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

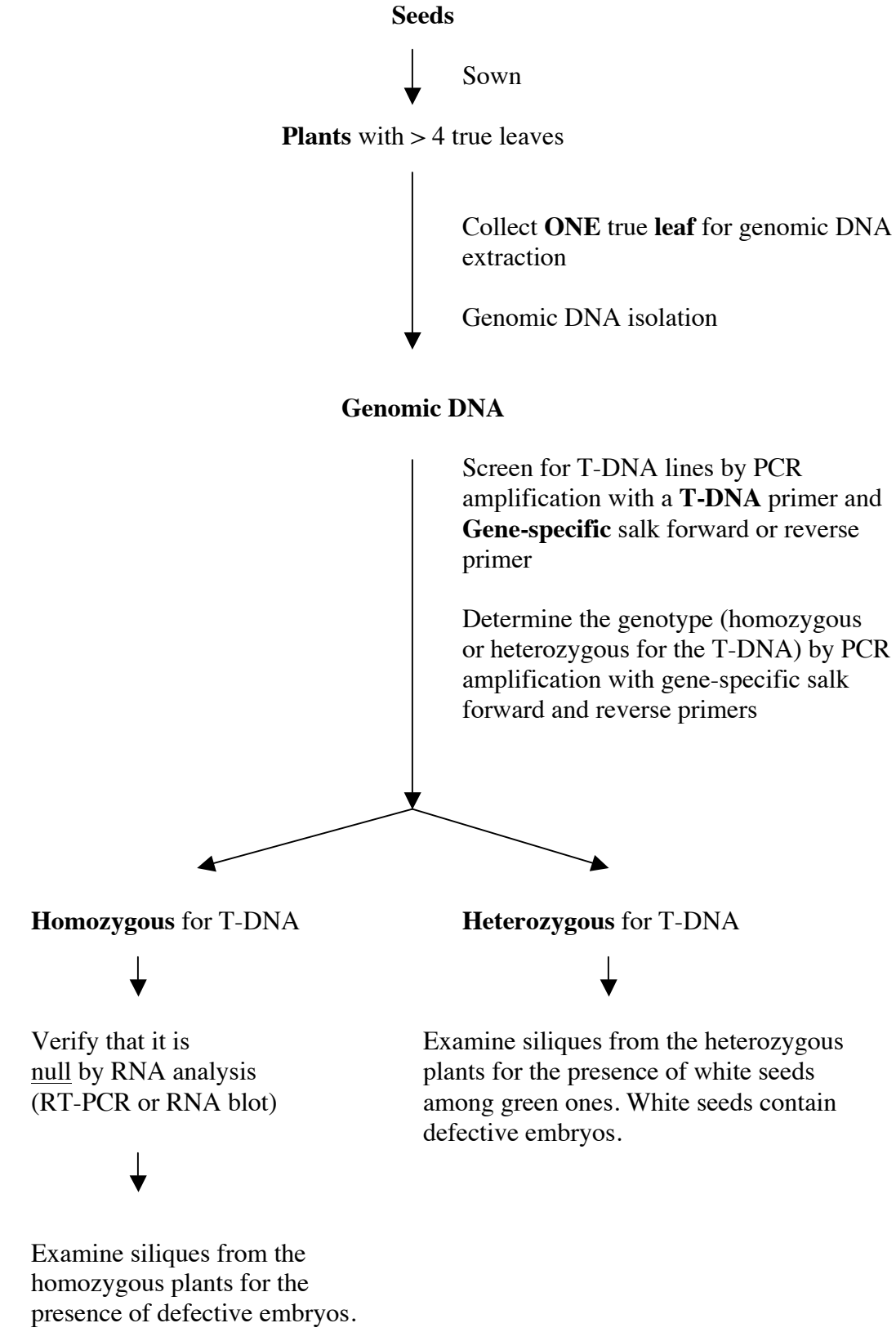
Purpose: To identify a knockout line for the gene of interest and characterize the phenotype of mutant plants.

References: University of Wisconsin - Madison Knockout Facility
Ohio State University - Arabidopsis Biological Resource Center

STRATEGY

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

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 lines by PCR amplification with a T-DNA primer and Gene-specific salk forward or reverse primer
Determine the genotype (homozygous or heterozygous for the T-DNA) by PCR amplification with gene-specific salk forward and reverse primers
Homozygous for T-DNA
Heterozygous for T-DNA
Verify that it is null by RNA analysis (RT-PCR or RNA blot)
Examine siliques from the heterozygous plants for the presence of white seeds among green ones. White seeds contain defective embryos.
Examine siliques from the homozygous plants for the presence of defective embryos.

I. SOWING SEEDS AND GROWING PLANTS

Purpose: To generate seedlings for genomic DNA extraction.

Reference: Arabidopsis Biological Resource Center http://abrc.osu.edu/

Materials Needed:

- Tubes of knockout seeds from the Arabidopsis Biological Resource Center
- Microcentrifuge tube rack
- Sheet of white Xerox paper cut into quarters
- Black sharpie (ultra-fine or fine)
- Plastic yellow tags (5-6 tags per knockout line)
- Pointed-end forceps
- Key to the Plant Growth Center (PGC)
- BruinCard with access to PGC
- Black plastic trays (in PGC)
- Black rectangular pots in sheets (12 pots/sheet; in PGC)
- Clear plastic covers for black trays (in PGC)
- Soil (in PGC)
- One or two pairs of latex gloves

PROCEDURE

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

1. Obtain **tubes of seeds** to be grown from the cold room and put them on a microcentrifuge tube rack. *For example, S_112701 (Salk line for gene At5g11240) and wild type seeds (Columbia-0).*
2. Bring the **materials** to the **Plant Growth Center** (**PGC**).
3. 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 siliques 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 work.*

