EXPERIMENT 5 – 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 WHAT GENE YOU ARE STUDYING AND 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

Verify that it is null by RNA analysis (RT-PCR or RNA blot)

Examine siliques from the homozygous plants for the presence of defective embryos.

Heterozygous for T-DNA

Examine siliques from the heterozygous plants for the presence of white seeds among green ones. White seeds contain defective embryos.

Screening SALK T-DNA Mutagenesis Lines (Gene TWO) 5.2
I. SOWING SEEDS AND GROWING PLANTS

**Purpose:** To generate seedlings for genomic DNA extraction.

**Reference:** Arabidopsis Biological Resource Center [http://abrc.osu.edu/](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 the 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**. *Note: 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. *NOTE: 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

<table>
<thead>
<tr>
<th>Gene ID: At__ g __________</th>
<th>Salk line#: ____________</th>
<th>Date: ________</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers for PCR: __________</td>
<td>________________________</td>
<td></td>
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<tr>
<td>Size of PCR product: ______</td>
<td>________________________</td>
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<table>
<thead>
<tr>
<th>Pot #</th>
<th>Pot #</th>
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**Screening SALK T-DNA Mutagenesis Lines (Gene TWO)** 5.7
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
PROCEDURE

Attention: 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/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
   ➢ 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 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 by numbering them. Also make a note of any interesting phenotypes. (For example, some plants may be smaller than others.)

   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 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 *micropostle* until no more chunks of plant tissue are observed in the mixture. *Note: Do NOT dispose of the micropostle.*

14. Rinse the *micropostle* with 300 µL of Extraction Buffer into the microcentrifuge tube. Put the used micropostles in a beaker labeled “used micropostles” 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 that against which 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: 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 (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 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 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 200 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 briefly. **Note:** 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 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.

   **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**.

   **Note:** 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). **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 step d using a P-20 pipette.

   g. Record the identity of the sample in each well.
5. **13** Lane

<table>
<thead>
<tr>
<th>Sample</th>
<th>Expected Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Kb Plus DNA ladder</td>
<td>-</td>
</tr>
<tr>
<td>Genomic DNA from Plant #1</td>
<td></td>
</tr>
<tr>
<td>Genomic DNA from Plant #2</td>
<td></td>
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<tr>
<td>Genomic DNA from Plant #3</td>
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<tr>
<td>Genomic DNA from Plant #4</td>
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<tr>
<td>Genomic DNA from Plant #5</td>
<td></td>
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<tr>
<td>Genomic DNA from Plant #6</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>Expected Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 Kb Plus DNA ladder</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Genomic DNA from Plant #7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Genomic DNA from Plant #8</td>
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<tr>
<td>4</td>
<td>Genomic DNA from Plant #9</td>
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<tr>
<td>5</td>
<td>Genomic DNA from Plant #10</td>
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<tr>
<td>6</td>
<td>Genomic DNA from Plant #11</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Genomic DNA from Plant #12</td>
<td></td>
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</tbody>
</table>

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

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 demonstrate how to use the NanoDrop.*
Note: 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:

<table>
<thead>
<tr>
<th>Sample</th>
<th>DNA Concentration (ng/µL)</th>
<th>Sample</th>
<th>DNA Concentration (ng/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant #1</td>
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<td>Plant #7</td>
<td></td>
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<tr>
<td>Plant #2</td>
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<td>Plant #8</td>
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<td>Plant #3</td>
<td></td>
<td>Plant #9</td>
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<td>Plant #4</td>
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<td>Plant #10</td>
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<tr>
<td>Plant #5</td>
<td></td>
<td>Plant #11</td>
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<tr>
<td>Plant #6</td>
<td></td>
<td>Plant #12</td>
<td></td>
</tr>
</tbody>
</table>

36. Dilute 5 µL of original DNA solutions to a final concentration of 0.2 ng/µL with TE buffer.

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,

\[ V_i \times C_i = V_f \times C_f \]

where,

\[ V_i = \text{initial volume} \] (the volume of original DNA solution is 5 µL)

\[ C_i = \text{initial concentration} \] (reading from the spectrophotometer; example: 8 ng/µL)

\[ V_f = \text{final volume} \] (depends on the initial concentration)
C_f = final concentration (0.2 ng/µL)
then,

\[ V_f = (V_i \times C_i)/C_f = (5 \, \mu L \times 8 \, \text{ng/µL})/(0.2 \, \text{ng/µL}) = 200 \, \mu L \] of total volume

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

\[ V_{TE} = V_f - V_i = 200 \, \mu L - 5 \, \mu L = 195 \, \mu L \] of TE

a. Record the volume of TE and the final volume in the table below.

