A Gene Discovery Lab Manual For Undergraduates:

Searching For Genes Required To Make A Seed

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EXPERIMENT 1 – INTRODUCTION TO GENERAL MOLECULAR BIOLOGY TECHNIQUES

STRATEGY

I. **PIPETTING EXERCISE**

II. **SERIAL DILUTION EXPERIMENT**

I. PIPETTING EXERCISE

Purpose: To learn how to use pipettes

Taken From: DNA Science: A First Course, Second Edition
Laboratory 1: Measurements, Micropipetting, and Sterile Techniques
p. 327-328. (ISBN 978-087969636-8)

Solutions Needed:

- Four Dye Solutions Labeled I-IV
  - Solution I: Blue
  - Solution II: Red
  - Solution III: Yellow
  - Solution IV: Green

Materials Needed:

- Set of pipettes (P-10, P-20, P-200 & P-1000)
- Pipet tips (regular, non-filter tips)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack

PROCEDURE

A. Small Volume Pipette Exercise

This exercise simulates setting up a reaction, using a pipette with a range of 1-10 μL or 2-20 μL.

1. Use a permanent marker (sharpie) to label THREE 1.5 mL tubes **A**, **B** and **C** and **your initials**.
2. Use the table below as a **checklist** while adding solutions to each reaction tube.

Tube	Sol. I (Blue)	Sol. II (Red)	Sol. III (Yellow)	Sol. IV (Green)	Total Volume
A	4 µL	5 µL	1 µL	-	10 µL
B	4 µL	5 µL	-	1 µL	10 µL
C	4 µL	4 µL	1 µL	1 µL	10 µL

3. Set the pipette to **4 µL** and add **Solution I** to each reaction tube.
4. Use a *fresh tip* to add the appropriate volume of **Solution II** to a clean spot inside reaction tubes **A**, **B** and **C**.
5. Use a *fresh tip* to add 1 µL of **Solution III** to tubes **A** and **C**.
6. Use a *fresh tip* to add 1 µL of **Solution IV** to tubes **B** and **C**.
7. Close lids. Pool and mix reagents by using one of the following methods:
    a. Sharply tap the tube bottom on the bench top. Make sure that the drops have pooled into one drop at the bottom of the tube.

    Or

    b. Place the tubes in a microcentrifuge and apply a short, few-second pulse. Make sure that the **reaction tubes are placed** in a **balanced configuration** in the microcentrifuge rotor. Caution: *Spinning tubes in an unbalanced position will damage the microcentrifuge*.
8. A **total** of **10 µL** of reagents were added to each reaction tube. To check that the previous pipetting measurements were accurate, set the pipette to 10 µL and very carefully withdraw the solution from each tube.
    a. Is the tip just filled? What does this suggest?

    Or

    b. Is a small volume of fluid left in tube? What does this suggest?

    Or

    c. After extracting all the fluid, is an air space left in the tip end? What does this suggest? (The air can be displaced and the actual volume determined simply

by rotating the volume adjustment to push the fluid to the very end of the tip. Then, read the volume directly.)

9. If several measurements were inaccurate, repeat this exercise to obtain near-perfect results.

B. Large Volume Pipette Exercise

This exercise simulates a bacterial transformation or plasmid preparation, for which a P-1000 pipette is used. It is far easier to measure incorrectly when using a large-volume pipette. If the plunger is not released slowly, an air bubble may form or solution may be drawn into the piston.

1. Use a permanent marker to label TWO 1.5 mL microcentrifuge tubes **D** and **E** and **your initials**.
2. Use the matrix below as a **checklist** while adding solutions to each reaction tube.

Tube	Sol. I (Blue)	Sol. II (Red)	Sol. III (Yellow)	Sol. IV (Green)	Total Volume
D	100 µL	200 µL	150 µL	550 µL	1000 µL
E	150 µL	250 µL	350 µL	250 µL	1000 µL

3. Set the pipette to add the appropriate volume of Solutions I-IV to reaction tubes **D** and **E**. Follow the same procedure as for the Small Volume Pipette Exercise to add **Solutions I-IV** to each reaction tube.
4. Close lids. Pool and mix reagents by using one of the following methods:
    a. Sharply tap the tube bottom on the bench top. Make sure that the drops have pooled into one drop at the bottom of the tube.

    Or

    b. Place the tubes in a microcentrifuge and apply a short, few-second pulse. Make sure that the **reaction tubes are placed** in a **balanced configuration** in

the microcentrifuge rotor. Caution: *Spinning tubes in an unbalanced position will damage the microcentrifuge.*

5. A **total** of **1000 μL** of reagents were added to each tube. To check that the measurements were accurate, set the pipette to 1000 μL and very carefully withdraw the solution from each tube.
    a. *Is the tip just filled? What does this suggest?*
    Or
    b. *Is a small volume of fluid left in tube? What does this suggest?*
    Or
    c. *After extracting all the fluid, is an air space left in the tip end? (The air can be displaced and the actual volume determined simply by rotating the volume adjustment to push the fluid to the very end of the tip. Then, read the volume directly.)*
6. If several measurements were inaccurate, repeat this exercise to obtain near-perfect results.