4. In the PGC, prepare ONE flat with **12 pots** of soil for every TWO knockout lines being planted.
    a. Assemble each flat as follows:
        i. Obtain a black plastic tray without holes.
        ii. Obtain a sheet of 12 rectangular plastic pots.
        iii. Obtain a clear plastic cover.
        iv. Set the sheet of 12 pots in the black plastic tray.
        v. Fill the pots loosely with soil, without compressing. Use the sifter to add a layer of fine soil. *(Soil prepared by PGC staff, Mr. Weimin Deng)*.
        vi. Flatten the surface of the soil by scraping off excess soil with a metal plate.
    b. Remove **one pot** from the corner of the flat and put the soil back into the mound of soil. So, there are **only 11 pots**. *The empty space will make it easier to put the water in*.
    c. Bring the flat to the bench near the sink.
    d. Use the hand brush to clean up the soil bench.
    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. Cover the flat with a **clear plastic cover** to prevent the growth of air-borne molds and to protect the soil from **stray *Arabidopsis* seeds**.
    h. Wait **20 minutes or until the surface of the soil appears darker** due to water seeping up from the bottom of the pots.
5. Label **11 plastic yellow tags** with a black sharpie.
    a. For **knockout lines**: **Gene name**
Salk line #
Date
Pot # 1-10
    b. For **wild type**: **Columbia-0**
Date
Pot # 11

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. Fold each **quarter sheet** of white paper **in half**, lengthwise.
8. Gently pour out the **knockout seeds** from the microcentrifuge tube onto one of the folded pieces of paper.
9. Bring the folded paper with seeds close to the soil of each of the first 5 pots. Lower one end of the paper near the soil surface. Use the **forceps** to guide one seed off of the paper to a precise location in the pot without dumping all of the seeds from the paper.
10. Visually divide the pot into 4 quadrants, and sow a seed in each quadrant. Sow **4 seeds** per pot, for the first 5 pots. *Note: Planted seeds should not be covered with additional soil because Arabidopsis seeds need light for germination.*
11. Put the labeled tags for the **knockout line** into **each** of the **5 pots** containing knockout seeds.
12. Put the seeds that were not used back into the **appropriate knockout seed** microcentrifuge tube.
13. Repeat steps 7-12 with the seeds for the next knockout line and pots #6-10. Use a **new** folded piece of white paper for each line.
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 wild type seeds** 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**. Put the flat aside.
17. After all of the lines are sown, put the flats on a metal cart and take the elevator to the lower level.
18. Put the flats on the wire 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. Note: Turn the lights off when you leave the cold room.

19. Leave the flats in the cold room for **2-5 days** to **vernalize seeds.** <u>*Note:*</u> *This will eliminate any dormancy, improve the germination rate and synchronize seed germination*.
20. After 2-5 days in the cold room, put the flats on a metal cart and take the elevator to the upper level. Transfer the flats to a bench in the greenhouse room 125B.
    <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 knockout flats when the clear covers are removed*.
21. Slide the clear covers off the trays by **0.5-1 inch** so that warm air under the covers will not overheat the seedlings or create a warm environment favorable for algal and fungal growth. Sliding off the clear covers will also provide some aeration, but still maintain enough humidity for germination and also avoid seed desiccation.
22. Wait until most of the seedlings in the flats have **4 true leaves**. Then completely remove the clear covers from the flats.
23. Map the **positions of seedlings** in **each of the 11 pots** on a "Plant Layout Chart."

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 WEEK-OLD SEEDLINGS

Purpose: To isolate genomic DNA from seedling leaves for identifying plants containing a T-DNA insert within the gene of interest.

Recommendation:

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

Materials Needed:

- Seedlings with at least 4 true leaves (knockout lines and wild type)
- Ice bucket
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Pipettes
- Pipet tips
- One or two pairs of latex gloves
- Two pairs of pointed-end forceps
- Squirt bottle of 100% ethanol solution
- Kimwipes
- Black sharpie (ultra-fine or fine)
- Pen
- Plant layout chart
- Digital camera
- Key to the Plant Growth Center
- BruinCard with access to PGC
- Extraction buffer (0.2 M Tris-HCl, pH 9.0; 0.4 M LiCl; 25 mM EDTA; 1% SDS)
- Sterile blue micropestles
- Microcentrifuge
- Timer
- Isopropanol
- 80% ethanol solution
- SpeedVac (optional)
- TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0)
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain

- 6x Loading dye
- 50 ng/μL 1 Kb Plus DNA ladder solution
- Gel electrophoresis materials (Appendix 1A)
- NanoDrop spectrophotometer

PROCEDURE

Attention: *You will need to **assess** the **quality** of **isolated genomic DNA** later (at **step 34**). If you plan to do this on the same day as the genomic DNA isolation, use time efficiently by preparing a 0.7% agarose gel before you start the isolation of genomic DNA (see **Appendix 1A**). While the agarose mixture cools in the 55-60°C water bath for at least 10 minutes, go to the Plant Growth Center to collect leaves. When you come back to the lab, add 10,000x SYBR Safe DNA gel stain to the agarose mixture, swirl to mix, pour the gel, and let the agarose mixture solidify in the dark.*

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

   Tube #**1 - 5**: Seedlings #1 - 5 of **knockout** lines

   Tube #**6**: **1** Seedling/plant from **wild type** (Columbia-0)
3. Pipet **100 μL** of **Extraction Buffer** into each tube. Keep the tubes on ice. *Note: 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:
   - Bucket of ice
   - 1.5 mL microcentrifuge tubes containing 100 μL of Extraction Buffer
   - One or two pairs of latex gloves
   - Two pairs of pointed-end forceps
   - Squirt bottle of 100% ethanol solution
   - Kimwipes
   - Black sharpie (ultra-fine or fine)
   - Pen
   - Plant layout chart
   - Digital camera
   - Key to the Plant Growth Center
   - BruinCard with access to PGC
5. Go to the **Plant Growth Center (PGC)** and locate your flat with plants.

