EXPERIMENT 4 – SCREENING SALK T-DNA MUTAGENESIS LINES (GENE TWO)

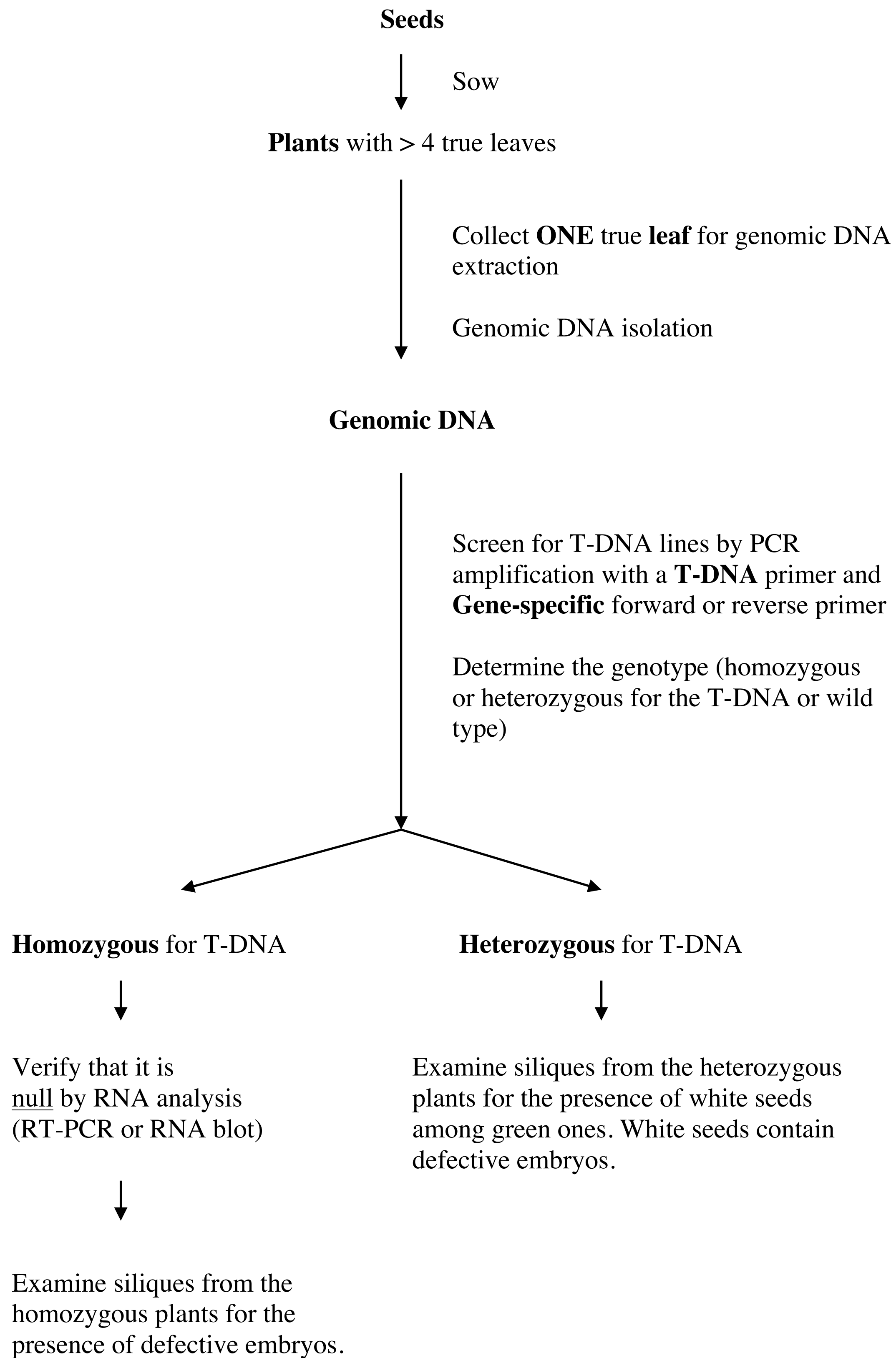
Purpose: To identify a knockout line for the gene of interest and characterize the phenotype of mutant plants.