II. SERIAL DILUTION EXPERIMENT

Purpose: To test the accuracy and precision of pipetting

Reference: Anhthu Bui

Introduction: ***Diluting*** is simply the **addition of a solution** (or plain solvent) to a **substance** in order to **decrease the concentration of the latter substance**. In this exercise, the **substance** is **DNA** and the **solution** is **TE Buffer**. By the end of this exercise, you will learn how to calculate the dilution factor and determine the accuracy of your pipetting technique as determined by gel electrophoresis and spectrophotometer readings.

Solutions Needed:

- DNA stock (known concentration)
- TE Buffer
- 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 (P-10 & P-20)
- Pipet tips (regular, non-filter tips)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- NanoDrop spectrophotometer
- Kimwipes
- 250 mL Erlenmeyer flask
- 25 mL Erlenmeyer flask
- Saran wrap
- Scale
- Microwave
- 55°C water bath
- Hot hand protector
- Gel cast
- Gel comb

- Round bubble level
- Gel box
- Cables
- Electrophoresis power supply
- Plastic container for carrying the gel
- Gel document system (Bio-Rad)

PROCEDURE

A. Serial Dilution of a DNA Stock

1. Label THREE 1.5 mL microcentrifuge tubes as:
   "**Dil #1**" for dilution #1
   "**Dil #2**" for dilution #2
   "**Dil #3**" for dilution #3
2. Pipet **15 µL** of **TE buffer** solution into each microcentrifuge tube in step 1. (Use the **P-20 pipette**)
3. Vortex the **DNA stock solution** for 5 seconds. Then, spin the tube for **10 seconds** to ensure that all of the solution is at the bottom of the tube.
4. Pipet **5 µL** of your **DNA stock solution** into the **Dil #1** microcentrifuge tube. (Use the **P-10** or **P-20 pipette**)
5. Vortex the contents of the tube for 5 seconds. Then, spin the tube for **10 seconds** to ensure that all of the solution is at the bottom of the tube.
6. Pipet **5 µL** of DNA solution from the **Dil #1** tube into the **Dil #2** tube.
7. Vortex the contents of the **Dil #2** tube for 5 seconds. Then, spin the tube for **10 seconds** to ensure that all of the solution is at the bottom of the tube.
8. Pipet **5 µL** of DNA solution from the **Dil #2** tube into the **Dil #3** tube.
9. Vortex the contents of the **Dil #3** tube for **5 seconds**. Then, spin the tube for **10 seconds** to ensure that all of the solution is at the bottom of the tube.

B. Determination of Pipetting Accuracy by Gel Electrophoresis *(See Appendix 1A)*

1. Label THREE microcentrifuge tubes with the letters "**A**," "**B**," "**C**" and "**D**."
2. Pipet **10 µL** of DNA solution to tubes A, B, C and D:

from	**DNA Stock**	**Dil #1**	**Dil #2**	**Dil #3**
to	Tube **A**	Tube **B**	Tube **C**	Tube **D**

3. Pipet **2 µL** of **6x loading dye** into tubes **A**, **B, C** and **D**. Mix by pipetting up and down 5 times. The total volume for each solution is **12 µL**.
4. Load the contents of tubes A, B, C and D into lanes 1, 2, 3 and 4.
5. Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the **anode** (positively charged) side of the gel box. (The **anode** is on the side **opposite** the wells.) Note*: Similar to ethidium bromide, SYBR Safe DNA Gel Stain is positively charged. Therefore, it migrates towards the negative side of the gel box, from anode to cathode. (Opposite the direction of DNA migration). Remember that DNA is negatively charged; so, it migrates to the positive end of the gel box. (DNA migrates from cathode to anode).*
6. Put the lid on the gel box and connect the electrodes to the power supply (**RED** to **RED** and **BLACK** to **BLACK**). *Note: SYBR Safe gel stain is unstable in UV or bright room light. If possible, run the gel in the* ***dark*** *by either turning off the lights, covering the gel with a cardboard box or aluminum foil, or run the gel inside of a drawer. Realistically, hours of constant UV or bright room light exposure are required to cause any significant loss of signal.*
7. Record the **identity of samples** loaded on the gel.

Lane	**Sample**
1	DNA Stock
2	Dilution #1
3	Dilution #2
4	Dilution #3

8. Run the gel at **105 volts** for **1-2 hours** or until the lower dye (bromophenol blue) has migrated one-half or two-thirds of the gel length.

   Time power supply turned ON:

   Time power supply turned OFF:

   How long was the gel run? ______ *hour(s) and* _______ *minutes*

10. After 1-2 hours of running the gel, turn **off** the power supply.
11. Remove the lid of the gel box. Put the gel in its gel cast into a small plastic container and bring the container to room 4128A2.

    Caution: *It is a* ***MUST*** *to put the gel into a plastic container so that the gel cannot slide off the gel cast, fall on the floor and be broken into pieces while walking.*
12. Take a picture of the gel using the Bio-Rad Gel Document System. Label the picture using the text program of the Gel Document System. (*Your TA will show you how.*)

    Alternatively: Print out the picture. Tape it to a piece of paper by putting a piece of white tape at a position immediately above the wells. Label the wells with the sample names.
13. Print out the picture. Store the labeled picture in your lab notebook.