6. Take pictures of the plants to document the phenotype. Take pictures of the yellow tags to identify the plants in the pictures.
7. Use the **Plant Layout Chart** to mark the **locations of the plants** and to indicate the plants that you will collect samples from. The **order of plants** should **correspond** to the **labeled tags** that were numbered when the seeds were planted. *Note: NOT all of the seeds will have germinated*.
8. Use a piece of Kimwipes to clean the forceps with ethanol. *Note: Two sets of forceps are used per plant. The forceps must be cleaned after the collection each leaf to avoid contamination*.
9. Using forceps, remove one **small leaf** from the **plant #1**.
10. Place this leaf in microcentrifuge **tube #1** containing the Extraction Buffer.
11. Repeat this process for the other plants.
    Note: MAKE SURE TO CLEAN THE FORCEPS BETWEEN LEAF SAMPLES!
12. Go back to the lab.
13. Homogenize or macerate the collected leaf in **tube #1** by crushing it with a **blue micropestle** until no more chunks of plant tissue are observed in the mixture. *Note: Do NOT dispose of the micropestle*.
14. Rinse the **micropestle** with **300 µL** of Extraction Buffer. Put the used micropestles in a beaker labeled "used micropestles" so that they can be washed. The **total volume** of Extraction Buffer in the microcentrifuge tube is now **400 µL**.
15. Vortex the **homogenate** for 5 seconds. Set the tube on ice.
16. Repeat steps 13-15 for the other tubes.
17. Centrifuge the tubes of homogenates at **room temperature** for **5 minutes** at **FULL speed**.
18. Meanwhile, **label** a set of **microcentrifuge tubes** with **Gene Name** and **tube #.**
19. Pipet **350 µL** of **isopropanol** to each of the new labeled tubes.
20. After centrifugation, transfer the tubes from the microcentrifuge onto a microcentrifuge tube rack. Organize tubes on the rack such that the **numbers** on the lids of the **NEW tubes match** with the **numbers** on the lids of the **tubes containing homogenates**.
21. Pipet **350 µL** of **supernatant** (**homogenate**) from the centrifuged tubes to the corresponding tubes containing isopropanol. Close the lids of the tubes.
    Note: AVOID disturbing the 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.

22. Mix the isopropanol and homogenate by inverting the tubes **5-10 times**.
23. Incubate the mixture at **room temperature** for **5 minutes** to precipitate **nucleic acids** *(both genomic DNA and total RNA)*.
24. Centrifuge the tubes at **room temperature** for **10 minutes** at **FULL** speed.
25. Pour off the supernatant into a glass beaker labeled "waste solution. *"Note: DNA is now in your pellet along with RNA. Therefore, be extremely careful when pouring off the isopropanol because the pellets are sometimes loose.*
26. 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 salt (in the extraction buffer) and isopropanol.*
27. Centrifuge the tubes at **room temperature** for **5 minutes**.
28. Pour off the supernatant into a glass beaker labeled "waste solution." Dab the tubes on Kimwipes to remove as much ethanol as possible. *Note: Be extremely careful when pouring off the ethanol solution because the pellets are sometimes loose.*
29. Put the tubes on a microcentrifuge tube rack with their lids open, allowing the ethanol to evaporate.
30. Dry pellets either in a **SpeedVac** at room temperature for **5-10 minutes** (TAs will show you how to do this step) or by leaving on the **bench at room temperature for up to 60 minutes**.
31. 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 a microcentrifuge tube rack **10-15 times** or **vortexing** the tubes for a few minutes until no pellet is visible.
32. Spin the tubes in a microcentrifuge for **1 minute** to bring liquid and any contaminants to the bottom of the tubes.
33. Store DNA solutions at **4°C** (on ice or in refrigerator) until use.

    Note:

    - *Keep DNA solutions cold as much as possible to prevent degradation of DNA because this is a crude extraction of genomic DNA, and there may be a trace amount of endonuclease present in the DNA solution.*
    - *Before using DNA solutions for PCR amplification after a long period of storage (more than 12 hours), spin the 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 contaminating 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 (step 34)*** *and* ***spectrophotometer reading (step 35)****, respectively.*

34. Analyze the **quality** of isolated genomic DNA by **gel electrophoresis** as follows:
    a. Prepare a **0.7%** agarose gel with a 20-tooth comb (0.7 g of agarose in 100 mL of 1x TAE buffer; see Appendix 1A). *Note: The agarose gel can be prepared before the collection of leaves for the extraction of genomic DNA.*
    b. Label the lids of 6 microcentrifuge tubes with **#1-6** and **your initials**, and set tubes on a microcentrifuge tube rack.
    c. Pipet **20 µL** of **isolated genomic DNA** solution into each of the labeled tubes.
    d. Add **2 µL** of **6x loading dye solution** to each tube and mix the contents by pipetting up and down 5 times.
    e. Load **10 µL** of **1 Kb Plus DNA ladder solution** into the first well.
    f. Load **20 µL** of each sample-dye mixture prepared in steps c and d using a P-20 pipette.
    g. Record the identity of the sample in each well.

Lane	Sample	Expected Size (bp)
1	1 Kb Plus DNA ladder	-
2	Genomic DNA from Plant #1	
3	Genomic DNA from Plant #2	
4	Genomic DNA from Plant #3	
5	Genomic DNA from Plant #4	
6	Genomic DNA from Plant #5	
7	Genomic DNA from Plant #6	

    h. Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the cathode.

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

Starting time:

Ending time:

j. 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?

35. Determine the concentration of DNA using the NanoDrop spectrophotometer. *Your TAs will demonstrate how to use the NanoDrop.*

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

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

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

Note: Dilution of DNA solutions will serve two purposes:

a. Contaminants, such as carbohydrates that bind nonspecifically to nucleic acids and proteins, will be diluted out. A tiny amount of contaminants in a PCR reaction will not interfere with the amplification of the target DNA.

b. Only a small amount of Arabidopsis genomic DNA (~0.4 ng) is needed for PCR amplification.

How to make a dilution?