<table>
<thead>
<tr>
<th>Plant #</th>
<th>Volume of Isolated Genomic DNA</th>
<th>Volume of TE Added</th>
<th>Final Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant #1</td>
<td>5 µL</td>
<td></td>
<td></td>
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<tr>
<td>Plant #2</td>
<td>5 µL</td>
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<tr>
<td>Plant #3</td>
<td>5 µL</td>
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<td>Plant #4</td>
<td>5 µL</td>
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<td>Plant #5</td>
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<td>Plant #6</td>
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<td>Plant #7</td>
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<td>Plant #8</td>
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<td>Plant #9</td>
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<td>Plant #10</td>
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<td>Plant #11</td>
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<tr>
<td>Plant #12</td>
<td>5 µL</td>
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</table>

*Note: If the volume of TE to be used in dilution is greater than 1.5 mL, it will not fit in a 1.5 mL microcentrifuge tube. In this case, dilute 2.5 µL of genomic DNA.*
b. Label the lids and sides of microcentrifuge tubes with the following information: 0.2 ng/µL gDNA, plant #, your initials and the date. Keep all tubes of DNA solutions on ice.

c. 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.

d. Add the appropriate volume of TE to each newly labeled tube.

e. Add 5 µL of original DNA solutions into each tube. Flick tubes to mix.

f. Spin the tubes in a microcentrifuge for 1 minute to bring liquid and any contaminants to the bottom of the tubes.

g. 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; 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 LBb1.3 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 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
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. 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 the **date**. 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. Pipet the reagents in order from top down (example: water, 10x Ex Taq buffer, dNTP mix, etc.) into the **Mmix A** tube.

<table>
<thead>
<tr>
<th>Reaction A</th>
<th>Mmix for ONE reaction</th>
<th>Mmix for 9 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>34.75 µL</td>
<td>312.75 µL</td>
</tr>
<tr>
<td>10x Ex Taq buffer</td>
<td>5.0 µL</td>
<td>45.0 µL</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>4.0 µL</td>
<td>36.0 µL</td>
</tr>
<tr>
<td>12 µM Gene-specific Salk Forward primer</td>
<td>1.0 µL</td>
<td>9.0 µL</td>
</tr>
<tr>
<td>12 µM Gene-specific Salk Reverse primer</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12 µM LBb1.3 primer</td>
<td>1.0 µL</td>
<td>9.0 µL</td>
</tr>
<tr>
<td>Ex Taq DNA polymerase (5 U/µL)</td>
<td>0.25 µL</td>
<td>2.25 µL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>46.0 µL</strong></td>
<td><strong>414.0 µL</strong></td>
</tr>
</tbody>
</table>

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.
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

---

### Reaction B

<table>
<thead>
<tr>
<th></th>
<th>Mmix for ONE reaction</th>
<th>Mmix for 9 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>34.75 µL</td>
<td>312.75 µL</td>
</tr>
<tr>
<td>10x Ex Taq buffer</td>
<td>5.0 µL</td>
<td>45.0 µL</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>4.0 µL</td>
<td>36.0 µL</td>
</tr>
<tr>
<td>12 µM Gene-specific Salk Forward primer</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12 µM Gene-specific Salk Reverse primer</td>
<td>1.0 µL</td>
<td>9.0 µL</td>
</tr>
<tr>
<td>12 µM LBr1.3 primer</td>
<td>1.0 µL</td>
<td>9.0 µL</td>
</tr>
<tr>
<td>Ex Taq DNA polymerase (5 U/µL)</td>
<td>0.25 µL</td>
<td>2.25 µL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>46.0 µL</td>
<td>414.0 µL</td>
</tr>
</tbody>
</table>