C. Determination of Pipetting Accuracy Using a Spectrophotometer

While running the gel, determine the **concentration** of DNA solutions in the tubes labeled “DNA Stock,” “Dil #1,” “Dil #2” and “Dil #3” by using the **NanoDrop Spectrophotometer** (Your instructor will demonstrate how to use the instrument).

What is a spectrophotometer? (See Appendix 1B)

1. For each tube, read the concentration at least TWICE, using a fresh drop each time.
2. Record the DNA concentration (in **ng/µL**) from each tube.

Sample	**Concentration (ng/µl)**
DNA Stock	
Dil #1	
Dil #2	

Dil #3	

D. Questions and Summary

1. *What did you expect to see on your gel?*
2. *How is your pipetting accuracy as determined by gel electrophoresis?*
3. *Does the gel result show what you expected? If not, what might be the problem?*
4. *What is the dilution factor in this exercise?*
5. *Given the stock DNA concentration is **1 µg/µL**, what is the expected DNA concentration in tubes "Dil #1," "Dil #2" and "Dil #3?"*

   Hint: Use the equation $\mathbf{V_i \times C_i = V_f \times C_f}$ where,

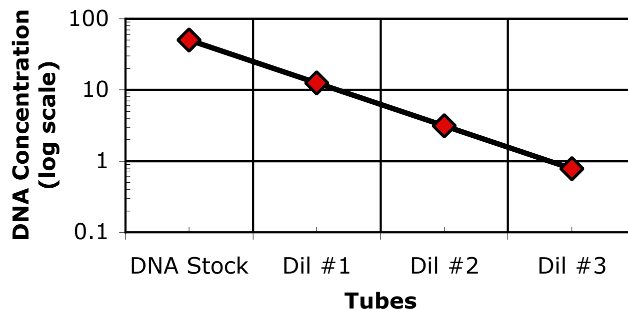
   $\mathbf{V_i}$ = **initial volume** (the volume of original DNA solution is **5 µL**)

   $\mathbf{C_i}$ = **initial concentration** (reading from the spectrophotometer; example: **1000 ng/µL**)

   $\mathbf{V_f}$ = **final volume** (the volume of Dil #1 is **20 µL**)

   $\mathbf{C_f}$ = **final concentration** (the concentration of Dil #1)

6. *Make a plot on log graph paper or Excel of the logarithm with base 2 of the expected DNA concentration (this will be your standard curve) as shown in the graph below:*
   The x-axis: Tubes (DNA stock, Dil #1, Dil #2 and Dil #3)
   The y-axis: The logarithm with base 2 of the expected DNA concentration



7. *Plot the logarithm with base 2 of the DNA concentration readings you obtained from the spectrophotometer.*

8. *How does your DNA concentration reading deviate from the expected DNA concentration?*

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

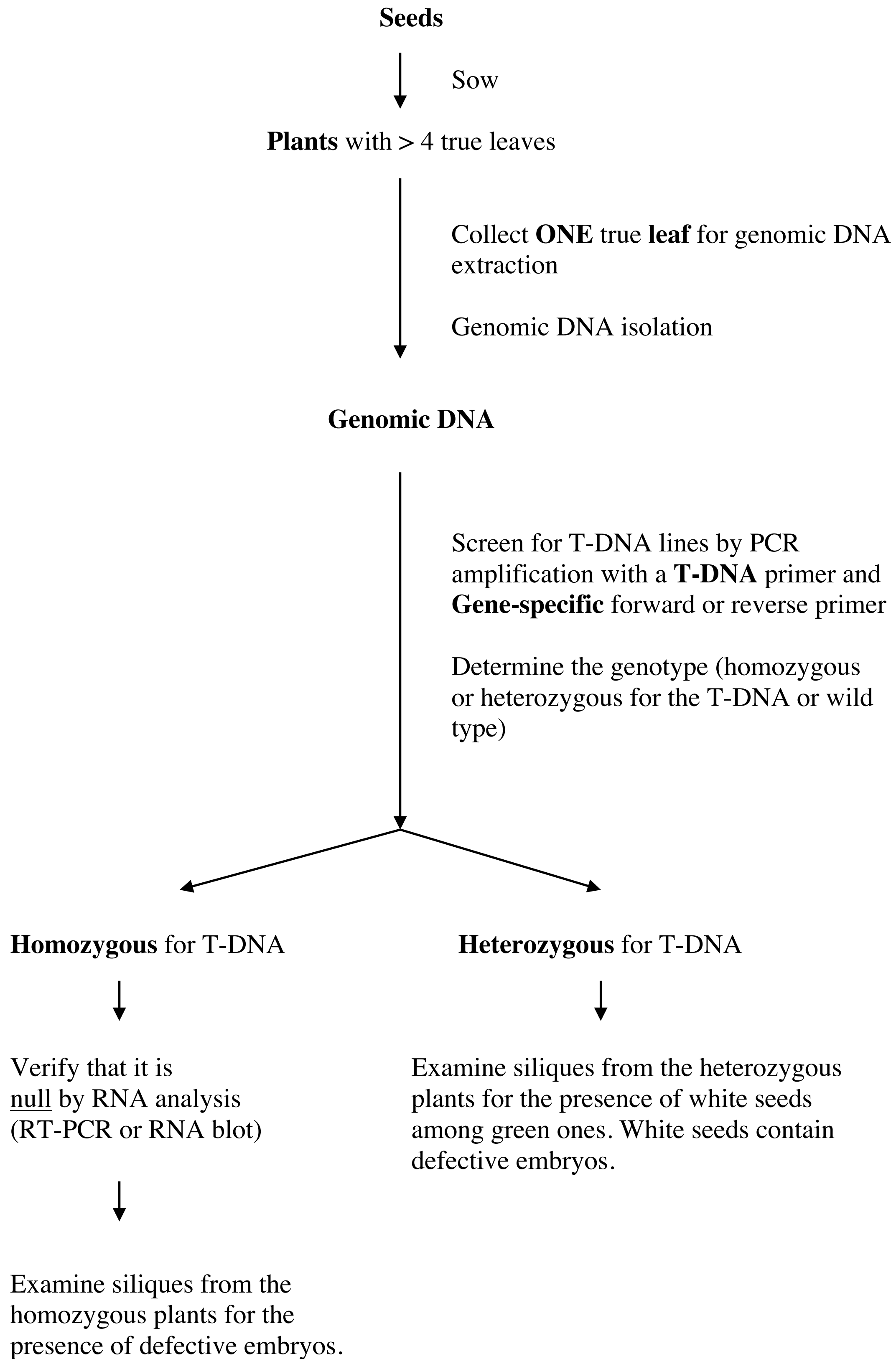
Purpose: To identify a knockout line for the gene of interest and characterize the phenotype of mutant plants.

