow-through solutions and the collection tubes.
25. Pipet **30 µL** 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.*
26. 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**

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 μM LBb1 primer (for T-DNA)
- 3 μM Gene-specific Forward primer
- 3 μM 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-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.
- 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 ng/4.5 ng/µL = 2.2 µL***

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

PROCEDURE

13. Get ice from the icemaker in room 2911 or 3906.
14. 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.
15. Label on the **lid** and **side** of a **1.5-mL microcentrifuge tube** as "**Mmix**" and **your initial**. Set the tube on ice.
16. 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** 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	**LBb1 primer**	**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**	0 µL	0 µL
3 µM Gene-specific Forward primer	0 µL	**1 µL**	0 µL
3 µM Gene-specific Reverse primer	0 µL	0 µL	**1 µL**
Total volume	**20 µL**	**20 µL**	**20 µL**

17. 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 96ºC, 10 sec. --> 50ºC, 5 sec. --> 60ºC, 4 min. Followed by 4ºC, ∞

    <u>or</u> **BioRad MyCycler** with a **Big Dye** protocol with the same profile as above.

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

6. Take the purified sequencing reaction to UCLA Sequencing Facility located on the 5th floor in Gonda Building. <u>*Note:*</u> *Make sure to copy down the* ***assigned file number*** *(example, # 5678); that is, automatically given by the Facility, after you enter the samples into the Facility computer.*

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.

25. From any computers in the lab, Log in to the UCLA Sequencing Retrieval System via http://www.genetics.ucla.edu/webseq/
26. Enter in the USER NAME field: **goldberg_r**
27. Enter in the PASSWORD field: **embryo**
28. 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".
29. Select sequences to be downloaded, and click "DOWNLOAD SELECTED" or click on "SEQUENCE FILE TO DOWNLOAD".
30. Select "SAVE TO DISK" and choose "THE DESKTOP".
31. Open the saved file using a SEQUENCE VIEWER PROGRAM (CHROMAS on Windows or EDITVIEW on Mac).
32. Copy DNA sequences to a Microsoft Word file. Note: Name the files according to the name of gene of interest (for example, At5g09250).

33. 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).
34. Determine if the DNA sequence corresponds to the gene of interest.
35. Print out the Blast results as hard-copy records.
36. Save the Blast results in the **pdf** format so that you can upload them in your webbook.

EXPERIMENT 8 – GENE EXPRESSION STUDY IN *ARABIDOPSIS THALIANA* (GENE TWO)

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

STRATEGY

Use the cDNA solutions that you generated in the Experiment 3 with the RT-PCR primers for gene TWO.

PROCEDURE

CARRYING OUT PCR AMPLIFICATION (RT-PCR) ANALYSIS

PROCEDURE

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 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
- 100-bp DNA ladder (Invitrogen)
- 6X Loading buffer containing ONLY **Xylene Cyanol**
- 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

16. Get ice from the icemaker in room 2911 or 3906.
17. Determine **how many RT reactions**, including **+RT's** and **-RT's**, will be amplified.
18. Make a **Table** with information sush 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

19. Label on the **lids** and **sides of SIX 0.2 mL PCR tubes** with **Number** and **your initial**.
20. Put the labeled tubes on a PCR rack sitting **on ice**.
21. 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 ONE 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	34.6 μL	242.2 μL
Ex-Taq DNA Polymerase (5 U/μL)	0.4 μL	2.8 μL
Total Volume	**48.0** μL	**336.0 μL**

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

22. 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**	0 µL	0 µL	0 µL	0 µL	0 µL
Leaf -RT	0 µL	**2 µL**	0 µL	0 µL	0 µL	0 µL
Silique +RT	0 µL	0 µL	**2 µL**	0 µL	0 µL	0 µL
Silique -RT	0 µL	0 µL	0 µL	**2 µL**	0 µL	0 µL
0.2 ng/µL Genomic DNA	0 µL	0 µL	0 µL	0 µL	**2 µL**	0 µL
Water	0 µL	0 µL	0 µL	0 µL	0 µL	**2 µL**
Total Volume	**50 µL**	**50 µL**	**50 µL**	**50 µL**	**50 µL**	**50 µL**

23. Carry out PCR reactions with the **RT-PCR program** containing the following profile: 1 cycle of 96 ºC, 3 min. → 40 cycles of 94 ºC, 10 sec./60 ºC, 30 sec./72 ºC, 45 sec. → 1 cycle of 72 ºC, 4 min. → 4 ºC, ∞.
24. Prepare **100 mL** of **1.5% agarose** gel in **1X TAE** buffer as usual (Use a **20-tooth** comb).

    <u>Note:</u> *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.*
25. Label 1.5 mL microcentrifuge tubes according to the PCR solutions being performed.

26. Add to the labeled 1.5 mL microcentrifuge tubes **20 µL** of **PCR solution** and **3 µL** of **6X loading dye containing ONLY xylene cyanol**. *Note: 20 µL of PCR solution was loaded so that you can see the PCR products clearly.*
27. Load samples on a **1.5%** agarose gel along with **10 µL** of **50 ng/µL 100-bp ladder** solution. Record RNA loading pattern.

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

28. 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:
29. Take a picture of the gel.
30. 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 Siliques?

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

EXPERIMENT 9 - IDENTIFYING FEATURES OF MUTANT EMBRYO USING NOMARSKI MICROSCOPY

Purpose: To introduce **D**ifferential **I**nterference **C**ontrast (**DIC**) or **N**omarski **I**nterference **C**ontrast (**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 (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
Acetic acid	0.5 mL
Total volume	5.0 mL

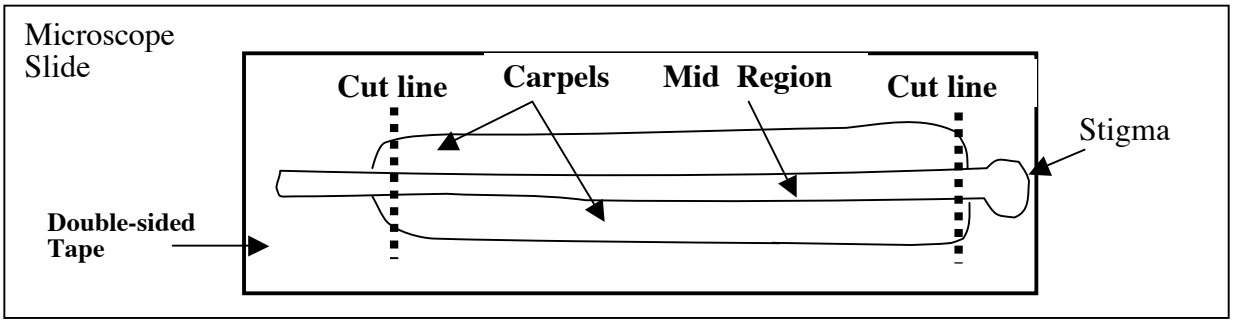
- Close the tube with its cap.
- Invert the tube to mix the content.

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).

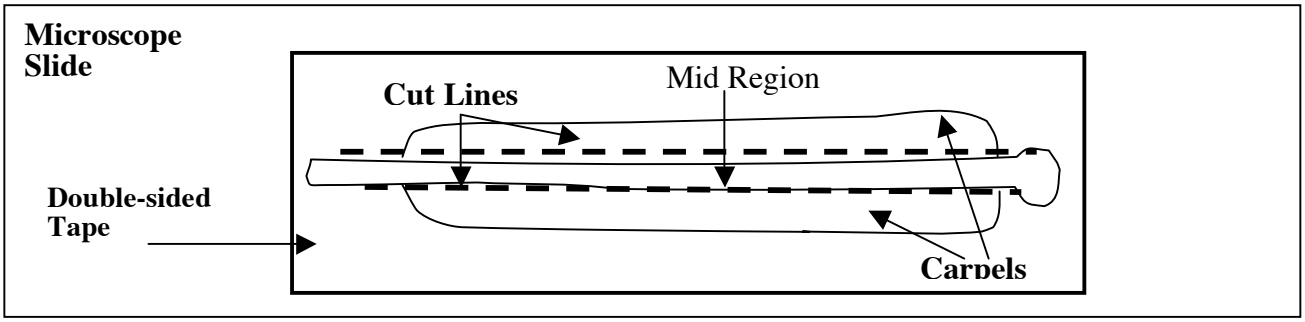
19. Measure and collect **3 siliques** for **each** of **two different lengths** (one short and one long) in a **range of 0.5-1.5 cm** from the wild type, a known T-DNA knockout (*raspberry 3*, *dicer-like, lec1*, or *titan*), and homozygous/heterozygous mutant. *Note: collect the **same length** of 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 Siliques 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 Siliques with mature green embryos, **dissect** the Siliques 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 Siliques --> 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 µL** 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
Double-distilled water	0.5 mL
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
Double-distilled water	1.5 mL
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).

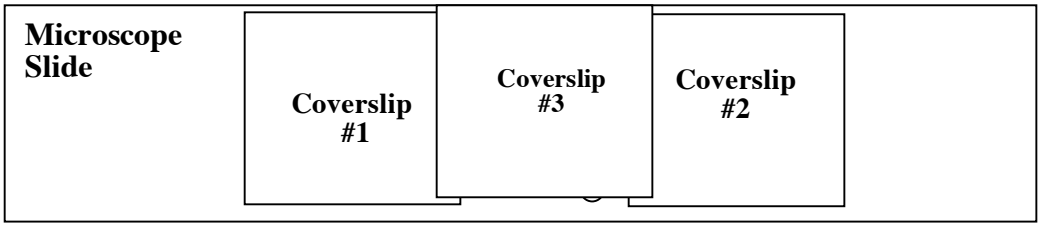
B. Observation of Seeds and Embryos

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 seed, 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.***

9. 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	2 mL
Total volume	3 mL

10. 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.
11. Set a new glass microscope slide on the bench.
12. Gently, pipet the clearing solution up and down for 5 times to mix up the seeds using a P-200 pipetman.
13. Dispense **100-150 µL** of the clearing solution with seeds in the center of the microscope slide.
14. Carefully, place two squared coverslips, one on each side of the solution. Then, place a third coverslip over the clearing solution (see below)



15. Observe the seeds under Nomarski optic using Leica CTR5000 microscope.

16. Take pictures of the embryos.

<u>Note:</u> For siliques, you need to dissect seeds out of the silique on a microscope slide under a dissecting microscope. Then, proceed to 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 55ºC 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 μL of Ethidium Bromide (EtBr) into the solution and swirl the flask GENTLY to mix. *<u>Note:</u> 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.

    <u>Note:</u> 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.

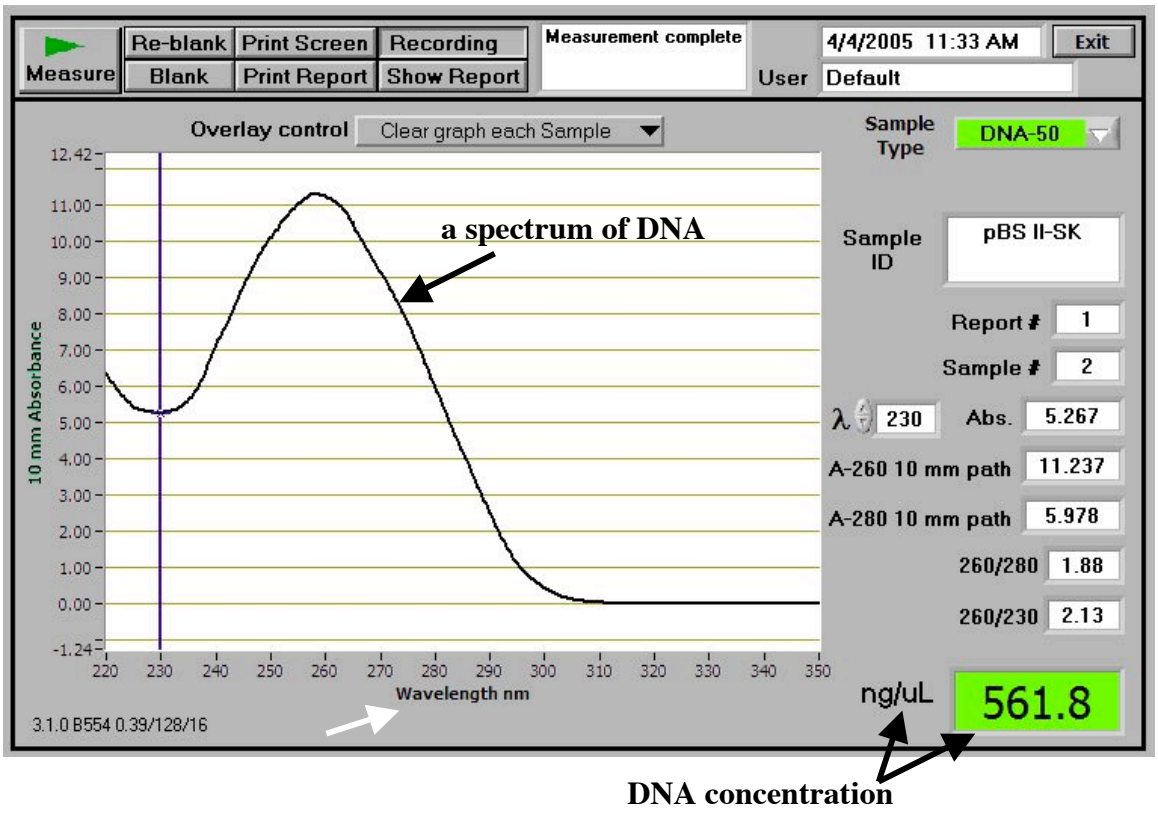
12. Pour ~600 mL of 1X TAE buffer into the gel box.