### Reaction C

<table>
<thead>
<tr>
<th></th>
<th>Mmix for ONE reaction</th>
<th>Mmix for 9 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>34.75 µL</td>
<td>312.75 µL</td>
</tr>
<tr>
<td>10x Ex Taq buffer</td>
<td>5.0 µL</td>
<td>45.0 µL</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>4.0 µL</td>
<td>36.0 µL</td>
</tr>
<tr>
<td>12 µM Gene-specific Salk Forward primer</td>
<td>1.0 µL</td>
<td>9.0 µL</td>
</tr>
<tr>
<td>12 µM Gene-specific Salk Reverse primer</td>
<td>1.0 µL</td>
<td>9.0 µL</td>
</tr>
<tr>
<td>12 µM LBr1.3 primer</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ex Taq DNA polymerase (5 U/µL)</td>
<td>0.25 µL</td>
<td>2.25 µL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>46.0 µL</td>
<td>414.0 µL</td>
</tr>
</tbody>
</table>
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 (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. Mix the contents by pipetting up and down 5 times or by flicking the tubes. Spin briefly.
6. Load 10 µL of 100 bp DNA ladder in the first well.
7. Very slowly load the ~28 µL sample-dye mixtures on the gel using a P-20 pipette (i.e. load ~15 µL first, then then remaining ~15 µL into the same well).
8. Load 10 µL of 1 Kb Plus DNA ladder in the last well.
<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>Primers</th>
<th>Expected Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 bp DNA ladder</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Genomic DNA from Plant #1</td>
<td>Reaction A: Salk Forward Forward primer and LBB1.3 primer</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Genomic DNA from Plant #2</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Genomic DNA from Plant #3</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Genomic DNA from Plant #4</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Genomic DNA from Plant #5</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Genomic DNA from Plant #6</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Col-0 Genomic DNA (from TAs)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Sterile water</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>100 bp DNA ladder</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Genomic DNA from Plant #1</td>
<td>Reaction B: LBB1.3 primer and Gene-specific Salk Reverse primer</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Genomic DNA from Plant #2</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Genomic DNA from Plant #3</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>Genomic DNA from Plant #4</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>Genomic DNA from Plant #5</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>Genomic DNA from Plant #6</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>Col-0 Genomic DNA (from TAs)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>Sterile water</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>100 bp DNA ladder</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>Genomic DNA from Plant #1</td>
<td>Reaction C: Gene-specific Salk Forward primer and Gene-specific Salk Reverse primer</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>Genomic DNA from Plant #2</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>Genomic DNA from Plant #3</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>Genomic DNA from Plant #4</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>Genomic DNA from Plant #5</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>Genomic DNA from Plant #6</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>Col-0 Genomic DNA (from TAs)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>27</td>
<td>Sterile water</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>28</td>
<td>1 Kb Plus DNA ladder</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
9. Add 5 µL of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.

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 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. You may take flowers back to the lab to observe the phenotype under a microscope.
## PHENOTYPE OBSERVATION RECORD

| Gene ID: At__g ___________ Salk line#: ______________ Date: ___________ |
|------------------------|-----------------|-----------------|

### LEAF

<table>
<thead>
<tr>
<th></th>
<th>Mutant</th>
<th>Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>What do the leaves look like, green or yellow, elongated or round?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>What is the range of their length in cm?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>How many leaves does each plant have?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Is the range of leaf sizes of the mutant plant smaller or larger or similar to wild type leaves?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### STEM

<table>
<thead>
<tr>
<th></th>
<th>Mutant</th>
<th>Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>What is the height of the main (or longest) stem?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>What is the thickness of the stem?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>How many stems (or branches including the main and side ones) does the plant have?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### FLOWERS

<table>
<thead>
<tr>
<th></th>
<th>Mutant</th>
<th>Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do the flowers have all FOUR floral organs (green sepals, white petals, yellow anthers, green pistils)?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>How many sepals are on each flower?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>How many petals are on each flower?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>How many anthers are on each flower?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>How many pistils are on each flower?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### SILIQUES, SEEDS AND EMBRYOS