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

STRATEGY

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

STRATEGY



I. SOWING SEEDS AND GROWING PLANTS

Purpose: To generate seedlings for genomic DNA extraction.

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

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

Materials Needed:

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

PROCEDURE

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

1. Obtain **tubes of seeds** to be grown from the cold room and put them on a microcentrifuge tube rack. *For example, S_112701 (Salk line for gene At5g11240) and wild type seeds (Columbia-0).*
2. Bring the **materials** to the **Plant Growth Center** (**PGC**).
3. At the PGC, put all of these items on **the bench** that runs along the **east wall**. This bench does NOT have any soil on it. *Note: Do NOT put tubes of seeds or plants full of mature siliques near the bench of soil because the prepared soil will be*

contaminated with these seeds, which could in turn, result in false mutant phenotypes for other people's work.

4. In the PGC, prepare ONE flat with **12 pots** of soil for every TWO knockout lines being planted.
   a. Assemble each flat as follows:
      i. Obtain a black plastic tray without holes.
      ii. Obtain a sheet of 12 rectangular plastic pots.
      iii. Obtain a clear plastic cover.
      iv. Set the sheet of 12 pots in the black plastic tray.
      v. Fill the pots loosely with soil, without compressing. Use the sifter to add a layer of fine soil. *(Soil prepared by PGC staff, Mr. Weimin Deng).*
      vi. Flatten the surface of the soil by scraping off excess soil with a metal plate.
   b. Remove **one pot** from the corner of the flat and put the soil back into the mound of soil. So, there are **only 11 pots**. *The empty space will make it easier to put the water in.*
   c. Bring the flat to the bench near the sink.
   d. Use the hand brush to clean up the soil bench.
   e. Make sure that the water hose is attached to the water pipeline labeled "**fertilizer-supplemented**."
   f. Fill each flat **2/3 of the way up** the tray with "fertilizer-supplemented" water.
   g. Cover the flat with a **clear plastic cover** to prevent the growth of air-borne molds and to protect the soil from **stray *Arabidopsis* seeds**.
   h. Wait **20 minutes or until the surface of the soil appears darker** due to water seeping up from the bottom of the pots.
5. Label **11 plastic tags** with a black sharpie.
   a. For **knockout lines**: **Gene name**
Salk line #
Date
Pot # 1-10
   b. For **wild type**: **Columbia-0**

Date
Pot # 11

6. Bring the flat over to the bench where the seeds and planting tools are located (or any other bench removed from the soil).
7. Fold each **quarter sheet** of white paper **in half**, lengthwise.
8. Gently pour out the **knockout seeds** from the microcentrifuge tube onto one of the folded pieces of paper.
9. Bring the folded paper with seeds close to the soil of each of the first 5 pots. Lower one end of the paper near the soil surface. Use the **forceps** to guide one seed off of the paper to a precise location in the pot without dumping all of the seeds from the paper.
10. Visually divide the pot into 4 quadrants, and sow a seed in each quadrant. Sow **4 seeds** per pot, for the first 5 pots. *Note: Planted seeds should not be covered with additional soil because Arabidopsis seeds need light for germination.*
11. Put the labeled tags for the **knockout line** into **each** of the **5 pots** containing knockout seeds.
12. Put the seeds that were not used back into the **appropriate knockout seed** microcentrifuge tube.
13. Repeat steps 7-12 with the seeds for the next knockout line and pots #6-10. Use a **new** folded piece of white paper for each line.
14. For pot **#11**, pour out wild type seeds onto a **new** folded piece of white paper. Visually divide the pot into 4 quadrants, and sow a wild type seed in each quadrant. **Four wild type seeds** should be sown in **pot #11.**
15. Put a **wild type labeled tag** into pot #11.
16. Cover the flat with the **clear plastic cover**. Put the flat aside.
17. After all of the lines are sown, put the flats on a metal cart and take the elevator to the lower level.
18. Put the flats on the wire racks in the cold room (the first room on the right after entering the double doors across from the elevator).
    CAUTION: Make sure the clear covers completely cover the flats so that no air-borne molds in the cold-room get in the soil. Note: Turn the lights off when you leave the cold room.