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



I. SOWING SEEDS AND GROWING PLANTS

Purpose: To generate seedlings for genomic DNA extraction.

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

Note: TAs sowed seeds 2-3 weeks before class started

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 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 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."

PLANT LAYOUT CHART

GENOTYPING ARABIDOPSIS PLANTS

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

<u>Attention:</u> *You will need to **assess** the **quality** of **isolated genomic DNA** later (at **step 36**). 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 from **wild type** (Columbia-0)
3. Pipet **100 µL** of **Extraction Buffer** into each tube. Keep the tubes on ice. <u>*Note:*</u> *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
   - This protocol
   - 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 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 by numbering them. Also make a note of any interesting phenotypes. (For example, some plants may be smaller than others.) *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 steps 8-10 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 into the microcentrifuge tube. 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**. *Note: Position the tubes in the centrifuge so that the hinge of the microcentrifuge tubes faces the outside of the microcentrifuge. This way after centrifugation you know to look for your pellet on the side of the microcentrifuge tube that has the hinge.*
18. Meanwhile, **label** a set of **microcentrifuge tubes** with **Gene Name, plant #,** "**gDNA**," **your initials** and the **date.**
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: Use your pipet to draw off liquid from the side of the tube opposite to the side where the plant material is pelleted. Start at the top and move downward as the liquid level drops. 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 or pipet each isopropanol supernatant into a waste container. *Note: Be extremely careful when pouring off the isopropanol because the pellets are sometimes loose. DNA is now in your white or clear pellet along with RNA.*
26. Add **1 mL** of **80% ethanol** solution to each pellet. Close the lid of the tube and invert **5 times**. *This step is to wash off any residual salt (from the extraction buffer) and isopropanol.*
27. Centrifuge the tubes at **room temperature** for **5 minutes**.
28. Pour or pipet each ethanol supernatant into a waste container. Dab the tubes upside down 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. *Note: You may use a P-200 pipette to carefully draw off excess ethanol from the side of the tube opposite that against which the nucleic acid is pelleted. Be very careful not to pipet the pellet.*
30. Dry pellets either in a **SpeedVac** at room temperature for **5-10 minutes** (Your instructor will operate the SpeedVac) or by leaving on the **bench at room temperature for up to 60 minutes**.

31. After drying the pellets, resuspend each pellet by adding **200 µL** of **TE** buffer, closing the lids of the tubes, and **raking** the tubes over a microcentrifuge tube rack **10 times** <u>or</u> **vortexing** the tubes briefly. <u>*Note:*</u> *If you vortex genomic DNA vigorously or for a long time, it will degrade.*
32. Spin the tubes in a microcentrifuge for **1 minute** to bring the liquid and any contaminants to the bottom of the tubes.
33. Store DNA solutions at **4°C** (on ice or in refrigerator) until use.
    <u>*Note:*</u> *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 that can degrade DNA.*

    <u>***Attention:***</u> *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**.
    <u>*Note:*</u> *First, 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 lids as well as any contaminating plant debris and/or carbohydrates in the solutions to the bottom of the tubes.*
    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). <u>*Note:*</u> *The agarose gel can be prepared before the collection of leaves for the extraction of genomic DNA.*
    b. Label the lids of SIX 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 step 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	

Lane	Sample	Expected Size (bp)
1	1 Kb Plus DNA ladder	-
2	Genomic DNA from Plant #7	
3	Genomic DNA from Plant #8	
4	Genomic DNA from Plant #9	
5	Genomic DNA from Plant #10	
6	Genomic DNA from Plant #11	
7	Genomic DNA from Plant #12	

h. Add **10 μL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode (near the bottom of the gel).

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?

   Do you observe any other bands? What do you think they are?