<table>
<thead>
<tr>
<th></th>
<th>Mutant</th>
<th>Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>How many siliques are on each plant?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you see a difference in the lengths of siliques?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>How many seeds are in EACH silique?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>What is the average number of seeds in FIVE siliques?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you see different COLORED seeds within a single silique?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>If yes, what colors are the seeds? How many seeds of each color?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>What stage of embryos (globular, heart, torpedo, cotyledon, mature green, or post mature green) do you see?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
IV. DETERMINING WHAT GENE YOU ARE STUDYING AND THE T-DNA INSERTION SITE

**Purpose:** To determine the identity of the gene you are studying, and 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/](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
PROCEDURE

1. Label the lids of TWO 1.5 mL microcentrifuge tubes with “T-DNA” or “WT” and your initials.
2. Pipet ~25 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. Measure the exact volumes of solution.
4. 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.
5. 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.
6. Place TWO QIAquick spin columns (lilac) in the provided 2 mL collection tubes. Label the lids of the columns with “T-DNA” or “WT” and your initials.
7. Pipet the sample mixtures in step 4 to the QIAquick spin columns. Spin the columns in the collection tubes 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 tubes by pouring it into a waste container. Blot the collection tubes on Kimwipes. Put the QIAquick columns back into the same collection tubes.
9. Add 750 µL of Buffer PE to the QIAquick spin columns and spin at FULL speed for 1 minute.
10. Discard the flow-through solution in the collection tubes by pouring it into a waste container. Blot the collection tubes on Kimwipes. Put the QIAquick columns back into the same collection tubes.
11. 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.

12. While spinning, label TWO 1.5 mL microcentrifuge tubes “PCR Purified T-DNA” or “PCR Purified WT,” your initials and the date.

13. Transfer the QIAquick columns to the appropriate NEWLY labeled microcentrifuge tubes. Discard the flow-through solutions and the collection tubes.

14. 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.* **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:**

Screening SALK T-DNA Mutagenesis Lines (Gene TWO) 5.28
Remaining PCR solutions (~25 µL)
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
- Timer

**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. *Note: 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. *Note: 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.

Note: If the desired bands were faint, 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” or the “WT DNA fragment.”
   c. Load 10 µL of 1 Kb Plus DNA ladder into the first well.
   d. Very slowly load the ~28 µL sample-dye mixtures on the gel using a P-20 pipette.
   e. Record the identity of the sample in each well.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>Primers</th>
<th>Expected Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 Kb Plus DNA ladder</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

   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 1.5 - 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.

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

8. Pipet Elution Buffer into a 1.5 mL microcentrifuge tube labeled with “EB” and your initials. Pipet 30 µL x (number of samples +1). Warm the 1.5 mL microcentrifuge tube with elution buffer to 65°C in a heat block. This will be used in step 25 to remove the DNA from the membrane.

9. Centrifuge the gel slice at full speed for 1 minute.

10. 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.

11. 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.

12. 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.

13. 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.

14. 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” or “WT” and your initials.

Screening SALK T-DNA Mutagenesis Lines (Gene TWO) 5.31
15. 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 15-17.

16. Centrifuge the tubes for 1 minute. This step allows DNA binding to the membrane.

17. Discard the flow-through solution in the collection tubes by pouring it into a waste container. Blot the collection tubes on Kimwipes. Put the QIAquick columns back into the same collection tubes. Keep collection tube for use in steps 17-19.

18. 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.

19. Add 750 µL Buffer PE to the columns and let the tubes stand for 2-5 minutes. Centrifuge the tubes at FULL speed for 1 minute. Discard the flow-through solution. This step washes the column.

20. Repeat step 19 two more times.

21. 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.

22. While spinning the tubes, label the lids and sides of TWO new 1.5 mL microcentrifuge tubes with “Gel Purified T-DNA” or “Gel Purified WT” and your initials.

23. After spinning, transfer the spin columns to the labeled microcentrifuge tubes. Discard the collection tubes and flow-through.