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:



OPERATION OF NANODROP SPECTROPHOTOMETER ND-1000

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

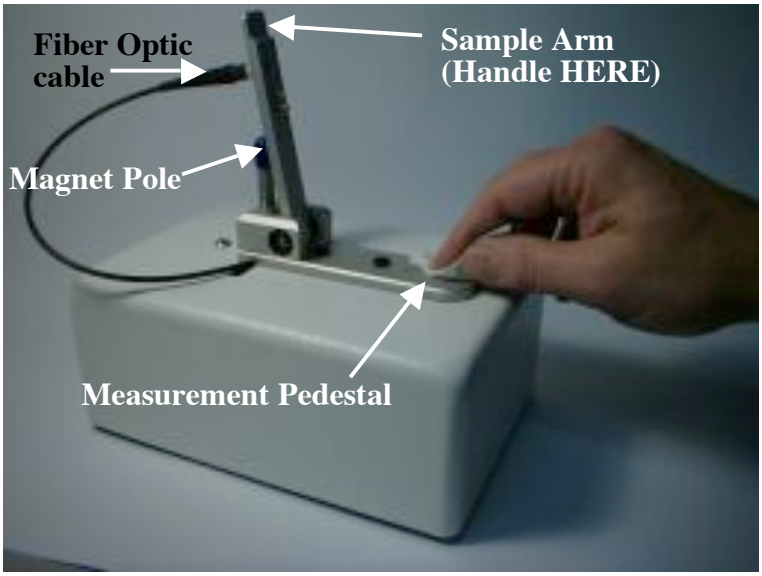
1. **Turn ON** the **Computer (laptop)** that connects to the Nanodrop.
2. **Turn ON** the **Nanodrop** by clicking an "**ND-1000 v3.1.0**" icon on the computer desktop. Wait for a few seconds for the Nanodrop to be up. You see the Nanodrop 3.1.0 Diagnose panel with **User field** as **Default.**
3. **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**.

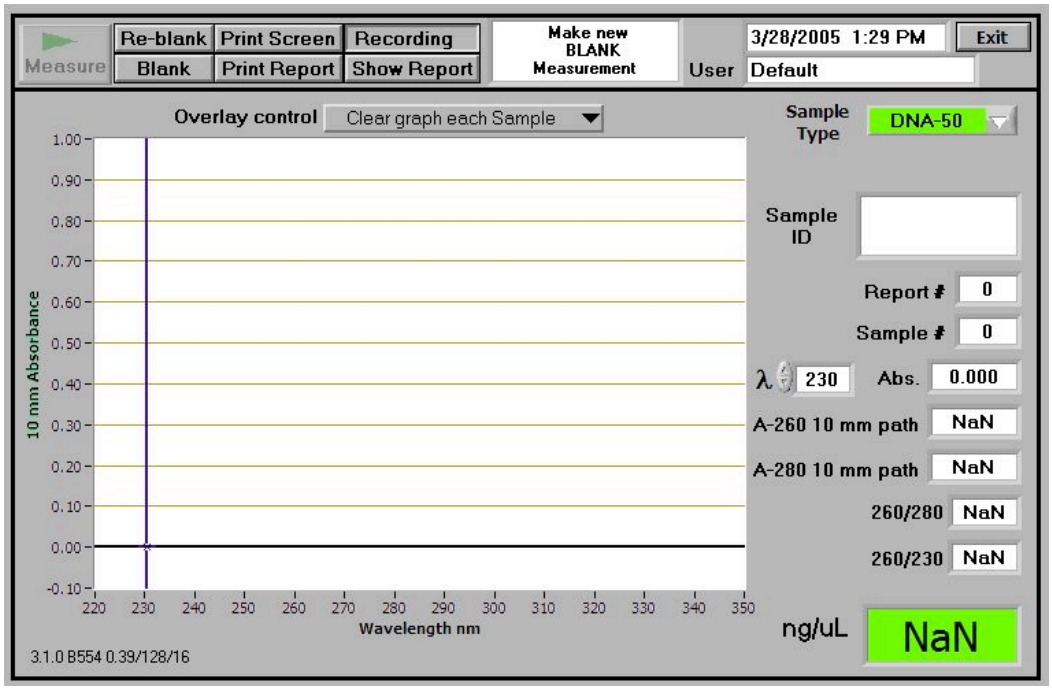
Note: Even though the Nanodrop Inc. claims that the Nanodrop can read as low as **1 µL**, the concentration reading is NOT consistent at this volume. Therefore, the **minimal** volume for the concentration reading is **1.5 µ□**

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 □L 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 □L 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/□L**) 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)

Invitrogen
life technologies

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 ^{32}P 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

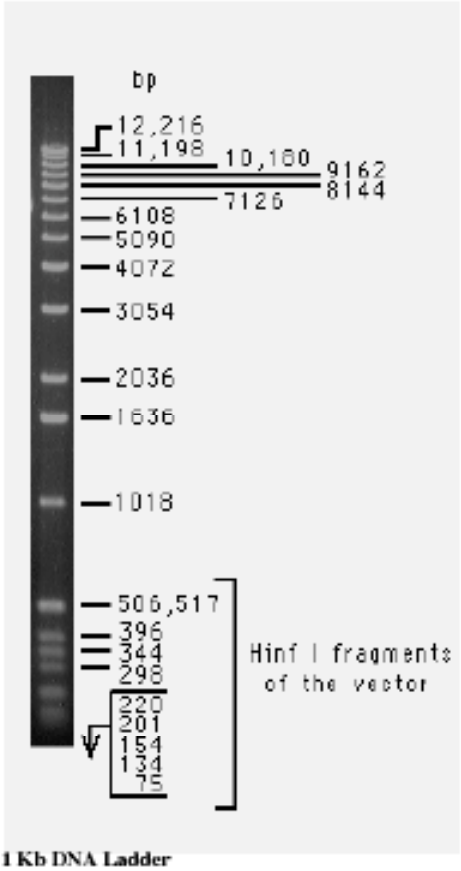
Recommended Procedure:
Invitrogen recommends the use of 10X BlueJuice® Gel Loading Buffer (10816-015) at a concentration of 2X [for electrophoresis of this ladder on agarose gels]. Alternatively, the DNA ladder can be diluted in a buffer such that the final concentration of NaCl is 20 mM. Apply approximately 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

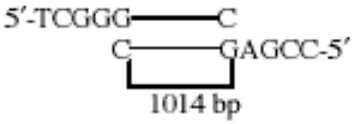
This product is distributed for laboratory research only. CAUTION: Not for diagnostic use. The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

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



1 Kb DNA Ladder
0.5 µg/lane
0.9% agarose gel
stained with ethidium bromide

Structure of Fragment (1):



Notes:

During 1.2% agarose gel electrophoresis with Tris-acetate (pH 7.6) as the running buffer, bromophenol blue migrates together with the 506/517 bp doublet band.

The 1636 bp band and all bands less than 1000 bp are generated from pBR322.

If the ionic strength of the sample is too low, blurring of the bands can occur.

Cat. No. 15615-016

Appendix 1D

iProof High-Fidelity DNA Polymerase (Taken from Bio-Rad Website)

BIO-RAD

iProof™ High-Fidelity DNA Polymerase

2 units/µl, 10 µl	20U	172-5300
2 units/µl, 50 µl	100U	172-5301
2 units/µl, 250 µl	500U	172-5302

For research purposes only
Store at -20°C

iProof is a high-fidelity DNA polymerase that offers extreme performance for all PCR applications. Incorporating an exciting new and patented technology, iProof DNA polymerase brings together a novel *Pyrococcus*-like enzyme with a processivity enhancing domain. This allows for the generation of long templates with an accuracy and speed previously unattainable with a single enzyme. The extreme fidelity of iProof makes it a superior choice for cloning. The error rate of iProof polymerase is determined to be 4.4 x 10^{-7} in iProof HF buffer, which is approximately 50-fold lower than that of *Thermus aquaticus*, and 6-fold lower than that of *Pyrococcus furiosus*.

Storage and Stability

Store iProof™ High-Fidelity DNA Polymerase at -20°C in a constant temperature freezer. When stored under these conditions, the polymerase is stable for one year after the ship date.

Kit Contents

Reagent	20U	100U	500U	Description
iProof Polymerase	10 µl	50 µl	250 µl	iProof™ High Fidelity DNA Polymerase, 2 units/µl
iProof HF Buffer	1.5 ml	1.5 ml	4 x 1.5 ml	5X HF Buffer, 7.5 mM $MgCl_2$
iProof GC Buffer	1.5 ml	1.5 ml	4 x 1.5 ml	5X GC Buffer, 7.5 mM $MgCl_2$
$MgCl_2$	1.5 ml	1.5 ml	2 x 1.5 ml	50 mM $MgCl_2$ solution
DMSO	500 µl	500 µl	500 µl	100% DMSO solution

iProof DNA polymerase is unlike other enzymes. Please read the *QuickGuide* to modify your protocol for optimal results.

QuickGuide (See *Notes About Cycling Conditions* for details)

- Use 98°C for denaturation.
- Anneal at T_m +3°C (>20nt oligo).
- Use 15–30 sec/kb for extension times. Do not exceed 1 min/kb.
- Use iProof at 0.5–1.0 U per 50 µl reaction. Do not exceed 2 U/50 µl.
- Use 200 µM dNTPs. Do not use dUTP.
- iProof produces blunt end DNA products.

10002298 Rev A

Reaction Setup

Important Note – Please Read Before Starting

Spin all tubes before opening to improve recovery. Reactions should be set up on ice. Pipet all components in the order given below. Always add iProof DNA Polymerase last to the reaction as primer degradation may occur in the absence of dNTPs. It is recommended that you prepare a master mix for the appropriate number of samples to be amplified.

Component	Volume for 50 µl reaction	Volume for 20 µl reaction	Final Conc.
5X iProof HF Buffer*	10 µl	4 µl	1X
10 mM dNTP mix	1 µl	0.4 µl	200 µM each
Primer 1**	x µl	x µl	0.5 µM
Primer 2**	x µl	x µl	0.5 µM
DNA template	x µl	x µl	
Sterile H_20	x µl	x µl	
iProof DNA Polymerase	0.5 µl	0.2 µl***	0.02 U/µl
Total Volume	50 µl	20 µl	

* For difficult or GC-rich templates, 5X iProof GC Buffer can be used.
** Recommended final primer concentration is 0.5 µM; can range between 0.2–1.0 µM.
*** Enzyme should be diluted to avoid pipeting errors.

Notes About Reaction Components

1. **iProof DNA Polymerase**
   The optimal amount of enzyme depends on the amount of template and the length of the PCR product. Usually 1 unit of iProof DNA polymerase per 50 µl reaction will give good results, but optimal amounts could range from 0.5–2 units per 50 µl reaction depending on amplicon length and difficulty. **Do not exceed 2 U/50 µl (0.04 U/µl), especially for amplicons that are > 5kb**.

2. **Buffers**
   Two buffers are provided: 5x iProof HF buffer and 5x iProof GC buffer. The error rate of iProof polymerase in HF buffer (4.4×10^{-7}) is lower than that in GC buffer (9.5×10^{-7}). Therefore, the HF buffer should be used as the default buffer for high fidelity amplification. However, the GC buffer can improve iProof performance on certain difficult or long templates, i.e. GC rich templates or those with complex secondary structures. Only use GC buffer when amplification with HF buffer does not provide satisfactory results.

3. **Mg^{2+} and dNTP**
   Mg^{2+} concentration is critical since iProof is a Mg^{2+}-dependent enzyme. Excessive Mg^{2+} stabilizes dsDNA, preventing complete denaturation, and can also promote inaccurate priming. Conversely, insufficient amounts of Mg^{2+} can lead to low product yield. The optimal Mg^{2+} concentration also depends on dNTP concentration, the specific DNA template and the sample buffer composition. The optimal Mg^{2+} concentration is 0.5 to 1 mM over the total dNTP concentration for standard PCR. For optimization, increase or decrease Mg^{2+} concentration in 0.2 mM increments.

   Only high quality dNTPs should be used. Use of dUTP or other dUTP-derivatives or analogs is not recommended. Due to the increased processivity of iProof, there is no advantage to increasing dNTP amounts. For optimal results, use 200 µM dNTPs.

4. **DNA Template**
   General guidelines are 1 pg–10 ng of DNA template in a 50 µl reaction for low complexity DNA (e.g. plasmid, lambda, or BAC DNA). For high complexity DNA (e.g. genomic DNA), 50–500 ng of template DNA should be used in a 50 µl reaction.

5. **PCR Additives**
   The recommended reaction conditions for GC-rich templates include the addition of 3% DMSO which aids in template denaturation. Further optimization of DMSO should be made in 2% increments. In some cases, DMSO may be used to help relax supercoiled plasmid DNA. High DMSO concentrations (10%) will require lowering the annealing temperature by 5.5–6.0°C. Other PCR additives such as formamide, glycerol, and betaine are also compatible with iProof.

Cycling Conditions

Important Note – Please Read

Due to the novel nature of iProof DNA polymerase, optimal reaction conditions may differ from standard PCR protocols. iProof works better at elevated denaturation and annealing temperatures due to higher salt concentration in the reaction buffer.

Typical Thermal Cycling Protocol

Cycle Step	Temp.	Time	Number of Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	5–10 s	
Annealing	45–72°C	10–30 s	25–35
Extension	72°C	15–30 s / kb	
Final Extension	72°C	5–10 min	1

Notes About Cycling Conditions

1. **Denaturation**
   Template denaturation should be performed at 98°C. Due to the high thermostability of iProof, denaturation temperatures greater than 98°C can be used. A 30 s initial denaturation time is recommended, but this can be extended to 3 min for difficult DNA templates. Subsequent denaturation should be performed for 5–10 s at 98°C.
2. **Annealing**
   When using iProof, a general rule is to anneal primers (>20 nt) for 10–30 s at +3°C above the primer with the lowest T_m. Primer T_m should be calculated using the nearest-neighbor method as results can vary significantly depending on the method used. For primers $\leq$ 20 nt, use an annealing temperature equal to the primer with the lowest T_m.
3. **Extension**
   Template extension should be performed at 72°C and extension time depends on amplicon length and complexity. For low complexity DNA (e.g. plasmid, lambda, or BAC DNA) use 15 s per kb. For high complexity DNA (e.g. genomic DNA) use 30 s per kb. **Do not exceed 1 min per kb for amplicons that are >5 kb.**

Component Specifications

Storage buffer
20 mM Tris-HCl (pH 7.4 at 25°C), 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, 0.5% Tween 20, 0.5% Nonidet P 40, 200 µg/ml BSA and 50% Glycerol

Unit Definition
One unit is defined as the amount of enzyme required to incorporate 10 nmoles of dNTPs into acid-insoluble form at 74°C in 30 minutes under the stated assay conditions.

