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

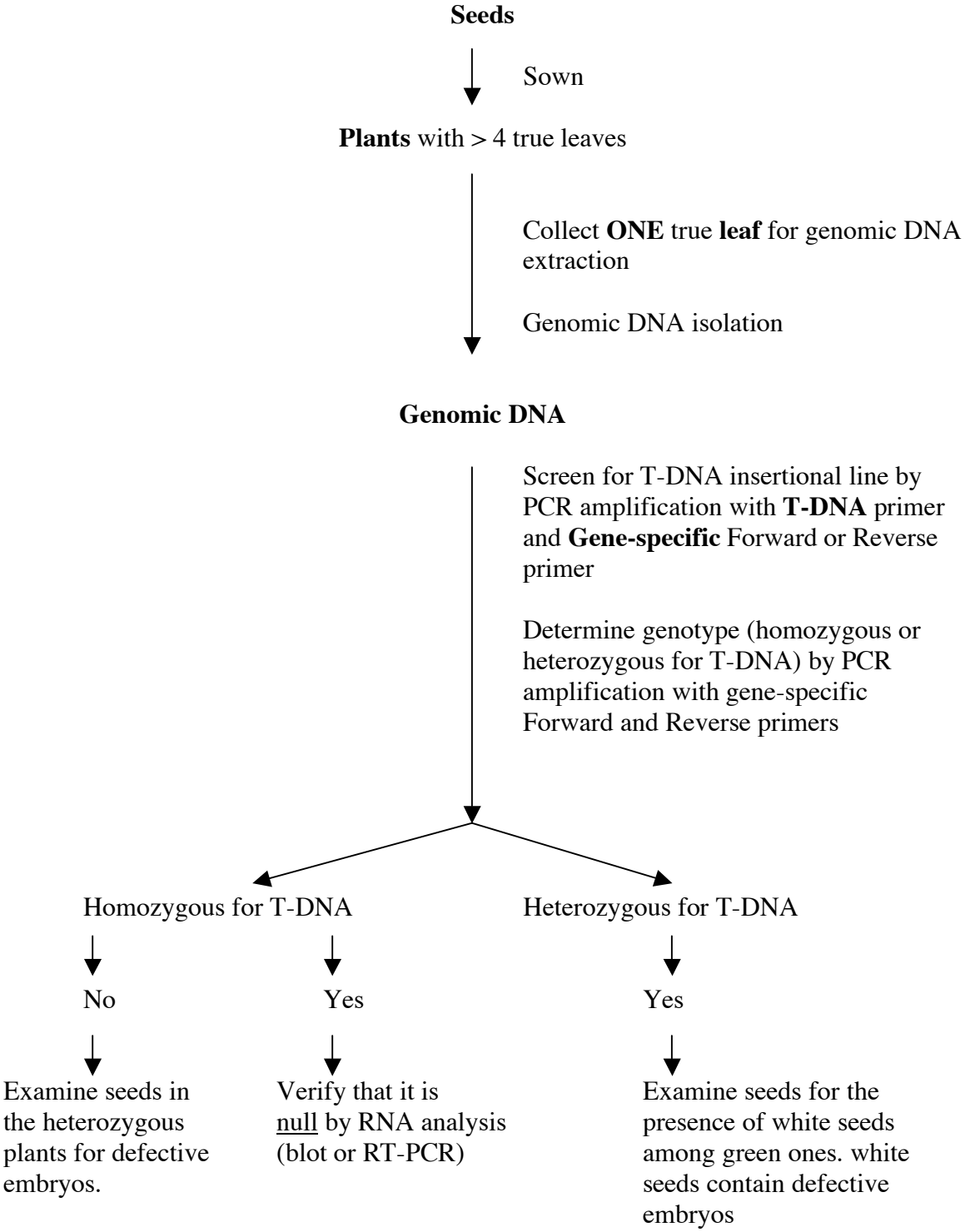
Purpose: To identify a knockout line for the gene of interest and characterize phenotype of mutant plant(s).

Reference: University of Wisconsin - Madison Knockout Facility

STRATEGY

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

STRATEGY


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

I. SOWING SEEDS AND GROWING PLANTS

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

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

Materials Needed:

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

PROCEDURE

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

c. Remove **one pot** from the corner of the flat and put the soil back into the same mount of soil. So, there are **only 11 pots**. *The empty space will make it easier to put the water in.*
d. Bring the flat to the bench near the sink.
e. Make sure that the water hose is attached to the water pipeline labeled "**fertilizer-supplemented**".
f. Fill each flat **2/3 of the way up** the tray with "fertilizer-supplemented" water.
g. Wait **15 minutes or until** the **surface of the soil appears darker** due to water sipping up from the bottom of the pots.
h. Cover the flat with **clear plastic cover** to prevent growth of air-borne molds and to protect from **strayed *Arabidopsis* seeds** from other plants.