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

$\mathbf{V_i \times C_i = V_f \times C_f}$

where,

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

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

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

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

$\mathbf{V_f = (V_i \times C_i)/ C_f}$ = (**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 µL - 5 µL = 195 µL** of **TE**

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

	Volume of Isolated Genomic DNA	**Volume of TE Added**	**Final Volume**
Plant #1	5 µL		
Plant #2	5 µL		
Plant #3	5 µL		
Plant #4	5 µL		
Plant #5	5 µL		
Plant #6	5 µL		

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

Purpose: To identify plants containing the T-DNA insert and to determine the genotypes of T-DNA-tagged plants (heterozygous or homozygous for the T-DNA).

STRATEGY

A. Polymerase Chain Reaction (PCR)

B. Gel Electrophoresis Analysis of PCR Product

C. Label T-DNA tagged plants

Solutions Needed:

- Ex Taq DNA polymerase (Takara)
- 10x Ex Taq buffer (Takara; comes with the Ex Taq DNA polymerase)
- dNTP mix (Takara; comes with the Ex Taq DNA polymerase)
- Sterile water
- 12 μM Gene-specific Salk Forward primer
- 12 μM Gene-specific Salk Reverse primer
- 12 μM LBb1 primer (anneals to the Left Border (LB) region of the T-DNA)
- 0.2 ng/μL genomic DNA extracted from the plants to be genotyped (including WT)
- 0.2 ng/μL genomic DNA extracted from wild type seedlings by TAs
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain
- 6x Loading dye
- 50 ng/μL 1 Kb Plus DNA ladder solution

Materials Needed:

- Pipettes
- Filter pipet tips for PCR
- 0.2 mL PCR tubes
- 1.5 mL microcentrifuge tubes
- Rack for 0.2 mL PCR tubes
- Rack for 1.5 mL microcentrifuge tubes
- Ice bucket
- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- Microcentrifuge
- Gel electrophoresis materials (Appendix 1A)
- Key to the Plant Growth Center

- BruinCard with access to PGC
- Wooden sticks
- Tape
- Black sharpie (ultra-fine or fine)
- Pen
- Plant Layout Chart
- Phenotype Observation Record
- Digital Camera

A. Polymerase Chain Reaction (PCR)

PROCEDURE

*Note: Because you do not know the orientation of the T-DNA insertion, and therefore the direction of the LBb1 primer, you will need to set up **THREE** PCR reactions for **each** sample.*

*Reaction A: **Gene-specific Salk Forward** primer and **LBb1** primer*

*Reaction B: **LBb1** primer and **Gene-specific Salk Reverse** primer*

*Reaction C: **Gene-specific Salk Forward** primer and **Gene-specific Salk Reverse** primer*

Note: ALWAYS wear gloves and use filter tips to prevent contamination when preparing PCR reactions.

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

1. Get ice from the icemaker in room 4128.
2. Thaw tubes of **10x Ex Taq buffer, dNTP mix, 12 µM Gene-specific Salk Forward primer, 12 µM Gene-specific Salk Reverse primer** and **12 µM LBb1 primer** on a rack for 1.5 mL microcentrifuge tubes at **room temperature** for 5-10 minutes. Once the solutions are thawed, put the tubes on **ice** until needed.

3. Spin genomic DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed. Keep on ice.
4. Label the lids and sides of **24 PCR tubes** with **A1-A8, B1-B8, C1-C8** and **your initials** and put them on a PCR tube rack sitting on ice.
5. Prepare a **"Reaction A" master mix** for **9 PCR reactions** in a 1.5 mL microcentrifuge tube labeled "**Mmix A**" sitting on **ice**.

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

Reaction A	**Mmix for ONE** reaction	**Mmix for 9** reactions
Sterile water	34.75 µL	312.75 µL
10x Ex Taq buffer	5.0 µL	45.0 µL
dNTP mix	4.0 µL	36.0 µL
12 µM **Gene-specific Salk Forward** primer	1.0 µL	9.0 µL
12 µM Gene-specific Salk Reverse primer	-	-
12 µM **LBb1** primer	1.0 µL	9.0 µL
Ex Taq DNA polymerase (5 U/µL)	0.25 µL	2.25 µL
Total Volume	**46.0 µL**	**414.0 µL**

6. Mix the contents of **Mmix A** by flicking the tube several times or vortexing the tube at a **setting of 2-3** for **5 seconds**. Spin the tube in a microcentrifuge at FULL speed for **10 seconds**. Put the tube back **on ice**.
7. Pipet **46 µL** of **Mmix A** into each of **8 PCR tubes** labeled **A1-A8**.
8. Pipet **4 µL** of **0.2 ng/µL genomic DNA** extracted from each of **6 seedlings** into PCR tubes A1-A6. Pipet up and down 5 times to mix the contents.
9. Pipet **4 µL** of **0.2 ng/µL genomic DNA** extracted from **wild type** (Col-0) seedlings by TAs into tube **A7**. Pipet up and down 5 times to mix the contents.
10. Pipet **4 µL** of **sterile water** into tube **A8** (**negative control** without DNA template). Pipet up and down 5 times to mix the contents.
11. Keep these PCR reactions on ice while you prepare the next master mix. Repeat steps 5-11 for Reactions B and C.