Enzyme Stability
Each lot of DNA polymerase is tested for stability under normal storage conditions (-20°C). Enzyme stability is monitored at regular intervals for a two year period after the original assay date.

Appendix 1E

pENTR/D-TOPO Cloning Instruction Manual (Taken from Invitrogen Website)

pENTR™ Directional TOPO® Cloning Kits

Five-minute, directional TOPO® Cloning of blunt-end PCR products into an entry vector for the Gateway® System

Catalog nos. K2400-20, K2420-20, K2525-20, K2535-20, K2435-20, and K2635-20

Version G
6 April 2006
25-0434

A Limited Use Label License covers this product (see Purchaser Notification). By use of this product, you accept the terms and conditions of the Limited Use Label License.

User Manual

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits.

Kit	Size	Catalog no.
pENTR™/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2400-20
with One Shot® Mach1™-T1R Chemically Competent E. coli	20 reactions	K2435-20
pENTR™/SD/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2420-20
with One Shot® Mach1™-T1R Chemically Competent E. coli	20 reactions	K2635-20
pENTR™/TEV/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2525-20
with One Shot® Mach1™-T1R Chemically Competent E. coli	20 reactions	K2535-20

Shipping/Storage

Each pENTR™ Directional TOPO® Cloning Kit is shipped on dry ice. Each kit contains two boxes as described below. Upon receipt, store the boxes as detailed below.

Box	Item	Storage
1	pENTR™ TOPO® Reagents	-20°C
2	One Shot® Chemically Competent *E. coli*	-80°C

continued on next page

Kit Contents and Storage, continued

One Shot® Reagents

The following reagents are included with the One Shot® TOP10 or Mach1™-T1R Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is ≥ 1 x 10^9 cfu/µg plasmid DNA. Store Box 2 at -80°C.

Reagent	Composition	Amount
S.O.C. Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 or Mach1™-T1R cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of *E. coli* Strains

TOP10: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 ara*D139 Δ(*ara-leu*)7697 *galU galK rpsL* (Str^R) *end*A1 *nupG*

Mach1™-T1R: F- Φ80*lacZ*ΔM15 Δ*lacX74 hsdR*(r_k^-, m_k^+) Δ*recA*1398 *endA1 tonA* (confers resistance to phage T1)

Information for Non-U.S. Customers Using Mach1™-T1R Cells

The parental strain of Mach1™-T1R *E. coli* is the non-K-12, wild-type W strain (ATCC #9637, S.A. Waksman). Although the parental strain is generally classified as Biosafety Level 1 (BL-1), we recommend that you consult the safety department of your institution to verify the Biosafety Level.

Kit Contents and Storage, continued

pENTR™ TOPO® Reagents

The following reagents are supplied with each pENTR™ TOPO® vector (Box 1). Note that the user must supply a thermostable, proofreading polymerase and the appropriate PCR buffer.

Store Box 1 at -20°C.

Item	Concentration	Amount
pENTR™ TOPO® vector, TOPO®-adapted (pENTR™/D-TOPO® or pENTR™/SD/D-TOPO® or pENTR™/TEV/D-TOPO®)	15-20 ng/µl linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM bromophenol blue	20 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP in water, pH 8	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Water	---	1 ml
M13 Forward (-20) Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
M13 Reverse Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
Control PCR Primers	0.1 µg/µl each in TE Buffer, pH 8	10 µl
Control PCR Template	0.1 µg/µl in TE Buffer, pH 8	10 µl

Sequences of the Primers

The table below provides the sequences of the M13 Forward (-20) and M13 Reverse sequencing primers.

Primer	Sequence	pMoles Supplied
M13 Forward (-20)	5′-GTAAAACGACGGCCAG-3′	407
M13 Reverse	5′-CAGGAAACAGCTATGAC-3′	385

continued on next page

Introduction

Overview

Introduction

The pENTR™ Directional TOPO® Cloning Kits utilize a highly efficient, 5-minute cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a vector for entry into the Gateway® System or the MultiSite Gateway® System available from Invitrogen. Blunt-end PCR products clone directionally at greater than 90% efficiency, with no ligase, post-PCR procedures, or restriction enzymes required.

A choice of pENTR™ Directional TOPO® vectors is available for optimal expression of your PCR product after recombination with the Gateway® destination vector of interest (see table below).

Vector	Benefit
pENTR™/D-TOPO®	For efficient expression of your gene of interest after recombination with a Gateway® destination vector
pENTR™/SD/D-TOPO®	Contains a T7 gene 10 translational enhancer and a ribosome binding site (RBS) for optimal expression of native protein after recombination with a prokaryotic Gateway® destination vector Note: Also suitable for efficient expression of your gene of interest in other hosts after recombination with a Gateway® destination vector (*e.g.* mammalian, insect, yeast)
pENTR™/TEV/D-TOPO®	Contains a Tobacco Etch Virus (TEV) recognition site for efficient TEV protease-dependent cleavage of an N-terminal tag from your recombinant protein after recombination and expression from a Gateway® destination vector

The Gateway® Technology

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest using the Gateway® Technology, simply:

1. TOPO® Clone your blunt-end PCR product into one of the pENTR™ TOPO® vectors to generate an entry clone.
2. Generate an expression construct by performing an LR recombination reaction between the entry clone and a Gateway® destination vector of choice.
3. Introduce your expression construct into the appropriate host (*e.g.* bacterial, mammalian, yeast, insect) and express your recombinant protein.

For more information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual which is available for downloading from www.invitrogen.com or by contacting Technical Service (see page 35).

continued on next page

Overview, continued

MultiSite Gateway® Technology

The MultiSite Gateway® Technology uses modifications of the site-specific recombination reactions of the Gateway® Technology (see the previous page) to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation. The MultiSite Gateway® Three-Fragment Vector Construction Kit available from Invitrogen (Catalog no. 12537-023) facilitates simultaneous cloning of DNA fragments in three entry vectors to create your own expression clone. For more information about the MultiSite Gateway® Technology and the MultiSite Gateway® Three-Fragment Vector Construction Kit, refer to the MultiSite Gateway® Three-Fragment Vector Construction Kit manual which is available for downloading from our Web site or by contacting Technical Service.

Features of the pENTR™ TOPO® Vectors

The pENTR™/D-TOPO®, pENTR™/SD/D-TOPO®, and pENTR™/TEV/D-TOPO® vectors are designed to facilitate rapid, directional TOPO® Cloning of blunt-end PCR products for entry into the Gateway® System. Features of the vectors include:

- *att*L1 and *att*L2 sites for site-specific recombination of the entry clone with a Gateway® destination vector
- T7 gene 10 translation enhancer and ribosome binding site for efficient translation of the PCR product in prokaryotes (pENTR™/SD/D-TOPO® only)
- TEV recognition site for TEV protease-dependent cleavage of an N-terminal tag from your recombinant protein (pENTR™/TEV/D-TOPO® only)
- Directional TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products (see page 3 for more information)
- *rrn*B transcription termination sequences to prevent basal expression of the PCR product of interest in *E. coli*
- Kanamycin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*

How Directional TOPO® Cloning Works

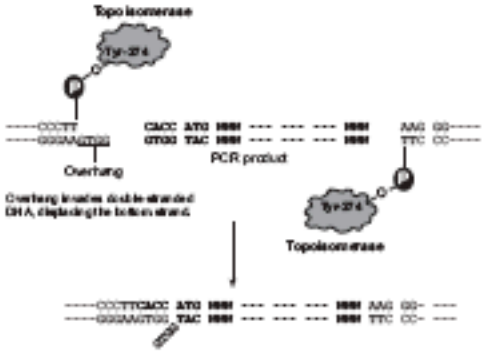
How Topoisomerase I Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites (CCCTT; see Note below) and cleaves the phosphodiester backbone in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products.

Directional TOPO® Cloning

Directional joining of double-strand DNA using TOPO®-charged oligonucleotides occurs by adding a 3' single-stranded end (overhang) to the incoming DNA (Cheng and Shuman, 2000). This single-stranded overhang is identical to the 5' end of the TOPO®-charged DNA fragment. At Invitrogen, this idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO®-charged DNA and adapting it to a 'whole vector' format.

In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5' end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



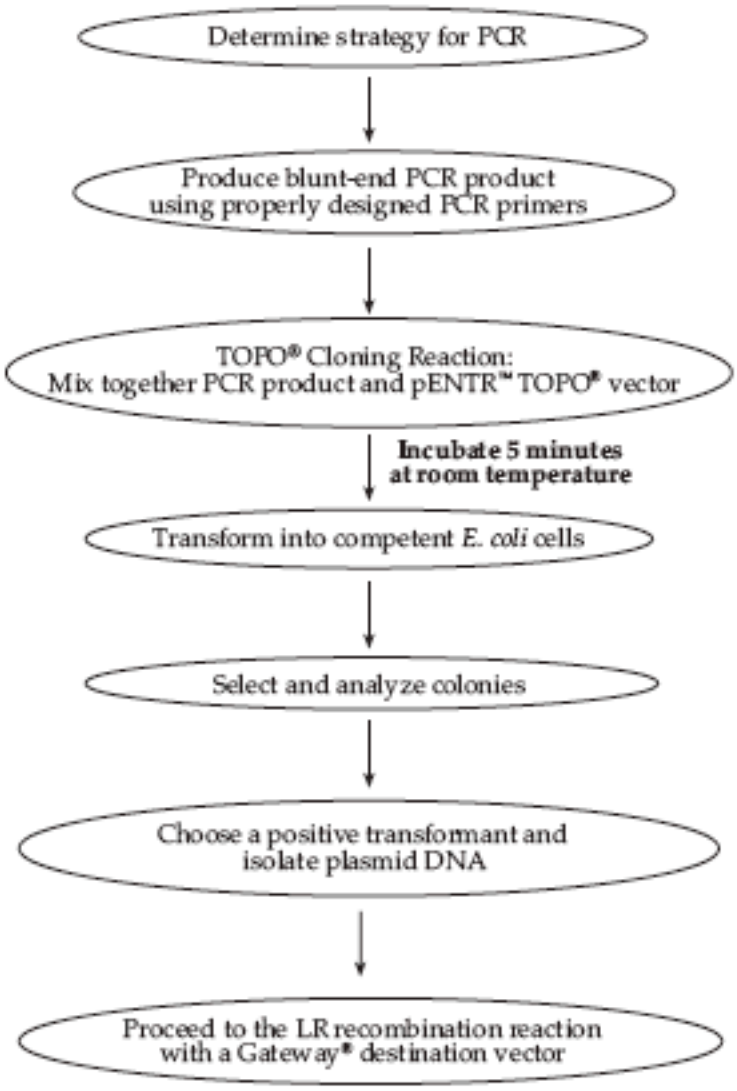
Note

The 5' TOPO® recognition site in pENTR™/TEV/D-TOPO® is encoded by the sequence TCCTT rather than CCCTT. This is because the 5' TOPO® recognition site directly follows the TEV recognition site, and studies have shown that TEV protease does not cleave efficiently if the first amino acid following the TEV recognition sequence is proline (Kapust *et al.*, 2002) as would be the case if the 5' TOPO® recognition site was encoded by CCCTT. By changing the sequence of the 5' TOPO® recognition site to TCCTT, the first amino acid following the TEV recognition site is now serine. This change does not affect TOPO® Cloning efficiency and allows efficient TEV cleavage.

Experimental Outline

Flow Chart

The flow chart below describes the general steps required to produce and clone your blunt-end PCR product.



Methods

Designing PCR Primers

Designing Your PCR Primers

The design of the PCR primers to amplify your gene of interest is critical for expression. Depending on the pENTR™ TOPO® vector you are using, consider the following when designing your PCR primers.

- Sequences required to facilitate directional cloning
- Sequences required for proper translation initiation of your PCR product
- Whether or not you wish your PCR product to be fused in frame with an N- or C-terminal tag after recombination of your entry clone with a Gateway® destination vector

Guidelines to Design the Forward PCR Primer

When designing your forward PCR primer, consider the following points below. Refer to pages 8-9 for diagrams of the TOPO® Cloning site for pENTR™/D-TOPO®, pENTR™/SD/D-TOPO®, and pENTR™/TEV/D-TOPO®.

- To enable directional cloning, the forward PCR primer must contain the sequence, CACC, at the 5′ end of the primer. The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in each pENTR™ TOPO® vector.
- If you plan to express your PCR product in mammalian cells as a native or C-terminal fusion-tagged protein (following recombination of the entry clone with a Gateway® destination vector), your sequence of interest should include a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is (G/A)NNA**T**GG. Other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is underlined.

  Note: If your sequence of interest does not contain an initiation codon within the context of a Kozak sequence, design the forward PCR primer to contain a Kozak sequence at the 5′ end of the primer (see Example on the next page).
- If you plan to express your PCR product in mammalian cells as an N-terminal fusion-tagged protein (following recombination of the entry clone with a Gateway® destination vector), your sequence of interest does not need to contain a Kozak translation initiation sequence. A Kozak sequence is provided by the appropriate destination vector. Note: In this case, internal initiation may occur if your PCR product contains an endogenous Kozak sequence.
- If you plan to express your PCR product in prokaryotic cells without an N-terminal fusion tag (following recombination of the entry clone with a Gateway® destination vector), you should TOPO® Clone your PCR product into pENTR™/SD/D-TOPO®. pENTR™/SD/D-TOPO® contains a T7 gene 10 translational enhancer and a ribosome binding site (RBS) to enable efficient translation of the PCR product in *E. coli*. **To ensure optimal spacing for proper translation, design your forward PCR primer so that the ATG initiation codon of your PCR product directly follows the CACC necessary for directional cloning** (see Example on the next page).

continued on next page

Designing PCR Primers, continued

Example of Forward Primer Design

Below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer. The ATG initiation codon is underlined.