6. Bring the flat over to the bench where the seeds and planting tools are located (or any other bench removed from the soil).
7. Cut the **sheet of white paper** into quarters
8. Fold **each quarter in half**, length-wise
9. Gently pour out seeds from the microcentrifuge tube onto one of the folded pieces of paper.
10. Bring the folded paper with seeds over each of the 12 pots. Lower one end of the paper near the soil surface. **Gently tap** the lower end of the paper to allow for one seed to slide down into the soil. The tweezers are a useful tool to guide one seed off of the paper to a precise location in the pot without dumping all of the seeds from the paper.
11. Sow 2 seeds per pot, for 11 of the pots.
12. Put the labeled tags for the **knockout line** into **each** of the **10 pots** containing knockout seeds.
13. Put the seeds that were not used back into the **appropriate knockout seed** microcentrifuge **tube**.
14. For pot **#11**, pour out wild-type seeds onto a **new** folded piece of white paper. Visually divide the pot into 4 quadrants, and sow a wild-type seed in each quadrant. **Four seeds** of **wild-type** should be sown in **pot #11.**
15. Put a **wild-type labeled tag** into pot #11.

16. Cover the flat with the **clear plastic cover**.
17. Put the flat aside.
18. Repeat seed sowing for other knockout lines.
19. After all of the lines are sown, put the flats on a metal car and take the elevator to the lower level.
20. Put the flats on wired-racks in the cold-room (the first room on the right after entering the double doors across from the elevator).

    CAUTION: Make sure the clear covers completely cover the flats so that no air-borne molds in the cold-room get in the soil.
21. Leave the flats in the cold-room for **2-3 days** to **vernalize seeds** and to enhance **synchronization of seed germination**.
22. After 2-3 days in the cold-room, transfer the flats to the white Percival growth chamber and leave them there for another 5-7 days.

    NOTE: Keep the clear plastic covers on the flats.
23. After a total of 7-10 days after planting, bring the flats of seedlings with 2 cotyledons to the glasshouse #3
24. Put the flats of seedlings on a table.

    NOTE: Choose a table that has no mature Arabidopsis plants bearing ripened seeds because these seeds could accidentally get in the soil of the seedling flats when the clear covers are removed.
25. Slide the clear covers off the trays by **0.5-1 inch** so that warm air under the covers will not cook the seedlings nor will the surface of the soil be too warm which is favorable for molds to grow.
26. Wait until most of seedlings in the flats have **4 true leaves**. Then remove the clear covers completely off the flats. Bring the clear covers to the washing room on the lower level of the PGC so that they will be washed by the PGC staff.
27. Map **positions of seedlings** in **each of 11 pots** on a sheet of "Plant Layout" chart.

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

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

GENOTYPING ARABIDOPSIS PLANTS

PLANT LAYOUT CHART

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

Pot #	**Pot #**
Pot #	**Pot #**
Pot #	**Pot #**
Pot #	**Pot #**
Pot #	**Pot #**
Pot #	**Pot #**

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

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

Recommendation:

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

Materials and Reagents Needed:

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

PROCEDURE

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

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

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

   Tube #**6**: **1** seedling/plant from **Wild type** (Columbia-0)
3. Pipet **100 μL** of **Extraction Buffer** into each tube.

   Note: I (Anhthu) found that it is ***not*** *necessary to keep tubes of* ***Extraction Buffer on ice*** *during collection of the leaf samples if genomic DNA will be isolated from samples within one hour.*
4. Gather together the following items on a plastic tray or container:
   - A pair of latex gloves
   - Two pairs of tweezers
   - A box of Kimwipes tissues
   - A squirt bottle of 100% Ethanol solution
   - A "Plant Layout" chart
   - Several sheets of white Xerox paper
   - A ruler with Metric system (mm and/or cm)
   - A pen
   - The Nikon 5400 digital Camera
   - The key to the Plant Growth Center
5. Go to the **Plant Growth Center (PGC)** and locate your flat with plants.
6. Use the "**Plant Layout Chart**" to mark the **locations of the plants** you will collect samples from. The **order of plants** should **correspond** to the **labeled tags** that were numbered when the seeds were planted.

   Note: NOT all of the seeds will have germinated.
7. Use a piece of Kimwipes to clean the tweezers with 95-100% ethanol solution.