35. Determine the concentration of DNA using the NanoDrop spectrophotometer. *Your instructor will operate the NanoDrop.*

<u>Note:</u> First, 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 lids as well as any contaminating plant debris and/or carbohydrates in the solutions to the bottom of the tubes.*

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

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

Sample	**DNA Concentration (ng/μL)**
Plant #7	
Plant #8	
Plant #9	
Plant #10	
Plant #11	
Plant #12	

36. Store DNA solutions at **4°C** (on ice or in refrigerator) until use.

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)
- dNTP mix (Takara)
- Sterile water
- 12 μM Gene-specific Salk Forward primer
- 12 μM Gene-specific Salk Reverse primer
- 12 μM LBb1.3 primer (anneals to the Left Border (LB) region of the T-DNA)
- Genomic DNA extracted from the plants to be genotyped (including WT)
- Genomic DNA extracted from wild type seedlings by TA
- 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 in strips of 8
- 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 LBb1.3 primer, you will need to set up ***THREE*** *PCR reactions for* ***each*** *sample.*

Reaction A: ***Gene-specific Salk Forward*** *primer and* ***LBb1.3*** *primer*

Reaction B: ***LBb1.3*** *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 LBb1.3 primer** on a rack for 1.5 mL microcentrifuge tubes at **room temperature** for 5-10 minutes.
3. **Vortex** each tube on a setting of 2-3 for 10 seconds. **Spin** in microcentrifuge at full speed for 5 seconds. Put the tubes on **ice** until needed.

4. Spin genomic DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed. Keep on ice.
5. Label the lids and sides of **24 PCR tubes** with **A1-A8, B1-B8, C1-C8** and **your initials** and the **date**. Put them on a PCR tube rack sitting on ice.
6. Prepare a **"Reaction A" master mix** for **9 PCR reactions** in a 1.5 mL microcentrifuge tube labeled "**Mmix A**" sitting on **ice**. Pipet the reagents in order from top down (example: water, 10x Ex Taq buffer, dNTP mix, etc.) into the **Mmix A** tube.

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 **LBb1.3** 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**

7. 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 **5 seconds**. Put the tube back **on ice**.
8. Pipet **46 μL** of **Mmix A** into each of **8 PCR tubes** labeled **A1-A8**.
9. Pipet **4 μL** of **genomic DNA** extracted from each of **6 seedlings** into PCR tubes A1-A6. Pipet up and down 5 times to mix the contents.
10. Pipet **4 μL** of **genomic DNA** extracted from **wild type** (Col-0) seedlings by TA into tube **A7**. Pipet up and down 5 times to mix the contents.
11. Pipet **4 μL** of **sterile water** into tube **A8** (**negative control** without DNA template). Pipet up and down 5 times to mix the contents.
12. 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 **LBb1.3** 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 LBb1.3 primer	-	-
Ex Taq DNA polymerase (5 U/µL)	0.25 µL	2.25 µL
Total Volume	**46.0 µL**	**414.0 µL**

13. Spin all PCR tubes in the minicentrifuge for PCR tubes for **5 seconds** to bring the liquid to the bottom of the tubes.
14. Turn **ON** the PCR machine (MyCycler). Wait for one minute for the machine to initialize. *Your instructor will operate the PCR machine*.
15. Put the PCR tubes in the wells of the 96-well hot plate of the MyCycler.
16. Select the "**Protocol Library**" by pressing "**F1**."
17. Select "**HC70AL**" by pressing the yellow arrowheads surrounding the "**ENTER**" button. Select the "**HC70AL KNOCKOUT**" protocol. Press "**ENTER**."
18. 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 ∞

19. Press "**F5**" for "**DONE.**" The "CHOOSE OPERATION" menu will appear. Press "**ENTER**" to **run the protocol**.
20. Enter the **volume** of the PCR reaction (50 μL). Press "**F5**" to "**Begin Run**." *Note: It will take about 3 hours for the PCR amplification to be completed.*
21. Once the PCR amplification is complete, remove the PCR tubes from the PCR machine and store them on ice or 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 (or 20-tooth comb depending on how many samples you have).
2. Record the identity of the sample in each lane. Use the table below as a guide.
3. Label **24 1.5 mL microcentrifuge** tubes (one for each sample) and set them on a microcentrifuge tube rack.
4. Add **3 μL** of **6x loading dye** to each tube.
5. Pipet **25 μL** of **PCR solution** to each tube. Spin briefly. *Note: Store remaining PCR product in the PCR tubes in the refrigerator.*
6. Slowly pipet **10 μL** of **100 bp DNA ladder** in the first well. *Note: Avoid pipetting bubbles into the well.*
7. Mix the contents of your first sample-dye mixture by pipetting up and down 5 times. Slowly pipet **~28 μL** of sample-dye mixture into the next well using a P-20 pipette *(i.e. load ~15 μL first, then then remaining ~15 μL into the same well)*. Repeat for the remaining sample-dye mixtures.
8. Slowly pipet **10 μL** of **1 Kb Plus DNA ladder** in the last well.