DNA sequence: 5′-ATG GGA TCT GAT AAA

Proposed Forward PCR primer: 5′-C ACC ATG GGA TCT GAT AAA

If you design the forward PCR primer as noted above, then:

- The ATG initiation codon falls within the context of a Kozak sequence (see boxed sequence), allowing proper translation initiation of the PCR product in mammalian cells.
- The ATG initiation codon is properly spaced from the RBS (in pENTR™/SD/D-TOPO® only), allowing proper translation of the PCR product in prokaryotic cells.

Note

The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a functional codon.

Guidelines to Design the Reverse Primer

When designing your reverse PCR primer, consider the following points below. Refer to pages 8-9 for diagrams of the TOPO® Cloning site for pENTR™/D-TOPO®, pENTR™/SD/D-TOPO®, and pENTR™/TEV/D-TOPO®.

- **To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer MUST NOT be complementary to the overhang sequence GTGG at the 5′ end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 50%, increasing the likelihood of your ORF cloning in the opposite orientation (see Example #1 on the next page).** We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch.
- If you wish to fuse your PCR product in frame with a C-terminal tag (following recombination of the entry clone with a Gateway® destination vector), then design the reverse PCR primer to remove the native stop codon in the gene of interest (see Example #2 on the next page).
- If you do not wish to fuse your PCR product in frame with a C-terminal tag (following recombination of the entry clone with a Gateway® destination vector), then include the native sequence containing the stop codon in the reverse primer or make sure the stop codon is upstream from the reverse PCR primer binding site (see Example #2 on the next page).

continued on next page

Designing PCR Primers, continued

Example #1 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. You want to fuse the protein in frame with a C-terminal tag (following recombination of the entry clone with a Gateway® destination vector). The stop codon is underlined.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′

One solution is to design the reverse PCR primer to start with the codon just upstream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4 bp overhang sequence. As a result, the reverse primer will be complementary to the 4 bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′

Proposed Reverse PCR primer sequence: TG AGC TGC TGC CAC AAA-5′

Another solution is to design the reverse primer so that it hybridizes just downstream of the stop codon, but still includes the C-terminus of the ORF. Note that you will need to replace the stop codon with a codon for an innocuous amino acid such as glycine, alanine, or lysine.

Example #2 of Reverse Primer Design

Below is the sequence for the C-terminus of a theoretical protein. The stop codon is underlined.

...GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TAG-3′

- To fuse the ORF in frame with a C-terminal tag (supplied by the destination vector after recombination), remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream. The reverse primer will be:

  5′-TGC AGT CGT CGA GTG CTC CGA CTT-3′

  This will amplify the C-terminus without the stop codon and allow you to join the ORF in frame with a C-terminal tag.

- If you don't want to join the ORF in frame with a C-terminal tag, simply design the reverse primer to include the stop codon.

  5′-CTA TGC AGT CGT CGA GTG CTC CGA CTT-3′

Important

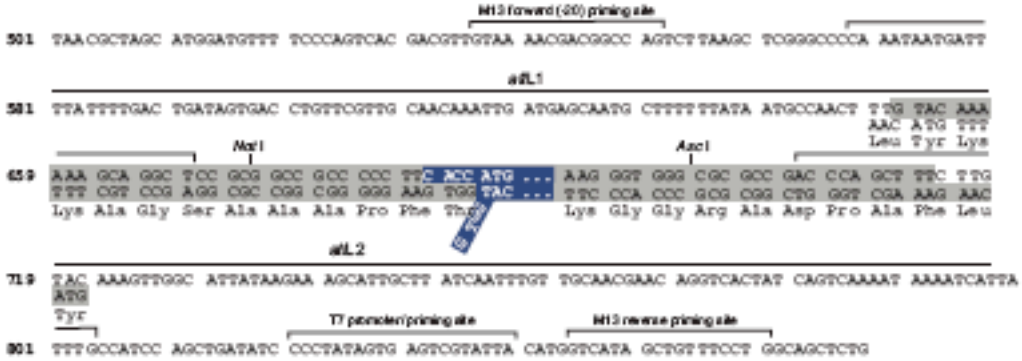
- Remember that the pENTR™ TOPO® vectors accept blunt-end PCR products.
- Do not add 5′ phosphates to your primers for PCR. This will prevent ligation into the pENTR™ TOPO® vectors.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).

continued on next page

Designing PCR Primers, continued

TOPO® Cloning Site for pENTR™/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pENTR™/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The shaded region corresponds to the DNA sequences that will be transferred from the entry clone into the destination vector following LR recombination. The sequence of pENTR™/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35). For more information about pENTR™/D-TOPO®, see pages 28-29.



TOPO® Cloning Site for pENTR™/SD/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pENTR™/SD/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The shaded region corresponds to the DNA sequences that will be transferred from the entry clone into the destination vector following LR recombination. The sequence of pENTR™/SD/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35). For more information about pENTR™/SD/D-TOPO®, see pages 30-31.

continued on next page

Producing Blunt-End PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, produce your blunt-end PCR product using any thermostable, proofreading polymerase. Follow the guidelines below to produce your blunt-end PCR product.

Materials Supplied by the User

You will need the following reagents and equipment for PCR. Note: dNTPs (adjusted to pH 8) are provided in the kit.

- Thermocycler and thermostable, proofreading polymerase
- 10X PCR buffer appropriate for your polymerase
- DNA template and primers to produce the PCR product

Producing Blunt-End PCR Products

Set up a 25 µl or 50 µl PCR reaction using the guidelines below.

- Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
- Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended.
- After cycling, place the tube on ice or store at –20°C for up to 2 weeks. Proceed to **Checking the PCR Product**, below.

Checking the PCR Product

After you have produced your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following outcomes below.

- Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations to optimize your PCR with the polymerase of your choice. Alternatively, you may gel-purify the desired product (see pages 26-27).
- Estimate the concentration of your PCR product. You will use this information when setting up your TOPO® Cloning reaction (see **Amount of PCR Product to Use in the TOPO® Cloning Reaction**, next page for details).

Setting Up the TOPO® Cloning Reaction

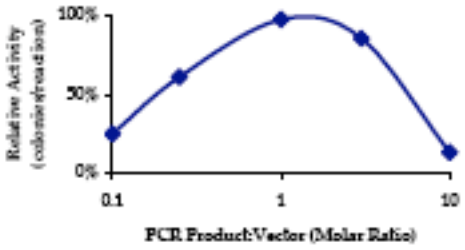
Introduction

Once you have produced the desired blunt-end PCR product, you are ready to TOPO® Clone it into the pENTR™ TOPO® vector and transform the recombinant vector into One Shot® competent *E. coli*. You should have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read this section and the section entitled **Transforming One Shot® Competent *E. coli*** (pages 13-14) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 23-25 in parallel with your samples.

Amount of PCR Product to Use in the TOPO® Cloning Reaction

When performing directional TOPO® Cloning, we have found that the molar ratio of PCR product:TOPO® vector used in the reaction is critical to its success. To obtain the highest TOPO® Cloning efficiency, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector (see figure below). Note that the TOPO® Cloning efficiency decreases significantly if the ratio of PCR product: TOPO® vector is <0.1:1 or >5:1 (see figure below). These results are generally obtained if too little PCR product is used (*i.e.* PCR product is too dilute) or if too much PCR product is used in the TOPO® Cloning reaction. If you have quantitated the yield of your PCR product, you may need to adjust the concentration of your PCR product before proceeding to TOPO® Cloning.

Tip: For pENTR™ TOPO® vectors, using 1-5 ng of a 1 kb PCR product or 5-10 ng of a 2 kb PCR product in a TOPO® Cloning reaction generally results in a suitable number of colonies.



continued on next page

Setting Up the TOPO® Cloning Reaction, continued

Using Salt Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page x for ordering information).

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO® Cloning reaction as directed below.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction must be reduced to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 200 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed below.

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli*. Reminder: For optimal results, be sure to use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector in your TOPO® Cloning reaction.

Note: The blue color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagents*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution (1:4)	--	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl
Final volume	6 µl	6 µl

*Store all reagents at –20°C when finished. Salt solution and water can be stored at room temperature or +4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield a sufficient number of colonies for analysis. Depending on your needs, 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) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® Competent *E. coli*,** next page.

   Note: You may store the TOPO® Cloning reaction at –20°C overnight.

Transforming One Shot® Competent *E. coli*

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pENTR™ TOPO® construct into competent *E. coli*. One Shot® TOP10 or Mach1™-T1^R Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation, however, you may also transform electrocompetent cells (see page x for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Materials Needed

In addition to general microbiological supplies (*i.e.* plates, spreaders), you will need the following reagents and equipment:

- TOPO® Cloning reaction (from Step 2, previous page)
- One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli* (supplied with the kit, Box 2)
- S. O.C. Medium (supplied with the kit, Box 2)
- pUC19 positive control (to verify transformation efficiency, if desired, Box 2)
- 42°C water bath (or electroporator with cuvettes, optional)
- 15 ml sterile, snap-cap plastic culture tubes (for electroporation only)
- LB plates containing 50 µg/ml kanamycin (two for each transformation)
- LB plates containing 100 µg/ml ampicillin (if transforming pUC19 control)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned in the correct orientation. Sequencing primers are included in the kit to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparing for Transformation

For each transformation, you will need one vial of One Shot® competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. Medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw on ice one vial of One Shot® cells from Box 2 for each transformation.

continued on next page

Transforming One Shot® Competent *E. coli*, continued

One Shot® Chemical Transformation Protocol

Use the following protocol to transform One Shot® TOP10 or Mach1™-T1® chemically competent *E. coli*.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 12 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**

   Note: If you are transforming the pUC19 control plasmid, use 10 pg (1 µl).
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. Medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 50-200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick 5-10 colonies for analysis (see **Analyzing Transformants**, page 16).

Transformation by Electroporation

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 or Mach1™-T1® chemically competent cells for electroporation.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 12 into a sterile microcentrifuge tube containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the cells to a 0.1 cm cuvette.
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see the next page.
3. Immediately add 250 µl of room temperature S.O.C. Medium.
4. Transfer the solution to a 15 ml snap-cap tube (*i.e.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the kanamycin resistance gene.
5. Spread 20-100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. Medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick 5-10 colonies for analysis (see **Analyzing Transformants**, page 16).

continued on next page

Transforming One Shot® Competent *E. coli*, continued

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5-10 colonies and culture them overnight in LB or SOB medium containing 50-100 µg/ml kanamycin.

   Note: If you transformed One Shot® MachI™-T1R competent *E. coli*, you may inoculate overnight-grown colonies and culture them for only 4 hours in pre-warmed LB medium containing 50 µg/ml kanamycin before isolating plasmid DNA. For optimal results, inoculate as much of a single colony as possible.

2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using Invitrogen's PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).

3. Analyze the plasmids by restriction analysis or PCR (see below) to confirm the presence and correct orientation of the insert.

Analyzing Transformants by PCR

Use the protocol below (or any other suitable protocol) to analyze positive transformants using PCR. For PCR primers, use a combination of the M13 Forward (-20) primer or the M13 Reverse primer and a primer that hybridizes within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template.

Materials Needed:

- PCR Super Mix High Fidelity (Invitrogen, Catalog no. 10790-020)
- Appropriate forward and reverse PCR primers (20 µM each)

Procedure:

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 5-10 colonies and resuspend them individually in 50 µl of the PCR SuperMix containing PCR primers (remember to make a patch plate to preserve the colonies for further analysis).
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

Sequencing

Once you have identified the correct clone(s), you may sequence your construct to confirm that your gene is cloned in the correct orientation. Use the M13 Forward (-20) and M13 Reverse included to help you sequence your insert (see the diagrams on pages 8-9 for the location of the priming sites in each pENTR™ TOPO® vector). For the complete sequence of each pENTR™ TOPO® vector, see our Web site (www.invitrogen.com) or call Technical Service (see page 35).

Note: The M13 Forward (-20) and M13 Reverse primers are available separately from Invitrogen (see page x for ordering information).

continued on next page

Analyzing Transformants, continued

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on page 23-25 or refer to the Troubleshooting section, page 21 for tips to help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colony on LB plates containing 50 µg/ml kanamycin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml kanamycin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Troubleshooting

TOPO® Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions (see pages 23-25) in parallel with your samples.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction and the transformation control gave colonies	Suboptimal ratio of PCR product:TOPO® vector used in the TOPO® Cloning reaction	Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Too much PCR product used in the TOPO® Cloning reaction	• Dilute the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence, CACC, at the 5′ end. • Make sure that the reverse PCR primer does not contain the sequence, CACC, at the 5′ end.
	Used *Taq* polymerase or a *Taq*/proofreading polymerase mixture for PCR	Use a proofreading polymerase for PCR.
	Large PCR product	• Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector. • Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Gel-purify the PCR product to remove primer-dimers and other artifacts.
	PCR reaction contains artifacts (*i.e.* does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of your choice. • Gel-purify your PCR product.

continued on next page

TOPO® Cloning Reaction and Transformation, continued

Problem	Reason	Solution
Few or no colonies obtained from sample reaction and the transformation control gave colonies, continued	Cloning large pool of PCR products or a toxic gene	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Incomplete extension during PCR	Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Large percentage of inserts cloned in the incorrect orientation	Incorrect PCR primer design	Make sure that the forward PCR primer contains the sequence, CACC, at the 5' end.
	Reverse PCR primer is complementary to the GTGG overhang at the 5' end	Make sure that the reverse PCR primer does not contain the sequence, CACC, at the 5' end.
Large number of incorrect inserts cloned	PCR cloning artifacts	• Gel-purify your PCR product to remove primer-dimers and smaller PCR products. • Optimize your PCR. • Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
	Incorrect PCR primer design	Make sure that the forward and reverse PCR primers are designed correctly.
Few or no colonies obtained from sample reaction and the transformation control gave no colonies	One Shot® competent *E. coli* stored incorrectly	Store One Shot® competent *E. coli* at -80°C. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add S.O.C. Medium and incubate the transformation mixture for 1 hour at 37°C before plating.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection.