24. 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. 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.

25. Determine the DNA concentrations using a NanoDrop spectrophotometer.

   What is the concentration of purified T-DNA PCR product? _____ ng/µL

   What is the size (in bp) of the T-DNA PCR product from gel electrophoresis? _____ bp
What is the concentration of purified WT PCR product? _____ ng/µL
What is the size (in bp) of the WT PCR product from gel electrophoresis? _____ bp

B. Sequencing Reaction with Big Dye v. 3

Purpose: To determine the identity of the gene you are studying, and 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/

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 LBb1.3 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:**

<table>
<thead>
<tr>
<th></th>
<th>ONE Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template *</td>
<td>x µL</td>
</tr>
<tr>
<td>Sterile water</td>
<td>y µL</td>
</tr>
<tr>
<td>20 µM Sequencing primer</td>
<td>1 µL</td>
</tr>
<tr>
<td>Big Dye v. 3</td>
<td>1 µL</td>
</tr>
<tr>
<td>5x Sequencing buffer</td>
<td>2 µL</td>
</tr>
<tr>
<td>Total volume</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

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. Alternatively: You may use the speed vac to concentrate your DNA.
Table: Amount of DNA to Use in a Sequencing Reaction

<table>
<thead>
<tr>
<th>Size of PCR Product (bp)</th>
<th>Amount of DNA Used in Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 200</td>
<td>20 ng</td>
</tr>
<tr>
<td>200 - 300</td>
<td>50 ng</td>
</tr>
<tr>
<td>&gt; 300</td>
<td>90 ng</td>
</tr>
</tbody>
</table>

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

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 two master mixes (Mmixs) for 3 reactions each (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:**

<table>
<thead>
<tr>
<th>Components</th>
<th>ONE reaction</th>
<th>3x Mmix for T-DNA allele</th>
<th>3x Mmix for WT allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td>x µL</td>
<td>3x µL</td>
<td>3x µL</td>
</tr>
<tr>
<td>Sterile water</td>
<td>y µL</td>
<td>3y µL</td>
<td>3y µL</td>
</tr>
<tr>
<td>Big Dye v. 3</td>
<td>1 µL</td>
<td>3 µL</td>
<td>3 µL</td>
</tr>
<tr>
<td>5x Sequencing buffer</td>
<td>2 µL</td>
<td>6 µL</td>
<td>6 µL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>9 µL</td>
<td>27 µL</td>
<td>27 µL</td>
</tr>
</tbody>
</table>

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 9 µL of WT Mmix into each of TWO 0.2 mL PCR tubes labeled with “WT,” your initials and “FW” or “RV.” Add 1 µL of 20 µM Gene-specific Salk Forward primer to one tube and 1 µL of 20 µM Gene-specific Salk Reverse primer to the other tube. Mix and spin briefly.

6. Pipet 9 µL of T-DNA Mmix into each of TWO 0.2 mL PCR tubes labeled with “T-DNA,” your initials, “LBb1.3” and either “FW” or “RV.” Add 1 µL of 20 µM LBb1.3 to one tube and 1 µL of either 20 µM Gene-specific Salk Forward primer or 20 µM Gene-specific Salk Reverse primer to the other tube. Mix and spin briefly.

7. 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.
8. 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.
   h. Centrifuge the columns at 3,000 rpm for 3 minutes at room temperature.

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.
9. 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.

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

C. Retrieving and Analyzing DNA Sequences

**Purpose:** To determine the identity of the gene you are studying, and 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/](http://signal.salk.edu/)

1. Log into WebSeq at [http://www.genetics.ucla.edu/webseq/](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 primers that you used for sequencing.

   **Example:** The **assigned file number** is **106203**, and the primer names are Gene A Fw and Gene A Rv. You would see the following files:
   - 106203GoldR Gene A Fw A12.ab1
   - 106203GoldR Gene A Rv B12.ab1

   What are the annotations?

   - **106203** = assigned file number; **GoldR** = user name; **Gene A Fw** = 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 (FINCH TV 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 what gene your sequence corresponds to.

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.