Lane	Sample	Primers	Expected Size (bp)
1	100 bp DNA ladder	-	-
2	Genomic DNA from Plant #1	Reaction A: **Gene-specific Salk Forward** primer and **LBb1.3** 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 TA)		-
9	Sterile water		-
10	100 bp DNA ladder		-
11	Genomic DNA from Plant #1	Reaction B: **LBb1.3** primer and **Gene-specific Salk Reverse** primer	
12	Genomic DNA from Plant #2		
13	Genomic DNA from Plant #3		
14	Genomic DNA from Plant #4		
15	Genomic DNA from Plant #5		
16	Genomic DNA from Plant #6		
17	Col-0 Genomic DNA (from TA)		-
18	Sterile water		-
19	100 bp DNA ladder		-
20	Genomic DNA from Plant #1	Reaction C: **Gene-specific Salk Forward** primer and **Gene-specific Salk Reverse** primer	
21	Genomic DNA from Plant #2		
22	Genomic DNA from Plant #3		
23	Genomic DNA from Plant #4		
24	Genomic DNA from Plant #5		
25	Genomic DNA from Plant #6		
26	Col-0 Genomic DNA (from TA)		
27	Sterile water		-
28	1 Kb Plus DNA ladder	-	-

9. Add **10 μL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode (near the bottom of the gel).
10. Run the gel at **105 volts** for **1-2 hours** or until the lower dye (bromophenol blue) travels two-thirds of the gel.
11. Stop the gel electrophoresis.
12. Take a picture of the gel and annotate it.
13. 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 **good scientific practice** to verify the exact location of the T-DNA insertion site by purifying the "T-DNA fragment" and sequencing it.*

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."*
    - c. *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.*
    - d. *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.

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 25 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 (~25 µL)

Materials Needed:

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

- Kimwipes

PROCEDURE

1. Write "**T-DNA**" and **your initials** on a 1.5 mL microcentrifuge tube.
2. Pipet **~25 μL** of the **PCR product solution** from one of the PCR tubes containing the T-DNA fragment into the labeled 1.5 mL microcentrifuge tube.
3. Measure the exact volume of solution.
4. Add **125 μL** of **Buffer PB** (or **5 volumes** of **Buffer PB** to **1 volume** of the PCR product solution) to the tube in step 2. Mix by vortexing for 5 seconds.
5. 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 tube rack.
6. Place a **QIAquick spin column** (lilac) in the provided **2 mL collection tube**. Label the lid of the column "**T-DNA**" and **your initials**.
7. Pipet the sample mixture in step 4 to the QIAquick spin column. Spin the column and collection tube in the microcentrifuge at **FULL speed** for **1 minute**. *This step allows the binding of DNA to the membrane.*
8. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube.
9. Add **750 μL** of **Buffer PE** to the QIAquick spin column and spin at **FULL speed** for **1 minute**.
10. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube.
11. Spin the column and collection tube 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.*

12. While spinning, label a 1.5 mL microcentrifuge tube "**PCR Purified T-DNA,**" **your initials** and the **date**.
13. Transfer the **QIAquick column** to the NEWLY labeled microcentrifuge tube. Discard the flow-through solution and the collection tube.
14. Pipet **30 μL** of **Buffer EB** to the **center** of the QIAquick column membrane. 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. Note: If some liquid remains on the column after centrifugation, remove it with a P10 pipette and dispense the liquid back onto the center of the QIAquick membrane. Centrifuge again at FULL speed for 1 minute.*
15. 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 25 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)