Reaction B	**Mmix for ONE** reaction	**Mmix for 9** reactions
Sterile water	34.75 µL	312.75 µL
10x Ex Taq buffer	5.0 µL	45.0 µL
dNTP mix	4.0 µL	36.0 µL
12 µM Gene-specific Salk Forward primer	-	-
12 µM **Gene-specific Salk Reverse** primer	1.0 µL	9.0 µL
12 µM **LBb1** primer	1.0 µL	9.0 µL
Ex Taq DNA polymerase (5 U/µL)	0.25 µL	2.25 µL
Total Volume	**46.0 µL**	**414.0 µL**

Reaction C	**Mmix for ONE** reaction	**Mmix for 9** reactions
Sterile water	34.75 µL	312.75 µL
10x Ex Taq buffer	5.0 µL	45.0 µL
dNTP mix	4.0 µL	36.0 µL
12 µM **Gene-specific Salk Forward** primer	1.0 µL	9.0 µL
12 µM **Gene-specific Salk Reverse** primer	1.0 µL	9.0 µL
12 µM LBb1 primer	-	-
Ex Taq DNA polymerase (5 U/µL)	0.25 µL	2.25 µL
Total Volume	**46.0 µL**	**414.0 µL**

12. Spin all PCR tubes in the minicentrifuge for PCR tubes for **5 seconds** to bring the liquid to the bottom of the tubes.
13. Turn **ON** the PCR machine (MyCycler). Wait for one minute for the machine to initialize.
14. Put the PCR tubes in the wells of the 96-well hot plate of the MyCycler.
15. Select the "**Protocol Library**" by pressing "**F1**."
16. Select "**HC70AL**" by pressing the yellow arrowheads surrounding the "**ENTER**" button. Select the "**HC70AL KNOCKOUT**" protocol. Press "**ENTER**."
17. The "CHOOSE OPERATION" menu will appear. Select "**VIEW PROTOCOL**." The **PCR profile** of the Knockout protocol is as follows:

94°C for 3 min
36 cycles of 94°C 15 sec
62°C 30 sec
72°C 2 min

72°C 4 min
4°C ∞

18. Press "**F5**" for "**DONE.**" The "CHOOSE OPERATION" menu will appear. Press "**ENTER**" to **run the protocol**.
19. Enter the **volume** of the PCR reaction. Press "**F5**" to "**Begin Run**." *Note: It will take about 3 hours for the PCR amplification to be completed.*
20. Once the PCR amplification is complete, remove the PCR tubes from the PCR machine and store them in the **refrigerator** until gel electrophoresis or leave them in the PCR machine at 4°C until you have a chance to put them away later.

B. Gel Electrophoresis Analysis of PCR Product

PROCEDURE

1. Prepare a **1% agarose gel** in 1x TAE buffer with a **30-tooth** comb.
2. Label **24 1.5 mL microcentrifuge** tubes and set them on a microcentrifuge tube rack.
3. Add **2 µL** of **6x loading dye** to each tube.
4. Pipet **20 µL** of **PCR solution** to each tube. Mix the contents by pipetting up and down at least 5 times.
5. Load **10 µL** of **1 Kb Plus DNA ladder** in the first well.
6. Load **20 µL** of the sample-dye mixtures on the gel using a P-20 pipette. Record the identity of the sample in each lane.

Lane	Sample	Primers	Expected Size (bp)
1	1 Kb Plus DNA ladder	-	-
2	Genomic DNA from Plant #1	Reaction A: **Gene-specific Salk Forward** primer and **LBb1** primer	
3	Genomic DNA from Plant #2		
4	Genomic DNA from Plant #3		
5	Genomic DNA from Plant #4		
6	Genomic DNA from Plant #5		
7	Genomic DNA from Plant #6		
8	Col-0 Genomic DNA (from TAs)		
9	Sterile water		-
10	Genomic DNA from Plant #1	Reaction B: **LBb1** primer and **Gene-specific Salk Reverse** primer	
11	Genomic DNA from Plant #2		
12	Genomic DNA from Plant #3		
13	Genomic DNA from Plant #4		
14	Genomic DNA from Plant #5		
15	Genomic DNA from Plant #6		
16	Col-0 Genomic DNA (from TAs)		
17	Sterile water		-
18	Genomic DNA from Plant #1	Reaction C: **Gene-specific Salk Forward** primer and **Gene-specific Salk Reverse** primer	
19	Genomic DNA from Plant #2		
20	Genomic DNA from Plant #3		
21	Genomic DNA from Plant #4		
22	Genomic DNA from Plant #5		
23	Genomic DNA from Plant #6		
24	Col-0 Genomic DNA (from TAs)		
25	Sterile water		-
26	1 Kb Plus DNA ladder	-	-

7. Add **10 μL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the cathode.
8. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) travels two-thirds of the gel.
9. Stop the gel electrophoresis.
10. Take a picture of the gel.
11. Analyze the 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?

    What is the orientation of the T-DNA relative to the gene?

    What are the genotypes of the 6 plants?

Note:

1. *Although the results of the PCR reactions should confirm the size of the so-called "T-DNA fragment," which contains part of the plant gene and part of the T-DNA, it is a* ***good scientific practice*** *to verify the exact location of the T-DNA insertion site.*

2. ***Depending on the results of the PCR*** *to genotype the plants, you can use one of the following procedures to purify the "T-DNA fragment."*
    a. *If a lane on the gel contains only a* ***single band*** *corresponding to the "T-DNA fragment," then the "T-DNA fragment" can be purified directly from the PCR solution by following the* ***QIAquick PCR Purification Procedure*** *below.*
    b. *If all lanes containing the "T-DNA fragment" also contain other bands (due to contamination or mispriming), then the "T-DNA fragment" must be purified from an agarose gel slice. Follow the* ***QIAquick Gel Extraction Procedure*** *below.*

C. Label T-DNA tagged plants

PROCEDURE

1. After determining the genotypes of the plants, make labels for the plants containing a T-DNA insert by putting a small piece of tape on a wooden stick. Write the **number** that corresponds to the **plant #** on the Plant Layout Chart and either "**homozygous for the T-DNA**" or "**heterozygous for the T-DNA**." You can also note the genotype of the plants on the Plant Layout Chart.
2. Go to the Plant Growth Center. Put the labeled wooden sticks next to the identified T-DNA tagged plants.
3. Observe T-DNA tagged plants for abnormal phenotypes. Write your observations on the **Phenotype Observation Record**. Take pictures of the plants to document the phenotype. Take pictures of the yellow tags to identify the plants in the pictures.