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using this product directly in a TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 50 µg/ml kanamycin.

Producing the Control PCR Product

Use your thermostable, proofreading polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer's recommendations for the proofreading polymerase you are using.

1. To produce the 750 bp control PCR product, set up the following 50 µl PCR:

Component	Amount
Control DNA Template (100 ng)	1 µl
10X PCR Buffer (appropriate for enzyme)	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile water	41.5 µl
Proofreading polymerase (1-2.5 U/µl)	1 µl
Total volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil, if required.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. Make sure that you see a single, discrete 750 bp band.
5. Estimate the concentration of the PCR product, and adjust as necessary such that the amount of PCR product used in the control TOPO® Cloning reaction results in an optimal molar ratio of PCR product:TOPO® vector (*i.e.* 0.5:1 to 2:1). Proceed to the Control TOPO® Cloning Reactions, next page.

continued on next page

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pENTR™ TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below. If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in place of the Salt Solution.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	–	1 µl
pENTR™/D-TOPO® vector	1 µl	1 µl
Total volume	6 µl	6 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® competent cells using the protocol on page 14.
4. Spread 50-200 µl of each transformation mix onto LB plates containing 50 µg/ml kanamycin. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
5. Incubate overnight at 37°C.

continued on next page

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. To analyze the transformations, isolate plasmid DNA and digest with the appropriate restriction enzyme as listed below. The table below lists the digestion patterns that you should see for inserts that are cloned in the correct orientation or in the reverse orientation.

Vector	Restriction Enzyme	Expected Digestion Patterns (bp)
pENTR™/D-TOPO®	*Not* I	Correct orientation: 127, 3203 Reverse orientation: 646, 2684 Empty vector: 2580
pENTR™/SD/D-TOPO®	*Not* I	Correct orientation: 148, 3203 Reverse orientation: 667, 2684 Empty vector: 2601
pENTR™/TEV/D-TOPO®	*EcoR* V/*Pst* I	Correct orientation: 757, 2602 Reverse orientation: 250, 3109 Empty vector: 2610

Greater than 90% of the colonies should contain the 750 bp insert in the correct orientation.

Relatively few colonies should be produced in the vector-only reaction.

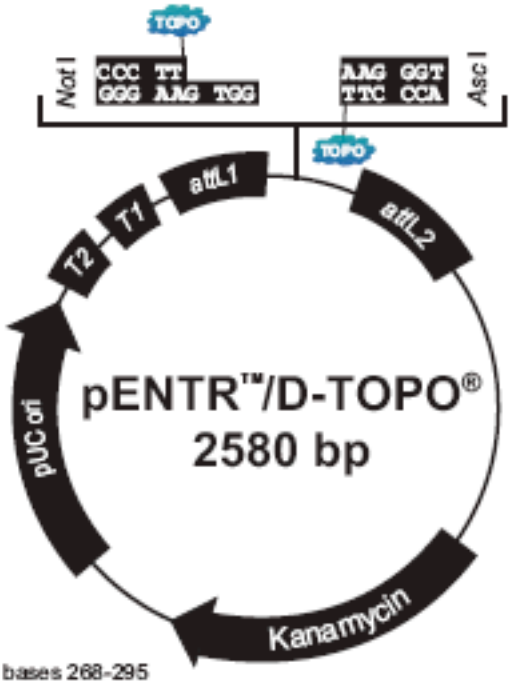
Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform one vial of One Shot® competent cells with 10 pg of pUC19 using the protocol on page 14. Plate 10 µl of the transformation mixture plus 20 µl of S.O.C. Medium on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ≥ 1 x 10^9 cfu/µg DNA.

Map and Features of pENTR™/D-TOPO®

pENTR™/D-TOPO® Map

The figure below shows the features of pENTR™/D-TOPO® vector. The complete sequence of pENTR™/D-TOPO® is available for downloading from www.invitrogen.com)or by contacting Technical Service (see page 35).



Comments for pENTR™/D-TOPO®
2580 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
attL1: bases 569-668 (c)
TOPO® recognition site 1: bases 680-684
Overhang: bases 685-688
TOPO® recognition site 2: bases 689-693
attL2: bases 705-804
T7 Promoter/priming site: bases 821-840 (c)
M13 reverse priming site: bases 845-861
Kanamycin resistance gene: bases 974-1783
pUC origin: bases 1904-2577

(c) = complementary sequence

continued on next page

Map and Features of pENTR™/D-TOPO®, continued

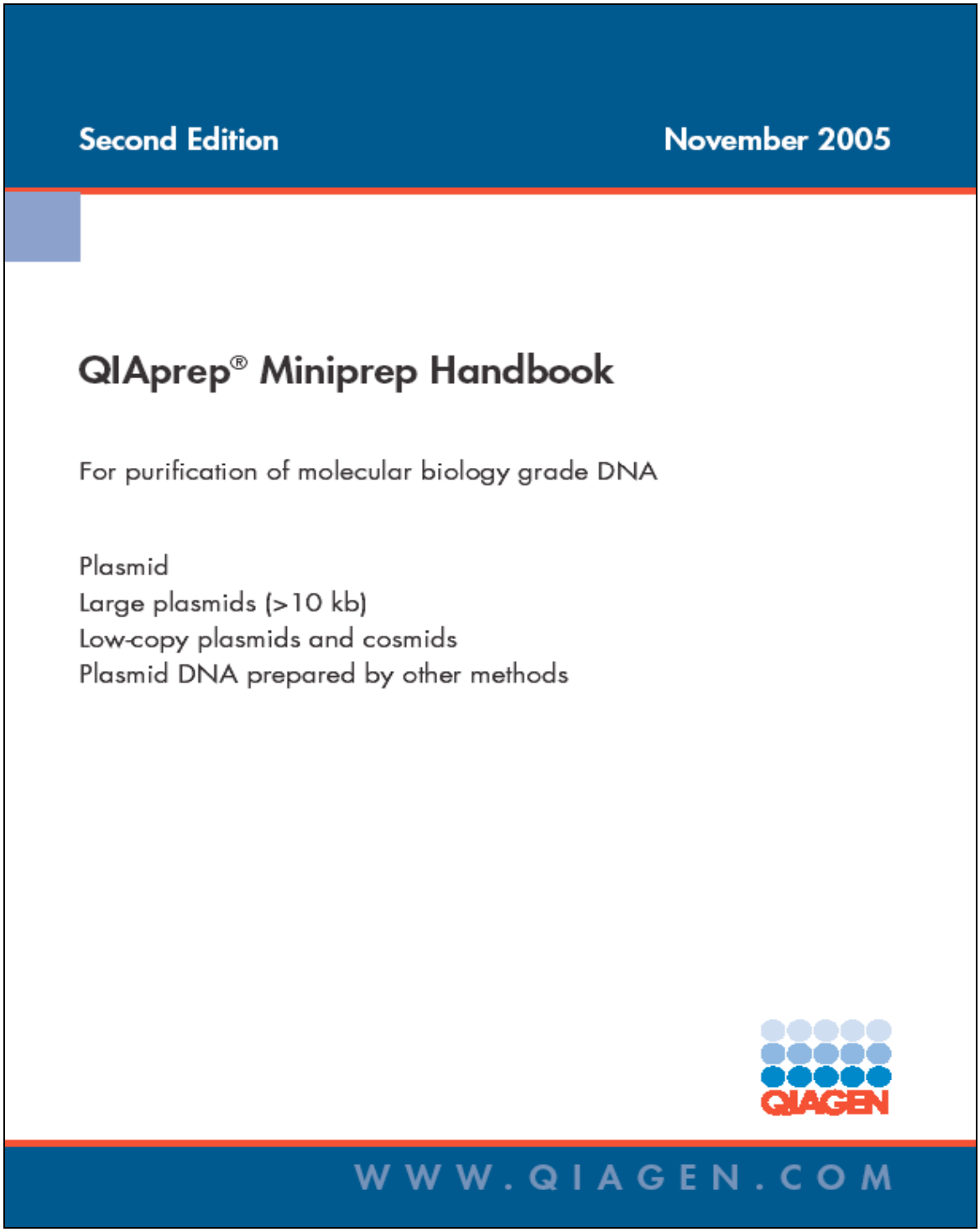
Features of pENTR™/D-TOPO®

pENTR™/D-TOPO® (2580 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription termination sequences	Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product.
M13 forward (-20) priming site	Allows sequencing of the insert.
attL1 and *attL2* sites	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the entry construct with a Gateway® destination vector (Landy, 1989).
TOPO® Cloning site (directional)	Allows rapid, directional cloning of your PCR product.
T7 promoter/priming site	Allows *in vitro* transcription, and sequencing of the insert.
M13 reverse priming site	Allows sequencing of the insert.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Allows high-copy replication and maintenance in *E. coli*.

Appendix 1F

QIAprep Miniprep Handbook (Taken from Qiagen Website)



Kit Contents

QIAprep Spin Miniprep Kit	(50)	(250)
Catalog no.	**27104**	**27106**
QIAprep Spin Columns	50	250
Buffer P1	20 ml	70 ml
Buffer P2	20 ml	70 ml
Buffer N3*	30 ml	140 ml
Buffer PB*	30 ml	150 ml
Buffer PE (concentrate)	2 x 6 ml	55 ml
Buffer EB	15 ml	55 ml
LyseBlue	20 ml	70 ml
RNase A†	200 µl	700 µl
Collection Tubes (2 ml)	50	250
Handbook	1	1

Storage

QIAprep Miniprep Kits should be stored dry at room temperature (15–25°C). Kits can be stored for up to 12 months without showing any reduction in performance and quality. For longer storage these kits can be kept at 2–8°C. If any precipitate forms in the buffers after storage at 2–8°C it should be redissolved by warming the buffers to 37°C before use.

After addition of RNase A and optional LyseBlue reagent, Buffer P1 is stable for 6 months when stored at 2–8°C. RNase A stock solution can be stored for two years at room temperature.

Quality Control

In accordance with QIAGEN's ISO-certified Total Quality Management System, each lot of QIAprep Miniprep Kit is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

QIAprep Miniprep Kits are developed, designed, and sold for research purposes only. They are not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department. We will credit your account or exchange the product — as you wish.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffers N3 and PB contain guanidine hydrochloride, which can form highly reactive compounds when combined with bleach.

If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

The following risk and safety phrases apply to QIAprep Miniprep Kits.

Buffer N3

Contains guanidine hydrochloride, acetic acid: harmful, irritant. Risk and safety phrases:* R22-36/38, S13-23-26-36/37/39-46.

Buffer P2

Contains sodium hydroxide: irritant. Risk and safety phrases:* R36/38, S13-26-36-46.

Buffer PB

Contains guanidine hydrochloride, isopropanol: harmful, flammable, irritant. Risk and safety phrases:* R10-22-36/38, S13-23-26-36/37/39-46

RNase A

Contains ribonuclease: sensitizer. Risk and safety phrases:* R42/43, S23-24-26-36/37.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

* R10: Flammable; R22: Harmful if swallowed; R36/38: Irritating to eyes and skin; R42/43: May cause sensitization by inhalation and skin contact; S13: Keep away from food, drink, and animal feedingstuffs; S23; Do not breathe spray; S24: Avoid contact with skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S36/37: Wear suitable protecting clothing and gloves; S36/37/39: Wear suitable protective clothing, gloves and eye/face protection; S46: If swallowed seek medical advice immediately and show the container or label.

Introduction

The QIAprep Miniprep system provides a fast, simple, and cost-effective plasmid miniprep method for routine molecular biology laboratory applications. QIAprep Miniprep Kits use silica membrane technology to eliminate the cumbersome steps associated with loose resins or slurries. Plasmid DNA purified with QIAprep Miniprep Kits is immediately ready for use. Phenol extraction and ethanol precipitation are not required, and high-quality plasmid DNA is eluted in a small volume of Tris buffer (included in each kit) or water. The QIAprep system consists of four products with different handling options to suit every throughput need.

Low throughput

The **QIAprep Spin Miniprep Kit** is designed for quick and convenient processing of 1–24 samples simultaneously in less than 30 minutes. QIAprep spin columns can be used in a microcentrifuge or on any vacuum manifold with luer connectors (e.g., QIAvac 24 Plus, or QIAvac 6S with QIAvac Luer Adapters).

Medium throughput

For medium throughput requirements the **QIAprep 8 Miniprep Kit and QIAprep 8 Turbo Miniprep Kit** utilize 8-well strips on QIAvac 6S allowing up to 48 minipreps to be performed simultaneously in approximately 40 and 30 minutes respectively. In addition, the **QIAprep 8 Turbo BioRobot® Kit** enables automated purification of up to 48 minipreps in 50 minutes on BioRobot systems.

High throughput

The **QIAprep 96 Turbo Miniprep Kit** enables up to 96 minipreps to be performed simultaneously in less than 45 minutes on the QIAvac 96. For automated high-throughput plasmid purification the **QIAprep 96 Turbo BioRobot Kit** enables up to 96 minipreps to be processed in 70 minutes.

Applications using QIAprep purified DNA

Plasmid DNA prepared using the QIAprep system is suitable for a variety of routine applications including:

- Restriction enzyme digestion
- Library screening
- In vitro translation
- Sequencing
- Ligation and transformation
- Transfection of robust cells

Principle

The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt (1). The unique silica membrane used in QIAprep Miniprep Kits completely replaces glass or silica slurries for plasmid minipreps.

The procedure consists of three basic steps:

- Preparation and clearing of a bacterial lysate
- Adsorption of DNA onto the QIAprep membrane
- Washing and elution of plasmid DNA

All steps are performed without the use of phenol, chloroform, CsCl, ethidium bromide, and without alcohol precipitation.

Preparation and clearing of bacterial lysate

The QIAprep miniprep procedure uses the modified alkaline lysis method of Birnboim and Doly (2). Bacteria are lysed under alkaline conditions, and the lysate is subsequently neutralized and adjusted to high-salt binding conditions in one step. After lysate clearing, the sample is ready for purification on the QIAprep silica membrane. For more details on growth of bacterial cultures and alkaline lysis, please refer to Appendix A on pages 39–42. In the QIAprep Spin and QIAprep 8 miniprep procedures, lysates are cleared by centrifugation, while the QIAprep 8 and 96 Turbo Miniprep kits provide TurboFilter strips or plates for lysate clearing by filtration.