   Note: The tweezers must be cleaned after collection each leaf to avoid cross-contamination, and two sets of tweezers are used per plant.
8. Remove one **small leaf** from the **first** plant.

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

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

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

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

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

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

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

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

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

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

f. Record **loading patterns** of samples

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

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

Starting time:

Ending time:

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

What do you observe on the gel?

What is the size of genomic DNA?

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

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

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

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

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

Answer: Two following reasons:

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

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

Table: Excitation and Excitation Spectra of Hoesch Dye H33258

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

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

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

References:

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

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

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

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

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

where,

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

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

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

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

then,

$\mathbf{V_f = (V_i \cdot C_i)/ C_f}$ = (**5 µL** x **8 ng/µL**)/(**0.2 ng/µL**) = **200 µL** of **total volume**

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

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

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

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

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

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

Materials and Reagents Needed:

- 12 μM Gene-specific Forward primer
- 12 μM Gene-specific Reverse primer
- 12 μM **LBb1** primer (Left Border (LB) region of T-DNA from **SALK** Lines)
- 10X Ex-Taq buffer
- dNTP Mix
- Ex-Taq DNA polymerase
- Sterile water
- 1-kb DNA ladder
- PCR Machine (Applied Biosystems GeneAmp 9700 or BioRad MyCycler)
- 0.2 mL PCR tubes
- 1.5 mL microcentrifuge tubes
- P-10, P-20, P-200 Pipetman
- PCR rack for 0.2 mL PCR tubes
- Rack for 1.5 mL microcentrifuge tubes
- Filtered Pipet tips for PCR
- Ice bucket
- Gloves
- Microcentrifuge
- Agarose
- Gel apparatus and power supply
- Bio-Rad Gel Documentation System

PROCEDURE

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

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

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

	Mmix for ONE reaction	**Mmix for 9** reactions
Sterile water	16.8 µL	151.2µL
10x Ex-Taq buffer	2.5 µL	22.5 µL
dNTP mix	2.0 µL	18.0 µL
12 µM **Gene-specific Forward** primer	0.5 µL	4.5 µL
12 µM **Gene-specific Reverse** primer	0.5 µL	4.5 µL
12 µM **LBb1** primer (for SALK lines)	0.5 µL	4.5 µL
Ex-Taq DNA polymerase (5 U/µL)	0.2 µL	1.8 µL
Total Volume	**23.0 µL**	**207.0 µL**

3. Mix the contents by flicking the tube five times or vortexing for the tube containing the master mix for **5 seconds**. Spin the tube in a microcentrifuge for **10 seconds**. Put the tube back **on ice**.
4. Pipet **23 µL** of the **Mmix** into each of **8 PCR tubes**.
5. Pipet **2 µL** of **0.2 ng/µL** genomic DNA extracted from each of **6** seedlings/plants into PCR tubes #1-6. Pipet up and down for five times to mix the contents. Put the first tube back on ice and work on the remaining tubes.
6. Pipet **2 µL** of **0.2 ng/µL genomic DNA** extracted (by TAs) from **wild type** (Col-0) seedlings into each of tubes **#7**. Pipet up and down for five times to mix the contents.
7. Pipet **2 µL** of **sterile water** to tube **#8** (**negative control** without DNA template). Pipet up and down for five times to mix the contents.
8. Spin PCR tubes in the microcentrifuge for PCR tubes for **5 seconds** to bring the liquid to the bottom of the tubes.

9. Put the tubes on the **wells** of the PCR machine.
10. Perform PCR with the "**KNOCKOUT**" program with the following profile:

    1 cycle of Hot start or 96ºC for 3 minutes
    36 cycles of 94 ºC, 15 seconds -> 60ºC, 30 seconds -> 72ºC, 2 minutes
    1 cycle of 72ºC, 4 minutes
    4ºC, ∞
11. Prepare a **1% agarose gel** in 1X TAE buffer with a **20-tooth** comb.
12. Label **8 1.5-mL microcentrifuge** tubes and set them on a rack.
13. Add **2 µL** of **loading dye** to each tube.
14. Pipet **10 µL** of **PCR solutions** to each tube.
15. Load samples on the **1% agarose gel** along with **10 µL** of **diluted DNA ladder** solution on each side of the loaded samples. Record sample loading pattern below:

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

16. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) is two-thirds of the gel.
17. Stop the gel electrophoresis.
18. Take a picture of the gel.
19. Analyze data.

    Do you observe PCR fragments?

    What are the sizes of these fragments?

    Do the sizes agree with expected sizes for the gene of interest and T-DNA insertion?