PHENOTYPE OBSERVATION RECORD

Gene ID: **At__ g _____ Salk line#: _____ Date: _____**

LEAF	**Mutant**	**Wild Type**
What do the leaves look like, green or yellow, elongated or round?		
What is the range of their length in cm?		
How many leaves does each plant have?		
Is the range of leaf sizes of the mutant plant smaller or larger or similar to wild type leaves?		

STEM	**Mutant**	**Wild Type**
What is the height of the main (or longest) stem?		
What is the thickness of the stem?		
How many stems (or branches including the main and side ones) does the plant have?		

FLOWERS	**Mutant**	**Wild Type**
Do the flowers have all FOUR floral organs (green sepals, white petals, yellow anthers, green pistils)?		
How many sepals are on each flower?		
How many petals are on each flower?		
How many anthers are on each flower?		
How many pistils are on each flower?		

SILIQUES, SEEDS AND EMBRYOS	**Mutant**	**Wild Type**
How many siliques are on each plant?		
Do you see a difference in the lengths of siliques?		
How many seeds are in EACH silique?		
What is the average number of seeds in FIVE siliques?		
Do you see different COLORED seeds within a single silique?		
If yes, what colors are the seeds? How many seeds of each color?		
What stage of embryos (globular, heart, torpedo, cotyledon, mature green, or post mature green) do you see?		

IV. DETERMING THE T-DNA INSERTION SITE

Purpose: To verify the location of the T-DNA insertion site in the gene of interest indicated by the Salk Institute Genomic Analysis Laboratory website http://signal.salk.edu/

STRATEGY

A. Purifying PCR Products

B. Sequencing Reaction with Big Dye v. 3

C. Retrieving and Analyzing DNA Sequences

A. Purifying PCR Products

QIAquick PCR Purification Procedure

Note: This procedure is used when you run 10 µL of PCR product on the gel and observe only one band.

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

Reference: QIAquick PCR Purification protocol (Qiagen; see Appendix 1F)

Solutions Needed:

- QIAquick PCR Purification Kit (Qiagen, Cat. # 28104)
- Remaining PCR solutions (~30 µL)

Materials Needed:

- Pipettes
- Filter pipet tips
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Microcentrifuge
- Vortex
- Timer
- NanoDrop spectrophotometer

- Kimwipes

PROCEDURE

1. Write on the **lids** and **sides** of TWO 1.5 mL microcentrifuge tubes "**T-DNA**" or "**WT**" and **your initials**.
2. Pipet **~30 µL** of the **PCR product solution** from the PCR tubes containing the T-DNA fragment or gene-specific DNA fragment into the labeled 1.5 mL microcentrifuge tubes.
3. Add **150 µL** of **Buffer PB** (or **5 volumes** of **Buffer PB** to **1 volume** of the PCR sample) to the tubes in step 2. Mix by vortexing for 5 seconds. Spin the tubes in the microcentrifuge at **FULL speed** for **10 seconds** to bring all the solution down to the bottom of the tubes. Set the tubes back on the microcentrifuge tube rack.
4. Place TWO **QIAquick spin columns** in the provided **2-mL collection tubes**. Label the collection tubes "**T-DNA**" or "**WT**" and **your initials**.
5. Apply the sample mixtures in step 3 to the QIAquick columns. Spin the columns 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 tubes. Put the QIAquick columns back into the same collection tubes.
7. Add **750 µL** of **Buffer PE** to the QIAquick spin columns and spin at **FULL speed** for **1 minute**.
8. Discard the flow-through solution in the collection tubes. Put the QIAquick columns back into the same collection tubes.
9. Spin the columns 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. If the residual ethanol is not removed from the column after spinning for 1 min, then spin for an additional 1-2 min.

10. While spinning, label the **lids** and **sides** of TWO **1.5 mL microcentrifuge** tubes "**Purified T-DNA PCR**" or "**Purified WT PCR,**" **your initials** and the **date**.
11. Transfer the **QIAquick columns** to the appropriate 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 membranes. Let the columns 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.

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 Gel Extraction Procedure

Note: This procedure is used when you run 10 µL of PCR product on the gel and observe more than a single band.

Purpose: To purify DNA (PCR product) from agarose gel for downstream applications such as sequencing reactions.

Reference: QIAquick Gel Extraction protocol (Qiagen; see Appendix 1F)

Reagents and Materials Needed:

- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain
- Remaining PCR solutions (~30 µL)
- 6x Loading dye
- Gel apparatus and a power supply
- 1.5 mL microcentrifuge tubes
- Razor blade
- Plastic (saran) wrap
- UV light box

- QIAquick Gel Extraction Kit (Qiagen, Cat. #28704)
- Isopropanol
- 50°C water bath
- Timer
- Microcentrifuge
- Scale
- Metal waste container for sharp objects

PROCEDURE

1. Label the **lids** of TWO 1.5 mL microcentrifuge tubes "**T-DNA**" or "**WT**" and **your initials**.
2. Place a NEW piece of plastic wrap on an ultraviolet (UV) light box. Then place your gel on the plastic wrap.
3. Put on a UV shield to protect your eyes and face.
4. Turn **on** the **UV box**. <u>Note:</u> *Turn **off** the UV box as soon as you are done excising the DNA band.*
5. Excise the desired DNA fragments from the gel using a razor blade. <u>Note:</u> *Trim off excess agarose surrounding the DNA band as much as possible. Your TAs will demonstrate.*
6. Place the agarose slices in the 1.5 mL microcentrifuge tubes.
7. Take a picture of the gel **after removing the agarose slices**. *This step serves as a record of the DNA fragment being collected.*

   <u>Note:</u> *If the desired bands were faint, run the remaining 15 µL of the desired PCR solutions on a new gel and excise those bands as well.*

   a. *Prepare a **1% agarose** gel with a **20-tooth comb** (see Appendix 1A).*
   b. *Add **3 µL** of **6x loading dye** to each tube of **~30 µL** PCR solutions containing the "T-DNA fragment" or the "WT DNA fragment."*
   c. *Load **10 µL of 1 Kb Plus DNA ladder** into the first well.*
   d. ***Very slowly** load the **33µL** sample-dye mixtures on the gel using a P-20 pipette.*
   e. *Record the identity of the sample in each well.*

Lane	*Sample*	*Primers*	*Expected Size (bp)*
1	*1 Kb Plus DNA ladder*	-	-
2			
3			

*f. Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the cathode.*

*g. Run the gel at **105 volts** for 1.5 - 2 hours in the dark.*

h. Take a picture of the gel.