LyseBlue reagent*

Use of LyseBlue is optional and is not required to successfully perform plasmid preparations. See "Using LyseBlue reagent" on page 14 for more information.

* LyseBlue reagent is only supplied with QIAprep Spin Miniprep Kits since multiwell or automated formats do not allow visual control of individual samples.

LyseBlue is a color indicator which provides visual identification of optimum buffer mixing. This prevents common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. This makes LyseBlue ideal for use by researchers who have not had much experience with plasmid preparations as well as experienced scientists who want to be assured of maximum product yield.

DNA adsorption to the QIAprep membrane

QIAprep columns, strips, and plates use a silica membrane for selective adsorption of plasmid DNA in high-salt buffer and elution in low-salt buffer. The optimized buffers in the lysis procedure, combined with the unique silica membrane, ensure that only DNA will be adsorbed, while RNA, cellular proteins, and metabolites are not retained on the membrane but are found in the flow-through.

Washing and elution of plasmid DNA

Endonucleases are efficiently removed by a brief wash step with Buffer PB. This step is essential when working with *endA*+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, to ensure that plasmid DNA is not degraded. The Buffer PB wash step is also necessary when purifying low-copy plasmids, where large culture volumes are used.

Salts are efficiently removed by a brief wash step with Buffer PE. High-quality plasmid DNA is then eluted from the QIAprep column with 50–100 µl of Buffer EB or water. The purified DNA is ready for immediate use in a range of applications — no need to precipitate, concentrate, or desalt.

Note: Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water for elution, make sure that the pH value is within this range. Store DNA at –20°C when eluted with water since DNA may degrade in the absence of a buffering agent.

DNA yield

Plasmid yield with the QIAprep miniprep system varies depending on plasmid copy number per cell (see page 39), the individual insert in a plasmid, factors that affect growth of the bacterial culture (see pages 39–42), the elution volume (Figure 1), and the elution incubation time (Figure 2). A 1.5 ml overnight culture can yield from 5 to 15 µg of plasmid DNA (Table 1, page 14). To obtain the optimum combination of DNA quality, yield, and concentration, we recommend using Luria-Bertani (LB) medium for growth of cultures (for composition see page 41), eluting plasmid DNA in a volume of 50 µl, and performing a short incubation after addition of the elution buffer.

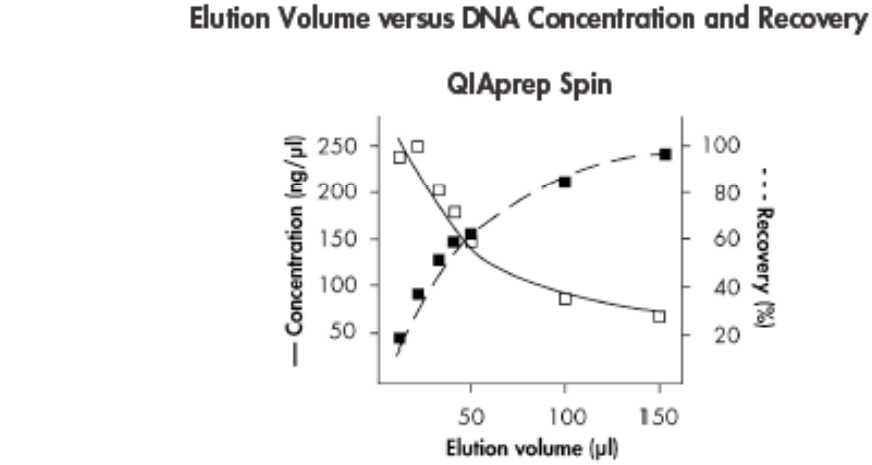


Figure 1 10 µg pUC18 DNA was purified using the QIAprep Spin protocol and eluted with the indicated volumes of Buffer EB. The standard protocol uses 50 µl Buffer EB for elution, since this combines high yield with high concentration. However the yield can be increased by increasing the elution volume.

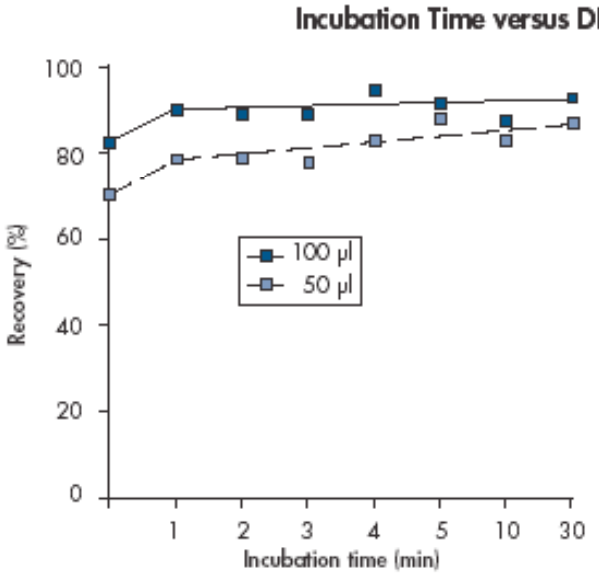


Figure 2 10 µg pBluescript DNA was purified using the QIAprep Spin Miniprep protocol and eluted after the indicated incubation times with either 50 µl or 100 µl Buffer EB. The graph shows that an incubation time of 1 minute and doubling the elution buffer volume increases yield.

Table 1. Effect of Different Compositions of Growth Medium LB on DNA Yield

Culture media	Yield
LB (containing 10 g/liter NaCl)	11.5 µg
LB (containing 5 g/liter NaCl)	9.5 µg

QIAprep Spin Miniprep Kit was used to purify DNA from 1.5 ml LB overnight cultures of XL1-Blue containing pBluescript®. Elution was performed according to the standard protocol (50 µl Buffer EB and 1 min incubation). Use of the recommended LB composition (with 10 g/liter NaCl, also see Appendix A, p. 43) provides optimal plasmid yield.

Using LyseBlue reagent

Using a simple visual identification system, LyseBlue reagent prevents common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, cell debris, and genomic DNA.

LyseBlue can be added to the resuspension buffer (Buffer P1) bottle before use. Alternatively, smaller amounts of LyseBlue can be added to aliquots of Buffer P1, enabling single plasmid preparations incorporating visual lysis control to be performed.

LyseBlue reagent should be added to Buffer P1 at a ratio of 1:1000 to achieve the required working concentration (e.g., 10 µl LyseBlue into 10 ml Buffer P1). Make sufficient LyseBlue/Buffer P1 working solution for the number of plasmid preps being performed.

LyseBlue precipitates after addition into Buffer P1. This precipitate will completely dissolve after addition of Buffer P2. Shake Buffer P1 before use to resuspend LyseBlue particles.

The plasmid preparation procedure is performed as usual. After addition of Buffer P2 to Buffer P1, the color of the suspension changes to blue. Mixing should result in a homogeneously colored suspension. If the suspension contains localized regions of colorless solution or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

Upon addition of neutralization buffer (Buffer N3), LyseBlue turns colorless. The presence of a homogeneous solution with no traces of blue indicates that SDS from the lysis buffer has been effectively precipitated.

Important Notes

Please read the following notes before starting any of the QIAprep procedures.

Growth of bacterial cultures in tubes or flasks

1. **Pick a single colony from a freshly streaked selective plate and inoculate a culture of 1–5 ml LB medium containing the appropriate selective antibiotic. Incubate for 12–16 h at 37°C with vigorous shaking.**

   Growth for more than 16 h is not recommended since cells begin to lyse and plasmid yields may be reduced. Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. **Harvest the bacterial cells by centrifugation at > 8000 rpm (6800 x *g*) in a conventional, table-top microcentrifuge for 3 min at room temperature (15–25°C).**

   The bacterial cells can also be harvested in 15 ml centrifuge tubes at 5400 x *g* for 10 min at 4°C. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained.

Cell Cultivation in a 96-Well Block for QIAprep Turbo 96

1. **Fill each well of a 96-well flat-bottom block with 1.3 ml of growth medium containing the appropriate selective agent. Inoculate each well from a single bacterial colony. Incubate the cultures for 20–24 h at 37°C with vigorous shaking.**

   The wells in the block may be protected against spill-over by covering the block with a plastic lid or adhesive tape. AirPore microporous tape sheets promote gas exchange during culturing (see ordering information, page 49). If non-porous tape is used, pierce 2–3 holes in the tape with a needle above each well for aeration.

2. **Harvest the bacterial cells in the block by centrifugation for 5 min at 2100 x *g* in a centrifuge with a rotor for microtiter plates (e.g., QIAGEN Centrifuge 4K15C, or Heraeus Minifuge® GL), preferably at 4–10°C. The block should be covered with adhesive tape during centrifugation. Remove media by inverting the block.**

   To remove the media, peel off the tape and quickly invert the block over a waste container. Tap the inverted block firmly on a paper towel to remove any remaining droplets of medium.

   WARNING: Ensure that the buckets on the rotor have sufficient clearance to accommodate the 2 ml flat-bottom blocks before starting the centrifuge.

Buffer notes

- Add the provided RNase A solution to Buffer P1, mix, and store at 2–8°C.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Check Buffers P2 and N3 before use for salt precipitation. Redissolve any precipitate by warming to 37°C. Do not shake Buffer P2 vigorously.
- Close the bottle containing Buffer P2 immediately after use to avoid acidification of Buffer P2 from CO_2 in the air.
- Buffers P2, N3, and PB contain irritants. Wear gloves when handling these buffers.
- Optional: Add the provided LyseBlue reagent to Buffer P1 and mix before use. Use one vial LyseBlue (spin down briefly before use) per bottle of Buffer P1 to achieve a 1:1000 dilution. LyseBlue provides visual identification of optimum buffer mixing thereby preventing the common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. For more details see "Using LyseBlue reagent" on page 14.

Centrifugation notes

- All centrifugation steps are carried out at 13,000 rpm (~17,900 x *g*) in a conventional, table-top microcentrifuge.

Vacuum notes

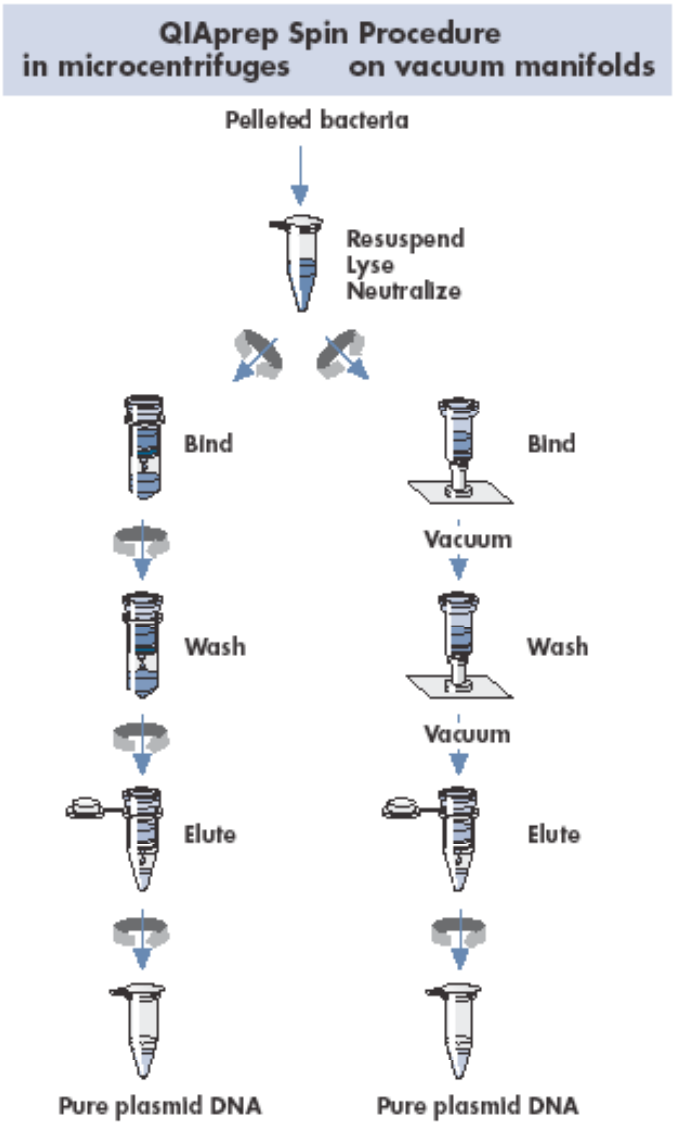
- Switch off vacuum between steps to ensure that a consistent, even vacuum is applied during manipulations.
- Wear safety glasses when working near a manifold under pressure.
- For safety reasons, do not use 96-well plates that have been damaged in any way.
- For QIAprep 8, QIAprep 8 Turbo, and QIAprep 96 Turbo miniprep procedures, the negative pressure (vacuum) should be regulated before beginning the procedure by applying the vacuum to the appropriate number of **empty** QIAprep modules (indicated in Table 2) on the QIAvac manifold.

  The vacuum pressure is the pressure differential between the inside of the manifold and the atmosphere (standard atmospheric pressure: 1013 millibar or 760 mm Hg) and can be measured using a vacuum regulator (see ordering information, page 48). Vacuum recommendations are given in negative units (Table 2) to indicate the required reduction in pressure with respect to the atmosphere. Table 3 provides pressure conversions to other units.
- Use of a vacuum pressure lower than recommended may reduce DNA yield and purity.

Elution notes

- Ensure that the elution buffer is dispensed directly onto the center of the QIAprep membrane for optimal elution of DNA. Average eluate volume is 48 µl from an elution-buffer volume of 50 µl (QIAprep spin procedures), and 60 µl from an elution-buffer volume of 100 µl (QIAprep multiwell procedures).
- For increased DNA yield, use a higher elution-buffer volume. For increased DNA concentration, use a lower elution-buffer volume (see "DNA yield", pages 13–14).
- If water is used for elution, make sure that its pH is between 7.0 and 8.5. Elution efficiency is dependent on pH and the maximum elution efficiency is achieved within this range. A pH <7.0 can decrease yield.