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

DETERMINATION OF T-DNA INSERTION SITE

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

Note:

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

2. ***Depending on the PCR results*** *on the* ***first screen*** *of* ***5 SALK plants****, you can use one of the following procedure to purify PCR products.*

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

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

A. PURIFICATION OF PCR PRODUCTS

QIAquick Gel Extraction Procedure

Reference: Qiagen QIAquick Gel Extraction protocol

Reagents and Materials Needed:

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

PROCEDURE

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

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

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

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

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

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

QIAquick PCR Purification Procedure

Materials and Reagents Needed:

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

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

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

NOT be completely removed unless the flow-through solution is discarded before this additional spin.

10. While spinning, label on the **lids** and **sides** of **1.5-mL microcentrifuge** tubes "**Purified T-DNA PCR**" or "**Purified WT PCR**", **your initial**, and **date**.
11. Transfer the appropriate **QIAquick columns** in the **NEWLY labeled microcentrifuge tubes**. Discard the flow-through solutions and the collection tubes.
12. Pipet **30 µL** of **Buffer EB** to the center of the QIAquick membrane. Let the column sit for **1 minute**, and then centrifuge at **FULL speed** for **1 minute**. *This step elutes the DNA from the QIAquick membrane*.
13. Determine DNA concentration using the Nanodrop spectrophotometer (measuring nucleic acids) or a DNA Fluorometer (measuring only DNA).

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

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

SEQUENCING REACTION WITH BIG DYE V. 3

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

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Reference: Perkin Elmer/Applied Biosystems

Solutions Needed:

- Applied Biosystems Big Dye version. 3 (Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- Dye Dilution Mix (Sigma, Cat. # S3938; also, obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- 3 μM LBb1 primer (for T-DNA)
- 3 μM Gene-specific Forward primer
- 3 μM Gene-specific Reverse primer
- Sterile water

Materials Needed:

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

Overview:

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

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

General Components of One Reaction:

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

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

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

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

- For **plasmid** DNA, use **250-500 ng**. We found that 500 ng of plasmid DNA gives the best read.
- For **PCR product**, use the amount of DNA according to the **table** on the next page (Taken from Perkin-Elmer Big Dye Protocol). *Note: Use the **maximum** amount of DNA in the reaction if there is more than enough DNA available. For example, for **PCR product** of **200 - 500 bp**, use **10 ng** of DNA.*

  Table: Amount of DNA Used in Sequencing Reactions Depending

on **Size of PCR Fragment**

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

For this exercise, there is **ONE DNA template**, i.e. the purified PCR product of the T-DNA fragment; but, there are **TWO primers**, LBb1 (T-DNA) primer and a gene-specific primer (either forward or reverse) depending on the orientation of the T-DNA Left Border (LB) inserted in the gene of interest (based on your analysis of the SALK line). The **sequencing reaction with** the **gene-specific primer** serves **as a control for the master mix** of Big Dye and Dye Dilution mix. Therefore, it is best to prepare a **master mix** with **all components**, **except** the **primers**, which will be added to individual reaction tubes.

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

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

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

Sample calculations:

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

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

*Hence, the **amount of PCR to be used** is **10 ng/4.5 ng/µL = 2.2 µL***

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

PROCEDURE

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

Master Mixes (Mmix) of Sequencing Reactions:

Components	**Mmix** for **ONE reaction**	**Mmix** for **2 reactions**
DNA template	**x** μL	**x** (x 3) μL
Sterile water	**y** μL	**y** (x 3) μL
Big Dye v. 3	**2.0** μL	**6.0** μL
Dye Dilution Mix (Sigma, S3938)	**2.0** μL	**6.0** μL
Total Volume	**19.0 μL**	**57.0 μL**

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

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

		either	**or**
Components	**LBb1 primer**	**Gene-specific <u>Forward</u> primer**	**Gene-specific <u>Reverse</u> primer**
Mmix	**19 µL**	**19 µL**	**19 µL**
3 µM LB1 primer	**1 µL**	0 µL	0 µL
3 µM Gene-specific Forward primer	0 µL	**1 µL**	0 µL
3 µM Gene-specific Reverse primer	0 µL	0 µL	**1 µL**
Total volume	**20 µL**	**20 µL**	**20 µL**

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

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

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

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

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

RETRIEVING AND ANALYZING DNA SEQUENCES

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

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

    Example: the ***assigned file number*** is **5677**, and the gene of interest is **At5g09250**.

    You would see the following files:

    5677 GOLDR_At5g09250Fw_080.ab1

    5677 GOLDR_At5g09250Rv_081.ab1

    What are the annotations?

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

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