Solutions Needed:

- Your agarose gel with bands containing PCR products
- QIAquick Gel Extraction Kit (Qiagen, Cat. #28704)
- Isopropanol
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain (Invitrogen)
- 50 ng/μL 1 Kb Plus DNA ladder (Invitrogen)

- 6x Loading Dye containing xylene cyanol and bromophenol blue dyes

Materials Needed:

- Pipettes
- Filter pipet tips
- Black ultra-fine sharpie pen
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Gel electrophoresis materials (Appendix 1A)
- Razor blade
- Metal waste container for sharp objects
- Saran wrap
- UV light box
- Scale
- Microcentrifuge
- Vortex
- 50°C water bath or heat block
- 65°C water bath or heat block
- Timer

PROCEDURE

1. Label a microcentrifuge tube "**T-DNA**" 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 T-DNA fragment from the gel using a razor blade. <u>Note:</u> *Trim off excess agarose surrounding the DNA band as much as possible. Your TA will demonstrate.*
6. Place the agarose slice in the 1.5 mL microcentrifuge tube.
7. Take a picture of the gel **after removing the agarose slice**. *This step serves as a record of the DNA fragment being collected.*

<u>Note:</u> *If the desired band is faint, you many collect all the T-DNA bands on the gel and pool them together. Alternatively, run the remaining 25 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 **~25 µL** PCR solutions containing the "T-DNA fragment."*

*c. Slowly pipet **10 µL of 1 Kb Plus DNA ladder** into the first well. <u>Note:</u> Avoid pipetting bubbles into the well.*

*d. Mix the contents of your first sample-dye mixture by pipetting up and down 5 times. Slowly pipet **~28 µL** of sample-dye mixture into the next well using a P-20 pipette (i.e. load ~15 µL first, then then remaining ~15 µL into the same well).*

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 anode.*

*g. Run the gel at **105 volts** for **1-2 hours** or until the lower dye (bromophenol blue) travels two-thirds of the gel.*

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**. <u>Note:</u> 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. <u>Note:</u> Trim off excess agarose surrounding the DNA band as much as possible. Your TA will demonstrate.

n. *Place the agarose slices in the 1.5 mL microcentrifuge tube from step 6 labeled "**T-DNA**" 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.*

Note: Be sure to preform steps 8-25 at room temperature.

8. Estimate the **gel volume** in the microcentrifuge tube using a **scale**. Use an empty 1.5 mL microcentrifuge tube as a blank. Write the **gel volume** on the side of the tube. *Note: **0.1 g** of **agarose gel** is **equivalent to 100 µL**.*
9. Add **3 gel volumes** of **Buffer QG** to the tube containing the gel 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.*
10. Incubate the tube at **50°C** for **10 minutes** or until the gel slice has **completely** dissolved. *Do **not** incubate at 50°C for more than 10 minutes! 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.*
11. Add **1 gel volume** of **isopropanol** to the mixture 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.*
12. Place a **QIAquick spin column** (lilac) in a provided **2 mL collection tube**. Label the lid of the spin column with "**T-DNA**" and **your initials**. *Note: If the gel bands were faint, use one column for multiple gel slices. This will increase the final concentration of purified DNA.*
13. Pipet the **mixture** from **step 12** into the **spin column**. *Do NOT pipet **more than 800 µL** of the mixture into the column. If the total volume is **more than 800 µL**, repeat **steps 13-15**.*
14. Centrifuge the spin column in the collection tube for **1 minute**. *This step allows DNA binding to the membrane.*
15. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube.