19. Leave the flats in the cold room for **2-5 days** to **vernalize seeds.** 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.”

PLANT LAYOUT CHART

GENOTYPING ARABIDOPSIS PLANTS

Gene ID: At__ g _________ Salk line#: ____________ Date: ________
Primers for PCR: ______________________________
Size of PCR product: __________

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

II. ISOLATING GENOMIC DNA FROM LEAVES OF AT LEAST 2-3 WEEK-OLD SEEDLINGS

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

Recommendation:

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

Materials Needed:

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

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

PROCEDURE

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 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 tags to identify the plants in the pictures.
7. Use the **Plant Layout Chart** to mark the **locations of the plants** and to indicate the plants that you will collect samples from by numbering them. Also make a note of any interesting phenotypes. (For example, some plants may be smaller than others.) *Note: NOT all of the seeds will have germinated.*
8. Use a piece of Kimwipes to clean the forceps with ethanol. *Note: Two sets of forceps are used per plant. The forceps must be cleaned after the collection each leaf to avoid contamination.*
9. Using forceps, remove one **small leaf** from the **plant #1**.
10. Place this leaf in microcentrifuge **tube #1** containing the Extraction Buffer.
11. Repeat steps 8-10 for the other plants.
    Note: MAKE SURE TO CLEAN THE FORCEPS BETWEEN LEAF SAMPLES!
12. Go back to the lab.
13. Homogenize or macerate the collected leaf in **tube #1** by crushing it with a **blue micropestle** until no more chunks of plant tissue are observed in the mixture. *Note: Do NOT dispose of the micropestle.*
14. Rinse the **micropestle** with **300 μL** of Extraction Buffer into the microcentrifuge tube. Put the used micropestles in a beaker labeled "used micropestles" so that they can be washed. The **total volume** of Extraction Buffer in the microcentrifuge tube is now **400 μL**.
15. Vortex the **homogenate** for 5 seconds. Set the tube on ice.
16. Repeat steps 13-15 for the other tubes.
17. Centrifuge the tubes of homogenates at **room temperature** for **5 minutes** at **FULL speed**. *Note: Position the tubes in the centrifuge so that the hinge of the microcentrifuge tubes faces the outside of the microcentrifuge. This way after centrifugation you know to look for your pellet on the side of the microcentrifuge tube that has the hinge.*
18. Meanwhile, **label** a set of **microcentrifuge tubes** with **Gene Name, plant #,** "**gDNA**," **your initials** and the **date.**
19. Pipet **350 μL** of **isopropanol** to each of the new labeled tubes.

20. After centrifugation, transfer the tubes from the microcentrifuge onto a microcentrifuge tube rack. Organize tubes on the rack such that the **numbers** on the lids of the **NEW tubes match** with the **numbers** on the lids of the **tubes containing homogenates**.
21. Pipet **350 µL** of **supernatant** (**homogenate**) from the centrifuged tubes to the corresponding tubes containing isopropanol. Close the lids of the tubes. *Note: Use your pipet to draw off liquid from the side of the tube opposite to the side where the plant material is pelleted. Start at the top and move downward as the liquid level drops. AVOID disturbing the plant debris on the bottom of the tubes as much as possible. However, it is okay if you accidentally transfer some plant debris into the isopropanol tube.*
22. Mix the isopropanol and homogenate by inverting the tubes **5-10 times**.
23. Incubate the mixture at **room temperature** for **5 minutes** to precipitate **nucleic acids** *(both genomic DNA and total RNA).*
24. Centrifuge the tubes at **room temperature** for **10 minutes** at **FULL** speed.
25. Pour or pipet each isopropanol supernatant into a waste container. *Note: Be extremely careful when pouring off the isopropanol because the pellets are sometimes loose. DNA is now in your white or clear pellet along with RNA.*
26. Add **1 mL** of **80% ethanol** solution to each pellet. Close the lid of the tube and invert **5 times**. *This step is to wash off any residual salt (from the extraction buffer) and isopropanol.*
27. Centrifuge the tubes at **room temperature** for **5 minutes**.
28. Pour or pipet each ethanol supernatant into a waste container. Dab the tubes upside down on Kimwipes to remove as much ethanol as possible. *Note: Be extremely careful when pouring off the ethanol solution because the pellets are sometimes loose.*
29. Put the tubes on a microcentrifuge tube rack with their lids open, allowing the ethanol to evaporate. *Note: You may use a P-200 pipette to carefully draw off excess ethanol from the side of the tube opposite that against which the nucleic acid is pelleted. Be very careful not to pipet the pellet.*
30. Dry pellets either in a **SpeedVac** at room temperature for **5-10 minutes** (Your instructor will operate the SpeedVac) or by leaving on the **bench at room temperature for up to 60 minutes**.