  Note: Store DNA at –20°C when eluted with water, as DNA may degrade in the absence of a buffering agent.
- DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.


QIAprep Spin Procedure
in microcentrifuges
on vacuum manifolds
Pelleted bacteria
Resuspend
Lyse
Neutralize
Bind
Bind
Vacuum
Wash
Wash
Vacuum
Elute
Elute
Pure plasmid DNA
Pure plasmid DNA

Protocol: Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of *E. coli* in LB (Luria-Bertani) medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations on page 44.

Please read "Important Notes" on pages 15–21 before starting.

Note: All protocol steps should be carried out at room temperature.

Procedure

1. **Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.**

   Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.

   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. **Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.**

   Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.

   If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

3. **Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.**

   To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥ 5 ml) may require inverting up to 10 times. The solution should become cloudy.

   If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

4. **Centrifuge for 10 min at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge.**

   A compact white pellet will form.

5. **Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.**
6. **Centrifuge for 30–60 s. Discard the flow-through.**
7. **Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.**

   This step is necessary to remove trace nuclease activity when using *endA*$^+$ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5α™ do not require this additional wash step.
8. **Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.**
9. **Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.**

   Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.
10. **Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.**

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocol(s) in this handbook or molecular biology applications (see back cover for contact information).

		Comments and suggestions
Low or no yield		
General		Low yields may be caused by a number of factors. To find the source of the problem, analyze fractions saved from each step in the procedure on an agarose gel (e.g., Figure 6, page 43). A small amount of the cleared lysate and the entire flow-through can be precipitated by adding 0.7 volumes isopropanol and centrifuging at maximum speed (13,000 rpm or ~17,000 x *g*) for 30 minutes. The entire wash flow-through can be precipitated by adding 0.1 volumes of 3 M sodium acetate, pH 5.0, and 0.7 volumes of isopropanol.
No DNA in the cleared lysate before loading		
a)	Plasmid did not propagate	Read "Growth of bacterial cultures" (pages 39–41) and check that the conditions for optimal growth were met.
b)	Lysate prepared incorrectly	Check storage conditions and age of buffers.
c)	Buffer P2 precipitated	Redissolve by warming to 37°C.
d)	Cell resuspension incomplete	Pelleted cells should be completely resuspended in Buffer P1. Do not add Buffer P2 until an even suspension is obtained.
DNA is found in the flow-through of cleared lysate		
a)	QIAprep membrane overloaded	If rich culture media, such as TB or 2x YT are used, culture volumes must be reduced. It may be necessary to adjust LB culture volume if the plasmid and host strain show extremely high copy number or growth rates. See "Culture media" on page 41.

b)	RNase A digestion omitted	Ensure that RNase A is added to Buffer P1 before use.
c)	RNase A digestion insufficient	Reduce culture volume if necessary. If Buffer P1 containing RNase A is more than 6 months old, add additional RNase A.

DNA is found in the wash flow-through

	Ethanol omitted from wash buffer	Repeat procedure with correctly prepared wash buffer (Buffer PE).

Little or no DNA in eluate

a)	Elution buffer incorrect	DNA is eluted only in the presence of low-salt buffer (e.g., Buffer EB [10 mM Tris·Cl, pH 8.5] or water). Elution efficiency is dependent on pH. The maximum efficiency is achieved between pH 7.0 and 8.5. When using water for elution, make sure that the pH value is within this range.
b)	Elution buffer incorrectly dispensed onto membrane	Add elution buffer to the center of the QIAprep membrane to ensure that the buffer completely covers the surface of the membrane for maximum elution efficiency.

Low DNA quality

DNA does not perform well in downstream applications

a)	Eluate salt concentration too high	For the QIAprep spin column, modify the wash step by incubating the column for 5 minutes at room temperature after adding 0.75 ml of Buffer PE and then centrifuging. For QIAprep 8 preparations and QIAprep 8 and 96 Turbo preparations, ensure that two wash steps are carried out prior to elution.
b)	Nuclease contamination	When using *endA*+ host strains such as HB101 and its derivatives, the JM series, or any wild-type strain, ensure that the wash step with Buffer PB is performed.
c)	Eluate contains residual ethanol	Ensure that step 9 in the QIAprep Spin Miniprep protocol and steps 9 and 10 in the QIAprep 8 Miniprep, QIAprep 8 Turbo Miniprep, or QIAprep 96 Turbo Miniprep protocols are performed.

		Comments and suggestions
RNA in the eluate		
a)	RNase A digestion omitted	Ensure that RNase A is added to Buffer P1 before use.
b)	RNase A digestion insufficient	Reduce culture volume if necessary. If Buffer P1 containing RNase A is more than 6 months old, add additional RNase A.
Genomic DNA in the eluate		
a)	Buffer P2 added incorrectly	The lysate must be handled gently after addition of Buffer P2 to prevent shearing. Reduce culture volume if lysate is too viscous for gentle mixing.
b)	Buffer N3 added incorrectly	Upon addition of Buffer N3 in step 3, mix immediately but gently.
c)	Lysis too long	Lysis in step 2 must not exceed 5 minutes.
d)	Culture overgrown	Overgrown cultures contain lysed cells and degraded DNA. Do not grow cultures for longer than 12–16 hours.

Appendix A: Background Information

Growth of bacterial cultures

Plasmids are generally prepared from bacterial cultures grown in the presence of a selective agent such as an antibiotic (3,4). The yield and quality of plasmid DNA may depend on factors such as plasmid copy number, host strain, inoculation, antibiotic, and type of culture medium.

Plasmid copy number

Plasmids vary widely in their copy number per cell (Table 5), depending on their origin of replication (e.g., pMB1, ColE1, or pSC101) which determines whether they are under relaxed or stringent control; and depending on the size of the plasmid and its associated insert. Some plasmids, such as the pUC series and derivatives, have mutations which allow them to reach very high copy numbers within the bacterial cell. Plasmids based on pBR322 and cosmids are generally present in lower copy numbers. Very large plasmids and cosmids are often maintained at very low copy numbers per cell.

Table 5. Origins of replication and copy numbers of various plasmids (3).

DNA construct	Origin of replication	Copy number	Classification
Plasmids			
pUC vectors	pMB1*	500–700	high copy
pBluescript vectors	ColE1	300–500	high copy
pGEM® vectors	pMB1*	300–400	high copy
pTZ vectors	pMB1*	>1000	high copy
pBR322 and derivatives	pMB1*	15–20	low copy
pACYC and derivatives	p15A	10–12	low copy
pSC101 and derivatives	pSC101	~5	very low copy
Cosmids			
SuperCos	ColE1	10–20	low copy
pWE15	ColE1	10–20	low copy

* The pMB1 origin of replication is closely related to that of ColE1 and falls in the same incompatibility group. The high-copy-number plasmids listed here contain mutated versions of this origin.

Host strains

Most *E. coli* strains can be used successfully to isolate plasmid DNA, although the strain used to propagate a plasmid has an effect on the quality of the purified DNA. Host strains such as DH1, DH5α, and C600 give high-quality DNA. The slower growing strain XL1-Blue also yields DNA of very high-quality which works extremely well for sequencing. Strain HB101 and its derivatives, such as TG1 and the JM series, produce large amounts of carbohydrates, which are released during lysis and can inhibit enzyme activities if not completely removed (4). In addition, these strains have high levels of endonuclease activity which can reduce DNA quality. The methylation and growth characteristics of the strain should also be taken into account when selecting a host strain. XL1-Blue and DH5α are highly recommended for reproducible and reliable results.

Inoculation

Bacterial cultures for plasmid preparation should always be grown from a single colony picked from a freshly streaked selective plate. Subculturing directly from glycerol stocks, agar stabs, and liquid cultures may lead to uneven plasmid yield or loss of the plasmid. Inoculation from plates that have been stored for a long time may also lead to loss or mutation of the plasmid.

The desired clone should be streaked from a glycerol stock onto a freshly prepared agar plate containing the appropriate selective agent so that single colonies can be isolated. This procedure should then be repeated to ensure that a single colony of an antibiotic-resistant clone can be picked. A single colony should be inoculated into 1–5 ml of media containing the appropriate selective agent, and grown with vigorous shaking for 12–16 hours. Growth for more than 16 hours is not recommended since cells begin to lyse and plasmid yields may be reduced.

Antibiotics

Antibiotic selection should be applied at all stages of growth. Many plasmids in use today do not contain the *par* locus which ensures that the plasmids segregate equally during cell division. Daughter cells that do not receive plasmids will replicate much faster than plasmid-containing cells in the absence of selective pressure, and can quickly take over the culture.

The stability of the selective agent should also be taken into account. Resistance to ampicillin, for example, is mediated by β-lactamase which is encoded by the plasmid-linked *bla* gene and which hydrolyzes ampicillin. Levels of ampicillin in the culture medium are thus continually depleted. This phenomenon is clearly demonstrated on ampicillin plates, where "satellite colonies" appear as the ampicillin is hydrolyzed in the vicinity of a growing colony. Ampicillin is also very sensitive to temperature, and when in solution should be stored frozen in single-use aliquots. The recommendations given in Table 6 are based on these considerations.

Table 6. Concentrations of Commonly Used Antibiotics

Antibiotic	Stock solutions Concentration	Storage	Working concentration (dilution)
Ampicillin (sodium salt)	50 mg/ml in water	–20°C	100 µg/ml (1/500)
Chloramphenicol	34 mg/ml in ethanol	–20°C	170 µg/ml (1/200)
Kanamycin	10 mg/ml in water	–20°C	50 µg/ml (1/200)
Streptomycin	10 mg/ml in water	–20°C	50 µg/ml (1/200)
Tetracycline HCl	5 mg/ml in ethanol	–20°C	50 µg/ml (1/100)

Culture media

Luria-Bertani (LB) broth is the recommended culture medium for use with QIAprep Kits, since richer broths such as TB (Terrific Broth) or 2x YT lead to extremely high cell densities, which can overload the purification system. It should be noted that cultures grown in TB may yield 2–5 times the number of cells compared to cultures grown in LB broth. If these media are used, recommended culture volumes must be reduced to match the capacity of the QIAprep membrane. If excess culture volume is used, alkaline lysis will be inefficient, the QIAprep membrane will be overloaded, and the performance of the system will be unsatisfactory. Furthermore, the excessive viscosity of the lysate will require vigorous mixing, which may result in shearing of bacterial genomic DNA and contamination of the plasmid DNA. Care must also be taken if strains are used which grow unusually fast or to very high cell densities. In such cases, doubling the volumes of Buffers P1, P2, and N3 may be beneficial. It is best to calculate culture cell density and adjust the volume accordingly.

Please note that a number of slightly different LB culture broths, containing different concentrations of NaCl, are in common use. Although different LB broths produce similar cell densities after overnight culture, plasmid yields can vary significantly.

Table 7. Recommended composition of Luria Bertani medium

Contents	Per liter
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

Preparation of cell lysates

Bacteria are lysed under alkaline conditions. After harvesting and resuspension, the bacterial cells are lysed in NaOH/SDS (Buffer P2) in the presence of RNase A (2, 5). SDS solubilizes the phospholipid and protein components of the cell membrane, leading to lysis and release of the cell contents while the alkaline conditions denature the chromosomal and plasmid DNAs, as well as proteins. The optimized lysis time allows maximum release of plasmid DNA without release of chromosomal DNA, while minimizing the exposure of the plasmid to denaturing conditions. Long exposure to alkaline conditions may cause the plasmid to become irreversibly denatured (2). This denatured form of the plasmid runs faster on agarose gels and is resistant to restriction enzyme digestion.

The lysate is neutralized and adjusted to high-salt binding conditions in one step by the addition of Buffer N3. The high salt concentration causes denatured proteins, chromosomal DNA, cellular debris, and SDS to precipitate, while the smaller plasmid DNA renatures correctly and stays in solution. It is important that the solution is thoroughly and gently mixed to ensure complete precipitation.

To prevent contamination of plasmid DNA with chromosomal DNA, vigorous stirring and vortexing must be avoided during lysis. Separation of plasmid from chromosomal DNA is based on coprecipitation of the cell wall-bound chromosomal DNA with insoluble complexes containing salt, detergent, and protein. Plasmid DNA remains in the clear supernatant. Vigorous treatment during the lysis procedure will shear the bacterial chromosome, leaving free chromosomal DNA fragments in the supernatant. Since chromosomal fragments are chemically indistinguishable from plasmid DNA under the conditions used, the two species will not be separated on QIAprep membrane and will elute under the same low-salt conditions. Mixing during the lysis procedure must therefore be carried out by slow, gentle inversion of the tube.

Appendix B: Agarose Gel Analysis of Plasmid DNA

The QIAprep Miniprep procedure can be analyzed using agarose gel electrophoresis as shown in Figure 6. Samples can be taken from the cleared lysate and its flow-through, precipitated with isopropanol and resuspended in a minimal volume of TE buffer. In Figure 6 the cleared lysate shows closed circular plasmid DNA and degraded RNase A-resistant RNA. The flow-through contains only degraded RNA and no plasmid DNA is present. The eluted pure plasmid DNA shows no contamination with other nucleic acids.

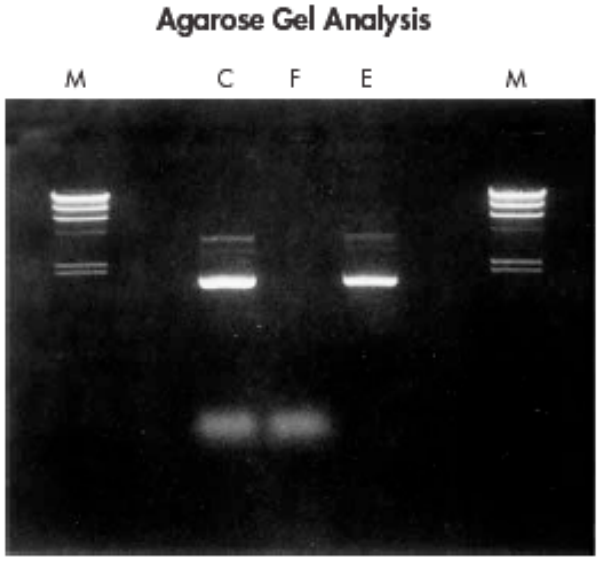


Figure 6 Agarose gel analysis of the QIAprep Miniprep procedure. **C**: cleared lysate; **F**: flow-through; **E**: eluted plasmid; **M**: markers.