*i. Verify the presence of the **expected size PCR product**.*

j. Place a NEW piece of plastic wrap on an ultraviolet (UV) light box. Then place your gel on the plastic wrap.

k. Put on a UV shield to protect your eyes and face.

*l. Turn **on** the **UV box**. Note: Turn **off** the UV box as soon as you are done excising the DNA band.*

m. Excise the desired DNA fragments from the gel using a razor blade. Note: Trim off excess agarose surrounding the DNA band as much as possible. Your TAs will demonstrate.

*n. Place the agarose slices in the 1.5 mL microcentrifuge tubes (in step 6) labeled "**T-DNA**" or "**WT**" and **your initials**.*

*o. Take a picture of the gel **after removing the agarose slices**. This step serves as a record of the DNA fragment being collected.*

8. Centrifuge the gel fragments at full speed for **1 minute**.
9. Estimate the **gel volume** in the microcentrifuge tubes using a **scale**. Use an empty 1.5 mL microcentrifuge tube as a blank. Write the **gel volume** on the side of the tubes. *Note: **0.1 g** of **agarose gel** is **equivalent to 100 µL**.*

10. Add **3 gel volumes** of **Buffer QG** to the tubes containing the gel slices. *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.*
11. Incubate the tubes at **50°C** for **10 minutes** or until the gel slice has **completely** dissolved. *To help dissolve the gel, you may vortex the tube for 5 seconds every 2-3 min during the incubation. This step solubilizes the agarose completely. Make sure the color of the mixture is yellow.*
12. Add **1 gel volume** of **isopropanol** to the mixtures and mix by **vortexing** for **5 seconds** or **inverting** the tubes **5-10 times**. *This increases the yield of DNA fragments. Note: Do not centrifuge the samples at this stage.*
13. Place TWO QIAquick **spin columns (purple)** in TWO **2 mL collection tubes**. Label the sides of the spin columns and collection tubes with "**T-DNA PCR**" or "**WT PCR**" and **your initials.**
14. Pipet the **mixtures** from **step 12** into the **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 14-16**.*
15. Centrifuge the tubes for **1 minute**. *This step allows DNA binding to the membrane.*
16. Separate the **spin columns** from the **collection tubes** and **pour off** the **flow-through solution** in the collection tubes. Put the spin columns back in the same collection tubes. *Keep collection tubes for use in steps 17-19.*
17. Add **500 µL** of **Buffer QG** to the **spin columns** and centrifuge for **1 minute**. Discard the flow-through solution. *This step removes all traces of agarose.*
18. Add **750** of **µL Buffer PE** to the columns and let the tubes stand for **2-5 minutes**. Centrifuge the tubes for **1 minute**. *This step washes the column.*
19. Discard the flow-through solution and centrifuge for an **additional minute** to remove all the ethanol from the columns. *Note: If any ethanol remains on the column, centrifuge for an additional 1-2 minutes.*
20. While spinning the tubes, label the lids and sides of TWO new 1.5 mL microcentrifuge tubes with "**T-DNA PCR**" or "**WT PCR**" and **your initials.**
21. After spinning, transfer the **spin columns** to the **labeled microcentrifuge tubes**. Discard the collection tubes and flow-through.

22. Add **30 µL** of **Buffer EB** to the **center of the membranes**. 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***.
23. Determine the DNA concentration using a NanoDrop spectrophotometer.

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

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

B. 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.

References: Applied Biosystems

UCLA WebSeq website http://genoseq.ucla.edu/action/view/Sequencing

Solutions Needed:

- Applied Biosystems Big Dye version 3 (Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- 5x Sequencing Buffer (Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building; or Sigma Cat. #S3938)
- 20 µM LBb1 primer
- 20 µM Gene-specific Salk Forward primer
- 20 µM Gene-specific Salk Reverse primer
- Sterile water

Materials Needed:

- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- 0.2 mL PCR tubes or strips of 8 tubes
- PCR tube rack
- Filter pipet tips
- Sequencing reaction purification columns (Qiagen DyeEx 2.0 Spin Kit; obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)

Overview:

Generally, **10 µL** reactions are set up with the following components in **0.2 mL PCR** tubes for a **single DNA template** (see table below). *Note: If you set up several sequencing reactions that share all, except one component, such as DNA template or primer, you should use a Master Mix (Mmix) to minimize the number of pipettings and mistakes of not adding some components into the individual reaction tubes.*

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

General Components of One Reaction:

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

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

y µL = the **remaining volume** to bring the **total volume** to **10 µL**

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

- For **plasmid** DNA, use **800 ng**. *Note: 250 ng of plasmid DNA will work, but more DNA gives the better reads.*
- For **PCR product**, use the amount of DNA according to the table below. (Taken from UCLA WebSeq website. Also, see Perkin-Elmer Big Dye Protocol).

Note: If the DNA concentration is too low, you may not be able to add the recommended amount of DNA. In this case, just add 6 µL.