31. After drying the pellets, resuspend each pellet by adding **200 µL** of **TE** buffer, closing the lids of the tubes, and **raking** the tubes over a microcentrifuge tube rack **10 times** <u>or</u> **vortexing** the tubes briefly. *<u>Note:</u> If you vortex genomic DNA vigorously or for a long time, it will degrade.*
32. Spin the tubes in a microcentrifuge for **1 minute** to bring the liquid and any contaminants to the bottom of the tubes.
33. Store DNA solutions at **4°C** (on ice or in refrigerator) until use.
    <u>Note:</u> Keep DNA solutions cold as much as possible to prevent degradation of DNA because this is a crude extraction of genomic DNA, and there may be a trace amount of endonuclease present in the DNA solution that can degrade DNA.

    <u>Attention:</u> *At this step, you need to assess the **quality** and **quantity** of isolated genomic DNA by **gel electrophoresis** (**step 34**) and **spectrophotometer reading** (**step 35**), respectively.*
34. Analyze the **quality** of isolated genomic DNA by **gel electrophoresis**.
    *<u>Note:</u> First, spin the tubes of DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed to bring down water condensation on the lids as well as any contaminating plant debris and/or carbohydrates in the solutions to the bottom of the tubes.*
    a. Prepare a **0.7%** agarose gel with a 20-tooth comb (0.7 g of agarose in 100 mL of 1x TAE buffer; see Appendix 1A). *<u>Note:</u> The agarose gel can be prepared before the collection of leaves for the extraction of genomic DNA.*
    b. Label the lids of SIX microcentrifuge tubes with **#1-6** and **your initials**, and set tubes on a microcentrifuge tube rack.
    c. Pipet **20 µL** of **isolated genomic DNA** solution into each of the labeled tubes.
    d. Add **2 µL** of **6x loading dye solution** to each tube and mix the contents by pipetting up and down 5 times.
    e. Load **10 µL** of **1 Kb Plus DNA ladder solution** into the first well.
    f. Load **20 µL** of each sample-dye mixture prepared in step d using a P-20 pipette.
    g. Record the identity of the sample in each well.

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

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

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

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

Starting time:

Ending time:

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

What do you observe on the gel?

What is the size of genomic DNA?

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

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

*<u>Note:</u> First, spin the tubes of DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed to bring down water condensation on the lids as well as any contaminating plant debris and/or carbohydrates in the solutions to the bottom of the tubes.*

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

Sample	**DNA Concentration (ng/μL)**	**Sample**	**DNA Concentration (ng/μL)**
Plant #1		Plant #7	
Plant #2		Plant #8	
Plant #3		Plant #9	
Plant #4		Plant #10	
Plant #5		Plant #11	
Plant #6		Plant #12	

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

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

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

STRATEGY

A. Polymerase Chain Reaction (PCR)

B. Gel Electrophoresis Analysis of PCR Product

C. Label T-DNA-Tagged Plants

Solutions Needed:

- Ex Taq DNA polymerase (Takara)
- 10x Ex Taq buffer (Takara)
- dNTP mix (Takara)
- Sterile water
- 12 μM Gene-specific Salk Forward primer
- 12 μM Gene-specific Salk Reverse primer
- 12 μM LBb1.3 primer (anneals to the Left Border (LB) region of the T-DNA)
- Genomic DNA extracted from the plants to be genotyped (including WT)
- Genomic DNA extracted from wild type seedlings by TA
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain
- 6x Loading dye
- 50 ng/μL 1 Kb Plus DNA ladder solution

Materials Needed:

- Pipettes
- Filter pipet tips for PCR
- 0.2 mL PCR tubes in strips of 8
- 1.5 mL microcentrifuge tubes
- Rack for 0.2 mL PCR tubes
- Rack for 1.5 mL microcentrifuge tubes
- Ice bucket
- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- Microcentrifuge
- Gel electrophoresis materials (Appendix 1A)
- Key to the Plant Growth Center

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

A. Polymerase Chain Reaction (PCR)

PROCEDURE

Note: *Because you do not know the orientation of the T-DNA insertion, and therefore the direction of the LBb1.3 primer, you will need to set up* ***THREE*** *PCR reactions for* ***each*** *sample.*

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

Reaction B: ***LBb1.3*** *primer and* ***Gene-specific Salk Reverse*** *primer*

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

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

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

1. Get ice from the icemaker in room 4128.
2. Thaw tubes of **10x Ex Taq buffer, dNTP mix, 12 µM Gene-specific Salk Forward primer, 12 µM Gene-specific Salk Reverse primer** and **12 µM LBb1.3 primer** on a rack for 1.5 mL microcentrifuge tubes at **room temperature** for 5-10 minutes.
3. **Vortex** each tube on a setting of 2-3 for 10 seconds. **Spin** in microcentrifuge at full speed for 5 seconds. Put the tubes on **ice** until needed.

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

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

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

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

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

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

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

72°C 4 min
4°C ∞

19. Press "**F5**" for "**DONE.**" The "CHOOSE OPERATION" menu will appear. Press "**ENTER**" to **run the protocol**.
20. Enter the **volume** of the PCR reaction (50 μL). Press "**F5**" to "**Begin Run**." *Note: It will take about 3 hours for the PCR amplification to be completed.*
21. Once the PCR amplification is complete, remove the PCR tubes from the PCR machine and store them on ice or in the **refrigerator** until gel electrophoresis or leave them in the PCR machine at 4°C until you have a chance to put them away later.

