EXPERIMENT 6 – SCREENING SALK T-DNA MUTAGENESIS LINES (GENE TWO)

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

Reference: University of Wisconsin - Madison Knockout Facility

Attention: ***By now, you have experience with the genotyping procedure. You can isolate genomic DNA from 12 or more plants. So, you use this protocol as the guide and adjust the volumes of reactions according to the number of plants that you are analyzing.***

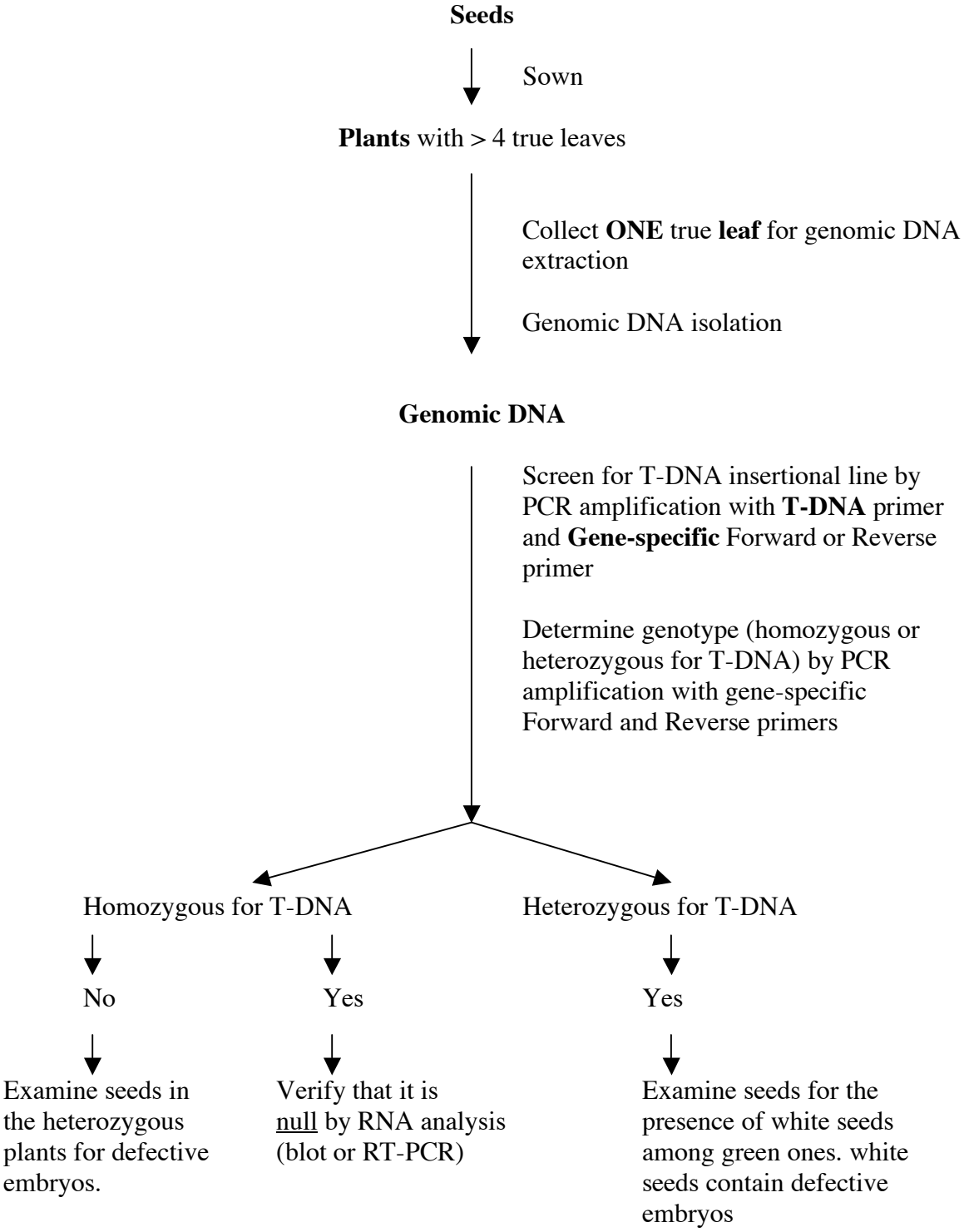
STRATEGY

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 of 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, remove one pot at the corner and 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: ***By now, you have experience with the genotyping procedure. You can isolate genomic DNA from 12 or more plants. So, you use this protocol as the guide and adjust the volumes of reactions according to the number of plants that you are analyzing.***

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	16.5 µL	148.5 µL
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)	0.5 µL	4.5 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.