16. Add **500 µL** of **Buffer QG** to the **spin column** and centrifuge at FULL speed for **1 minute**. Discard the flow-through solution. *This step removes all traces of agarose.*
17. Add **750 µL** of **Buffer PE** to the column and let the tube stand for **2-5 minutes**. Centrifuge the tube at FULL speed for **1 minute**. Discard the flow-through solution. *This step washes the column.*
18. Repeat step 17 **two more times**.
19. Discard the flow-through solution and centrifuge for an **additional minute** to remove all the ethanol from the column. *Note: If any ethanol remains on the column, centrifuge for an additional 1-2 minutes.*
20. While spinning the tube, label a new 1.5 mL microcentrifuge tube with "**Gel Purified T-DNA**", **your initials** and **the date.**
21. Transfer the **QIAquick column** to the NEWLY labeled microcentrifuge tube. Discard the flow-through solution and the collection tube.
22. Add **30 µL** of **Buffer EB** to the **center of the column membrane**. Let the column stand for **1 minute**, and then centrifuge for **1 minute**. *This step elutes the DNA from the membrane. **DNA** is in the **microcentrifuge tube**. Note: If some liquid remains on the column after centrifugation, remove it with a P10 pipette and dispense the liquid back onto the center of the column membrane. Centrifuge again at FULL speed for 1 minute.*
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 the T-DNA insertion site in the gene of interest of 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)
- 5x Sequencing Buffer (Obtained from UCLA Sequencing Facility or Sigma Cat. #S3938)
- 12 μM LBb1.3 primer
- 12 μM Gene-specific Salk Forward primer OR 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)

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
12 μM 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 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. Alternatively: You may use the speed vac to concentrate your DNA.

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**: LBb1.3 (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

<u>Sample calculations:</u>

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. Thaw **12 μM primers** and **5x sequencing buffer** on a microcentrifuge tube rack at room temperature for 5 minutes. After the tubes have thawed, vortex briefly and centrifuge at full speed for 5 seconds. Place tubes on ice.
3. Label the top or sides of **TWO 0.2 mL PCR tubes** with **your initials** and **"T-DNA"** or **"Gene"**. Set the tubes on a PCR tube rack sitting on ice.
4. Label a **1.5 mL microcentrifuge tube** as "**Mmix**" and **your initials**. Set the tube on ice.
5. 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. *<u>Note:</u> 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 contents to the bottom of the tube.
c. Set the tube back on ice.

6. Pipet **Mmix** and **primers** into the TWO labeled 0.2 mL PCR tubes. Pipet up and down 5 times to mix the contents.

either ***or***

Components	**LBb1.3 primer**	**Gene-specific Salk Forward primer**	**Gene-specific Salk Reverse primer**
Mmix	**9 µL**	**9 µL**	**9 µL**
12 µM LB1 primer	**1 µL**	-	-
12 µM Gene-specific Salk Forward primer	-	**1 µL**	-
12 µM Gene-specific Salk Reverse primer	-	-	**1 µL**
Total volume	**10 µL**	**10 µL**	**10 µL**

7. Spin PCR tubes in the minicentrifuge for PCR tubes for **5 seconds** to bring the liquid to the bottom of the tubes.
8. 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.

9. 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 flicking or gently vortexing upside down.

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 discard it. Place the spin column in a 2 mL collection tube.
d. Centrifuge at **3,000 rpm** for **3 minutes** at room temperature.
e. Meanwhile, clearly label a new set of 1.5 mL microcentrifuge tubes with your initials and the primer name.
f. Carefully transfer the spin columns to the new tubes.
g. **Slowly** apply the sequencing reactions to the gel beds of the appropriate columns.

   <u>Note:</u> 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.
h. Centrifuge at **3,000 rpm** for **3 minutes** at room temperature.
i. Discard the spin columns. *The eluate in the microcentrifuge tubes contains the purified DNA.*

10. Keep samples on ice or in the refrigerator. Take the purified sequencing reactions to the UCLA Sequencing Facility. *<u>Note:</u> Use your initials and 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.*
11. After one to two days, retrieve your sequences from the Sequencing Facility webpage.

C. Retrieving and Analyzing DNA Sequences

<u>Purpose:</u> To determine the identity of your gene that has been knockout out by the T-DNA and the exact location of the T-DNA insertion.

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 your gene of interest (for example, At5g09250).*
6. Determine the identity of your gene that has been knockout out by the T-DNA and the exact location of the T-DNA insertion 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. Print out the Blast results as hard-copy records for your lab notebook.
8. Save the Blast results in the **pdf** format so that you can upload them to your webbook.