B. Gel Electrophoresis Analysis of PCR Product

PROCEDURE

1. Prepare a **1% agarose gel** in 1x TAE buffer with a **30-tooth** comb (or 20-tooth comb depending on how many samples you have).
2. Record the identity of the sample in each lane. Use the table below as a guide.
3. Label **24 1.5 mL microcentrifuge** tubes (one for each sample) and set them on a microcentrifuge tube rack.
4. Add **3 μL** of **6x loading dye** to each tube.
5. Pipet **25 μL** of **PCR solution** to each tube. Spin briefly. *Note: Store remaining PCR product in the PCR tubes in the refrigerator.*
6. Slowly pipet **10 μL** of **100 bp DNA ladder** in the first well. *Note: Avoid pipetting bubbles into the well.*
7. Mix the contents of your first sample-dye mixture by pipetting up and down 5 times. Slowly pipet **~28 μL** of sample-dye mixture into the next well using a P-20 pipette *(i.e. load ~15 μL first, then then remaining ~15 μL into the same well)*. Repeat for the remaining sample-dye mixtures.
8. Slowly pipet **10 μL** of **1 Kb Plus DNA ladder** in the last well.

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

9. Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode (near the bottom of the gel).
10. Run the gel at **105 volts** for **1-2 hours** or until the lower dye (bromophenol blue) travels two-thirds of the gel.
11. Stop the gel electrophoresis.
12. Take a picture of the gel and annotate it.
13. Analyze the data.
    Do you observe PCR fragments?
    What are the sizes of these fragments?
    Do the sizes agree with expected sizes for the gene of interest and T-DNA insertion?
    What is the orientation of the T-DNA relative to the gene?
    What are the genotypes of the 6 plants?

Note:

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

2. ***Depending on the results of the PCR*** *to genotype the plants, you can use one of the following procedures to purify the "T-DNA fragment."*
    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.

IV. DETERMING THE T-DNA INSERTION SITE

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

STRATEGY

A. **Purifying PCR Products**
B. **Sequencing Reaction with Big Dye v. 3**
C. **Retrieving and Analyzing DNA Sequences**

A. Purifying PCR Products

QIAquick PCR Purification Procedure

Note: This procedure is used when you run 25 µL of PCR product on the gel and observe only one band.

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

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

Solutions Needed:

- QIAquick PCR Purification Kit (Qiagen, Cat. # 28104)
- Remaining PCR solutions (~25 µL)

Materials Needed:

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

- Kimwipes

PROCEDURE

1. Write "**T-DNA**" and **your initials** on a 1.5 mL microcentrifuge tube.
2. Pipet **~25 µL** of the **PCR product solution** from one of the PCR tubes containing the T-DNA fragment into the labeled 1.5 mL microcentrifuge tube.
3. Measure the exact volume of solution.
4. Add **125 µL** of **Buffer PB** (or **5 volumes** of **Buffer PB** to **1 volume** of the PCR product solution) to the tube in step 2. Mix by vortexing for 5 seconds.
5. Spin the tube in the microcentrifuge at **FULL speed** for **10 seconds** to bring all the solution down to the bottom of the tube. Set the tube back on the microcentrifuge tube rack.
6. Place a **QIAquick spin column** (lilac) in the provided **2 mL collection tube**. Label the lid of the column "**T-DNA**" and **your initials**.
7. Pipet the sample mixture in step 4 to the QIAquick spin column. Spin the column and collection tube in the microcentrifuge at **FULL speed** for **1 minute**. *This step allows the binding of DNA to the membrane.*
8. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube.
9. Add **750 µL** of **Buffer PE** to the QIAquick spin column and spin at **FULL speed** for **1 minute**.
10. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube.
11. Spin the column and collection tube at FULL speed for an **additional 1 minute** to get rid of residual ethanol in Buffer PE. <u>*Caution:*</u> *Residual ethanol from Buffer PE will NOT be completely removed unless the flow-through solution is discarded before this additional spin. If the residual ethanol is not removed from the column after spinning for 1 min, then spin for an additional 1-2 min.*

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

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

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

QIAquick Gel Extraction Procedure

Note: This procedure is used when you run 25 µL of PCR product on the gel and observe more than a single band.

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

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

Solutions Needed:

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

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

Materials Needed:

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

PROCEDURE

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

   Note: *If the desired band is faint, you many collect all the T-DNA bands on the gel and pool them together. Alternatively, run the remaining 25 μL of the desired PCR solutions on a new gel and excise those bands as well.*

*a. Prepare a **1% agarose** gel with a **20-tooth comb** (see Appendix 1A).*

*b. Add **3 µL** of **6x loading dye** to each tube of **~25 µL** PCR solutions containing the "T-DNA fragment."*

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

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

e. Record the identity of the sample in each well.

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

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

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

h. Take a picture of the gel.