Appendix C: Special Applications

Purification of low-copy plasmids and cosmids

All QIAprep miniprep protocols in this handbook can be used for preparation of low-copy-number plasmid or cosmids from 1–10 ml overnight *E. coli* cultures grown in LB medium.

Only two slight modifications to the protocols are required:

- The wash step with Buffer PB is required for all strains.
- When plasmid or cosmids are >10 kb, pre-heat Buffer EB (or water) to 70°C prior to eluting DNA from the QIAprep membrane. A 10 ml overnight LB culture typically yields 5–10 µg DNA.

Note: When using 10 ml culture volume, it is recommended to double the volumes of Buffers P1, P2, and N3 used.

Purification of very large plasmids (>50 kb)

Plasmids >50 kb elute less efficiently from silica than smaller plasmids, but do elute efficiently from QIAGEN anion-exchange resin. QIAGEN provides the anion-exchange-based QIAGEN Large-Construct Kit for efficient large-scale purification of ultrapure genomic DNA-free BAC, PAC, P1, or cosmid DNA. For high-throughput, small-scale purification of BACs, PACs, and P1s, an optimized alkaline lysis protocol in R.E.A.L.® Prep 96 Kits yields DNA suitable for sequencing and screening. Call QIAGEN Technical Services or your local distributor for more information on these kits, or see ordering information on page 47.

Purification of plasmid DNA prepared by other methods

Plasmid DNA isolated by other methods can be further purified using QIAprep modules and any of the QIAprep protocols in this handbook.

1. Add 5 volumes of Buffer PB to 1 volume of the DNA solution and mix (e.g., add 500 µl Buffer PB to 100 µl of DNA sample).
2. Apply the samples to QIAprep spin columns or to the wells of a QIAprep 8 strip or 96-well plate. Draw the samples through the QIAprep membrane by centrifugation or vacuum, and continue the appropriate protocol at the Buffer PE wash step. The optional wash step with Buffer PB is not necessary.

References

1. Vogelstein, B., and Gillespie, D. (1979) Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA **76**, 615–619.
2. Birnboim, H.C., and Doly, J. (1979) A rapid alkaline lysis procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**, 1513–1522.
3. Sambrook, J. et al., eds. (1989) Molecular cloning: a laboratory manual. 2nd ed., Cold Spring Harbor Laboratory Press.
4. Ausubel, F.M. et al., eds. (1991) Current protocols in molecular biology. Wiley Interscience, New York.
5. Birnboim, H.C. (1983) A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol. **100**, 243–255.

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)

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

Procedure:

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

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?

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)

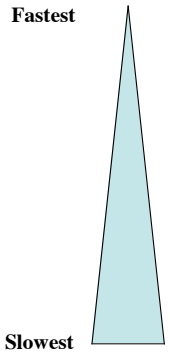
What does BLAST do?

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?



blastp - protein query vs protein db

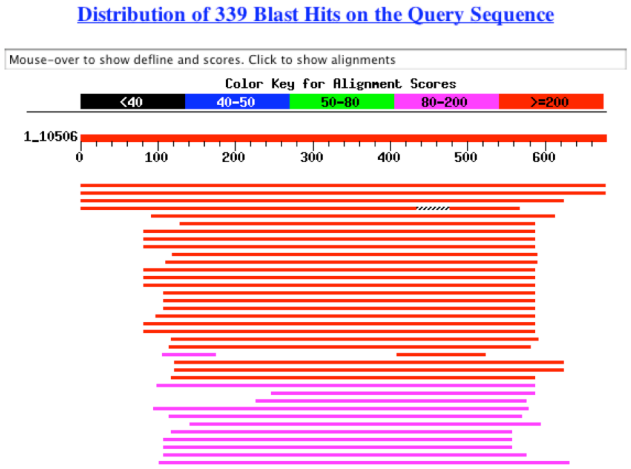
blastn - DNA query vs DNA db

blastx - translated DNA query vs protein db

tblastx - protein query vs translated DNA db

tblastn - translated DNA query vs translated DNA db

Anatomy of a BLAST Result -- Part I



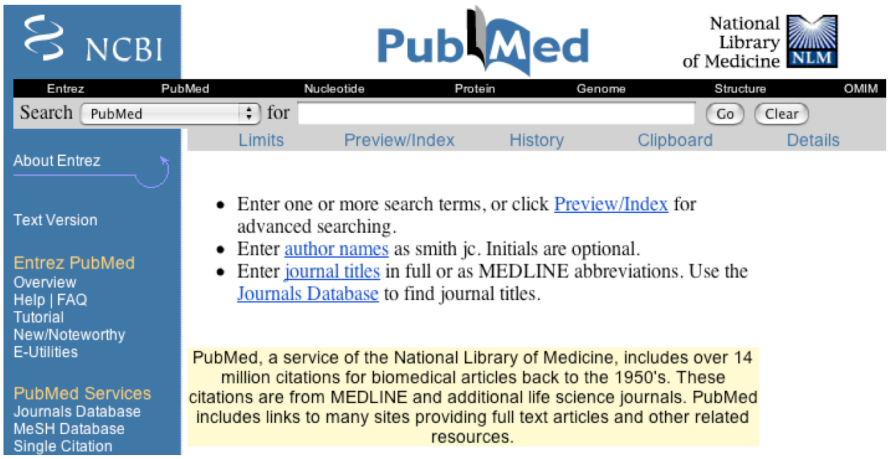
Anatomy of a BLAST Result -- Part II

Sequences producing significant alignments:	Score (bits)	E Value	
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| Sel1 (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| Sel1 (suppressor of lin-12) 1 ...	259	2e-67	L
gi|29612522|gb|AAH49959.1| Sel1h 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   MRILSYGIVILSLLVFSFIEFGVHARPVVLVXXXXXXXXXXXXXXVXXXXXXXXXXXXXX 60
           MRILSYGIVILSLLVFSFIEFGVHARPVVLV              V
Sbjct: 1   MRILSYGIVILSLLVFSFIEFGVHARPVVLVLSNDDLNSGGDDNGVGESSDFDEFGESEP 60

Query: 61  XXXXXLDPGSWRSIFEPDDSTVQAASPQYYSGLKKILSAASEGNFRLMEEAVDEIEAASS 120
                LDPGSWRSIFEPDDSTVQAASPQYYSGLKKILSAASEGNFRLMEEAVDEIEAASS
Sbjct: 61  KSEEELDPGSWRSIFEPDDSTVQAASPQYYSGLKKILSAASEGNFRLMEEAVDEIEAASS 120

Query: 121 AGDPHAQSIMGFVYGIGMMREKSKSKSFLHHNFAAAGGNMQSKMALAFTYLRQDMHDKAV 180
           AGDPHAQSIMGFVYGIGMMREKSKSKSFLHHNFAAAGGNMQSKMALAFTYLRQDMHDKAV
Sbjct: 121 AGDPHAQSIMGFVYGIGMMREKSKSKSFLHHNFAAAGGNMQSKMALAFTYLRQDMHDKAV 180

Query: 181 QLYAELAETAVNSFLISKDSPVVEPTRIHSGTEENKGALRKSRGEEDEDFQILEYQAQKG 240
           QLYAELAETAVNSFLISKDSPVVEPTRIHSGTEENKGALRKSRGEEDEDFQILEYQAQKG
Sbjct: 181 QLYAELAETAVNSFLISKDSPVVEPTRIHSGTEENKGALRKSRGEEDEDFQILEYQAQKG 240

Query: 241 NANAMYKIGLFYYFGLRGLRRDHTKALHWFLKAVDKGEPRSMELLGEIYARGAGVERNYT 300
           NANAMYK GLFYYFGLRGLRRDHTKALHWFLKAVDKGEPRSMELLGEIYARGAGVERNYT
Sbjct: 241 NANAMYKNGLFYYFGLRGLRRDHTKALHWFLKAVDKGEPRSMELLGEIYARGAGVERNYT 300
```

PubMed - Endless Resources



HC70AL Spring 2004

An Introduction to Bioinformatics -- Part II

By

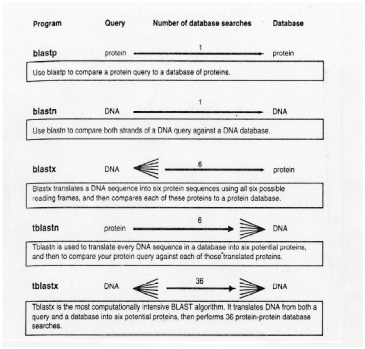
Brandon Le

April 8, 2004

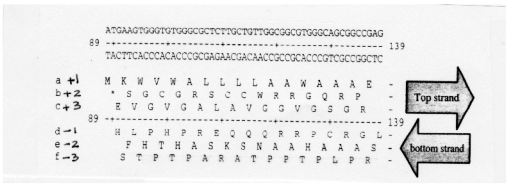
Review of BLAST Search

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 may BLAST program 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?



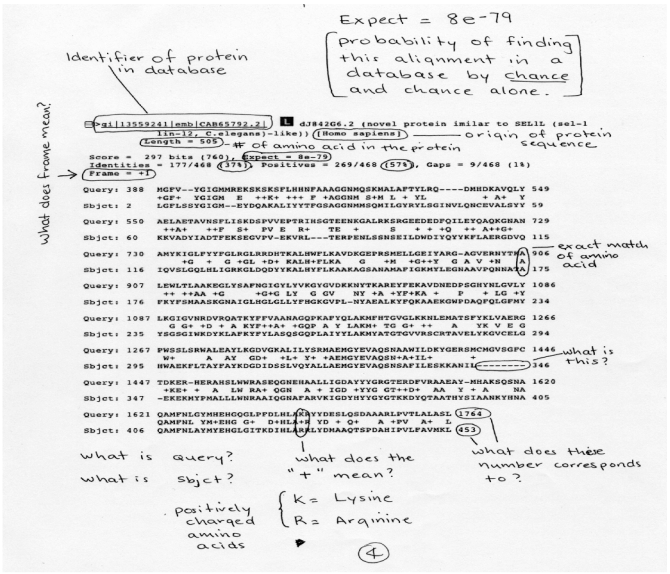
Question:

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?



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 b
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 Webboook

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)**

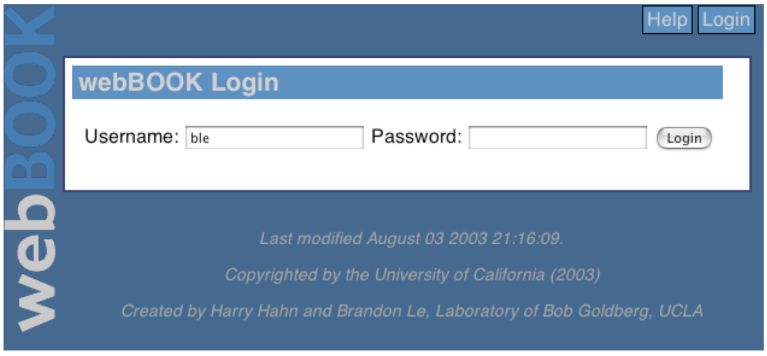
Calendar - **calendar to plant your experiments**

Browse - **search and look at other members experiments**

Contact - **email for help**

Logout - **will logout if idle for 30 min**

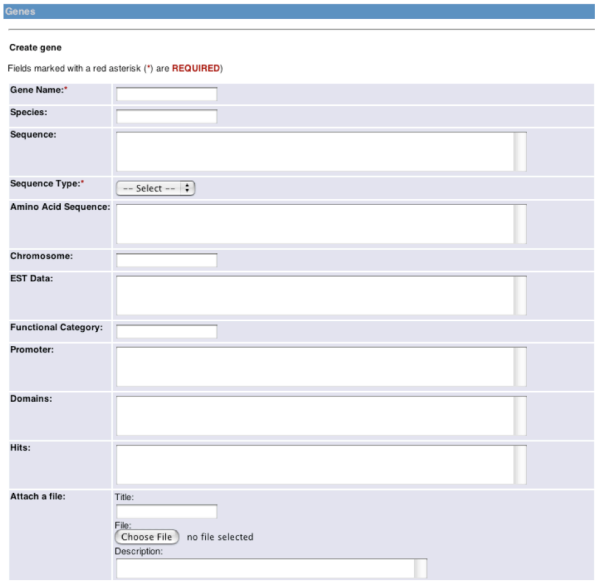
Webbook Login Page



Creating Projects / Experiments

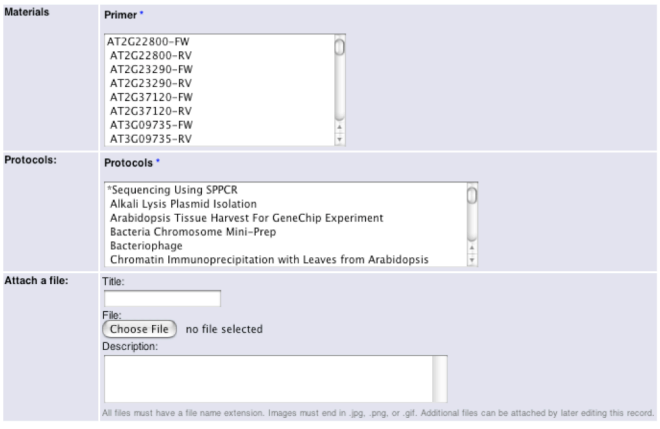
1. Title of project
2. Questions/Purpose of project
3. Summary of project (ideas)

Entering Gene Information



Entering Experiments Information
Part 1

Entering Experiment Information
Part II



Entering References Relating to your Gene