Table: Amount of DNA to Use in a Sequencing Reaction

Size of PCR Product (bp)	Amount of DNA Used in Reaction
< 200	20 ng
200 - 300	50 ng
> 300	90 ng

For this exercise, there is **ONE DNA template** (the purified PCR product of the T-DNA fragment), but there are **TWO primers**: LBb1 (T-DNA) primer and gene-specific salk primer. The gene-specific salk primer will be either forward or reverse depending on the orientation of the T-DNA Left Border (LB) relative to the gene of interest. The orientation of the T-DNA relative to the gene of interest was determined by your analysis of the genotyping PCR results. The **sequencing reaction with** the **gene-specific primer** serves **as a control for the master mix** of Big Dye and 5x Sequencing buffer. 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

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

Sample calculations:

Size of PCR product** is **400 bp** and **its concentration** is **20 ng/µL

***Want to use 90 ng** of purified PCR product (see table above)*

*Hence, the **volume of DNA to be used** is **90 ng ÷ 20 ng/µL = 4.5 µL***

PROCEDURE

1. Get ice from the icemaker in room 4128.
2. Label the **sides** of **TWO 0.2-mL PCR tubes** with **your initials** and **primer name**. Set the tubes on a PCR tube rack sitting on ice.
3. Label the **lid** and **side** of a **1.5 mL microcentrifuge tube** as "**Mmix**" and **your initials**. Set the tube on ice.

4. Prepare a **master mix (Mmix)** for **3 reactions** (2 reactions + 1 extra) by pipetting the following components into the **Mmix tube** as shown in the table below. *Note: Use the information on the previous page to fill in the volume of DNA solution to be added and calculate the volume of water to be added to the Mmix tube for 3 reactions.*

Master Mix (Mmix) for Sequencing Reactions:

Components	**Mmix** for **ONE reaction**	**Mmix** for **3 reactions**
DNA template	**x** μL	**3x** μL
Sterile water	**y** μL	**3y** μL
Big Dye v. 3	**1** μL	**3** μL
5x Sequencing buffer	**2** μL	**6** μL
Total Volume	**9 μL**	**27 μL**

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

5. Pipet **Mmix** and **primers** into TWO labeled 0.2 mL PCR tubes.

		either	***or***
Components	**LBb1 primer**	**Gene-specific Salk Forward primer**	**Gene-specific Salk Reverse primer**
Mmix	**9 μL**	**9 μL**	**9 μL**
20 μM LB1 primer	**1 μL**	-	-
20 μM Gene-specific Salk Forward primer	-	**1 μL**	-
20 μM Gene-specific Salk Reverse primer	-	-	**1 μL**
Total volume	**10 μL**	**10 μL**	**10 μL**

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

   USER: <<hc-lab>>
   PROGRAM: **HC70AL BIG DYE**
   The profile of the Big Dye program is:
   25 cycles of 96°C 10 sec
   55°C 5 sec
   60°C 4 min
   4°C ∞

   or **Bio-Rad MyCycler** with a **Big Dye** protocol with the same profile as above.

7. After the cycling reaction is finished, clean up the sequencing reactions using the DyeEx 2.0 Spin Columns (stored in the refrigerator drawer) as following:
   a. Resuspend the resin by inversion or gently vortexing.
   b. Loosen the cap of the column a **quarter turn**. *This is necessary to avoid a vacuum inside the spin column.*
   c. Snap off the bottom closure of the spin column, and place the spin column in a 2 mL collection tube.
   d. Centrifuge at **3,000 rpm** for **3 minutes** at room temperature.
   e. Meanwhile, label a new set of 1.5 mL microcentrifuge tubes according to your reactions.
   f. Carefully transfer the spin columns to the new tubes.
   g. **Slowly** apply the sequencing reactions to the gel beds of the appropriate columns.

      Note:

      - *Pipet the sequencing reaction directly onto the center of the slanted gel-bed surface. Do not allow the reaction mixture or the pipet tip to touch the sides of the column. The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel bed surface with the pipet tip.*
      - *For easier handling, more reproducible pipetting, and reduced error with small sample volumes, you may adjust the volume of your sequencing reaction to 20 µL using distilled water, before application to the gel bed.*

h. Centrifuge the columns at **3,000 rpm** for **3 minutes** at room temperature.

i. Remove the spin columns from the microcentrifuge tubes. *The eluate contains the purified DNA*.

8. Keep samples on ice or in the refrigerator. Take the purified sequencing reactions to the UCLA Sequencing Facility located on the 5th floor in the Gonda Building. *Note: Use the primer name as the name of your sequence. Make sure to copy down the* ***assigned file number*** *(example, #*106203*), which is automatically given by the computer after you enter the samples*.
9. After one to two days, retrieve your sequences from the Sequencing Facility webpage.

C. Retrieving and Analyzing DNA Sequences

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

1. Log into WebSeq at http://www.genetics.ucla.edu/webseq/
   a. Enter Username: **goldberg_r**
   b. Enter Password: **embryo**
   c. Click "LOGIN."
2. Find your sequence files by looking up the ***assigned file number*** and the name of the gene you are working on.

   Example: The ***assigned file number*** is **106203**, and the gene of interest is **At5g09250**. You would see the following files:

   106203GoldR At5g09250Fw A12.ab1

   106203GoldR At5g09250Rv B12.ab1

   What are the annotations?

   106203 = assigned file number; **GoldR** = user name; **At5g09250Fw** = name of sequence obtained with the Forward sequencing primer, **A12** = capillary position used in loading sequencing sample in the Sequencer (Biosystems 3730 Capillary DNA Analyzer), ab1 = ABI file format.

3. Check the boxes next to the sequences to be downloaded, and click "Download selected." Alternatively, click on each filename that you want to download.
4. Open the ab1 files in the "Downloads" window using a sequence viewer program (CHROMAS on Windows, or 4PEAKS on Mac).
5. Copy DNA sequences to a Microsoft Word file. *Note: Name the files according to the name of gene of interest (for example, At5g09250).*
6. Process the DNA sequences by "BLASTN" and "BLASTX" searches. See Appendix 2. *Note: Blast search may take a few minutes or longer to complete depending on how busy the NCBI server in Washington D.C. is.*
7. Determine if the DNA sequence corresponds to the gene of interest.
8. Print out the Blast results as hard-copy records for your lab notebook.
9. Save the Blast results in the **pdf** format so that you can upload them to your webbook.