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

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

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

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

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

n. *Place the agarose slices in the 1.5 mL microcentrifuge tube from step 6 labeled "**T-DNA**" and **your initials**.*
o. *Take a picture of the gel **after removing the agarose slices**. This step serves as a record of the DNA fragment being collected.*

<u>Note:</u> *Be sure to preform steps 8-25 at room temperature.*

8. Estimate the **gel volume** in the microcentrifuge tube using a **scale**. Use an empty 1.5 mL microcentrifuge tube as a blank. Write the **gel volume** on the side of the tube. <u>*Note:*</u> ***0.1 g** of **agarose gel** is **equivalent to 100 µL**.*
9. Add **3 gel volumes** of **Buffer QG** to the tube containing the gel slice. *For example, if the **weight** of **the agarose slice** is **0.15 g**, then its **gel volume** is **150 µL**. Therefore, add **450 µL** of **Buffer QG** to the tube.*
10. Incubate the tube at **50°C** for **10 minutes** or until the gel slice has **completely** dissolved. *Do **not** incubate at 50°C for more than 10 minutes! To help dissolve the gel, you may vortex the tube for 5 seconds every 2-3 min during the incubation. This step solubilizes the agarose completely. Make sure the color of the mixture is yellow.*
11. Add **1 gel volume** of **isopropanol** to the mixture and mix by **vortexing** for **5 seconds** or **inverting** the tubes **5-10 times**. *This increases the yield of DNA fragments. <u>Note:</u> Do not centrifuge the samples at this stage.*
12. Place a **QIAquick spin column** (lilac) in a provided **2 mL collection tube**. Label the lid of the spin column with "**T-DNA**" and **your initials**. *<u>Note:</u> If the gel bands were faint, use one column for multiple gel slices. This will increase the final concentration of purified DNA.*
13. Pipet the **mixture** from **step 12** into the **spin column**. *Do NOT pipet **more than 800 µL** of the mixture into the column. If the total volume is **more than 800 µL**, repeat **steps 13-15**.*
14. Centrifuge the spin column in the collection tube for **1 minute**. *This step allows DNA binding to the membrane.*
15. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube.

16. Add **500 µL** of **Buffer QG** to the **spin column** and centrifuge at FULL speed for **1 minute**. Discard the flow-through solution. *This step removes all traces of agarose.*
17. Add **750 µL** of **Buffer PE** to the column and let the tube stand for **2-5 minutes**. Centrifuge the tube at FULL speed for **1 minute**. Discard the flow-through solution. *This step washes the column.*
18. Repeat step 17 **two more times**.
19. Discard the flow-through solution and centrifuge for an **additional minute** to remove all the ethanol from the column. *Note: If any ethanol remains on the column, centrifuge for an additional 1-2 minutes.*
20. While spinning the tube, label a new 1.5 mL microcentrifuge tube with "**Gel Purified T-DNA**", **your initials** and **the date.**
21. Transfer the **QIAquick column** to the NEWLY labeled microcentrifuge tube. Discard the flow-through solution and the collection tube.
22. Add **30 µL** of **Buffer EB** to the **center of the column membrane**. Let the column stand for **1 minute**, and then centrifuge for **1 minute**. *This step elutes the DNA from the membrane. **DNA** is in the **microcentrifuge tube**. Note: If some liquid remains on the column after centrifugation, remove it with a P10 pipette and dispense the liquid back onto the center of the column membrane. Centrifuge again at FULL speed for 1 minute.*
23. Determine the DNA concentration using a NanoDrop spectrophotometer.

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

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

B. Sequencing Reaction with Big Dye v. 3

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

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

Solutions Needed:

- Applied Biosystems Big Dye version 3 (Obtained from UCLA Sequencing Facility)
- 5x Sequencing Buffer (Obtained from UCLA Sequencing Facility or Sigma Cat. #S3938)
- 12 μM LBb1.3 primer
- 12 μM Gene-specific Salk Forward primer OR Gene-specific Salk Reverse primer
- Sterile water

Materials Needed:

- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- 0.2 mL PCR tubes or strips of 8 tubes
- PCR tube rack
- Filter pipet tips
- Sequencing reaction purification columns (Qiagen DyeEx 2.0 Spin Kit)

Overview:

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

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

General Components of One Reaction:

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

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

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

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

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

Note: If the DNA concentration is too low, you may not be able to add the recommended amount of DNA. In this case, just add 6 μL. Alternatively: You may use the speed vac to concentrate your DNA.

Table: Amount of DNA to Use in a Sequencing Reaction

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

For this exercise, there is **ONE DNA template** (the purified PCR product of the T-DNA fragment), but there are **TWO primers**: LBb1.3 (T-DNA) primer and gene-specific salk primer. The gene-specific salk primer will be either forward or reverse depending on the orientation of the T-DNA Left Border (LB) relative to the gene of interest. The orientation of the T-DNA relative to the gene of interest was determined by your analysis of the genotyping PCR results. The sequencing reaction with the gene-specific primer serves as a control for the master mix of Big Dye and 5x Sequencing buffer. Therefore, it is best to prepare a **master mix** with **all components except** the **primers**, which will be added to individual reaction tubes.

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

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

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

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

<u>Sample calculations:</u>

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

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

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

PROCEDURE

1. Get ice from the icemaker in room 4128.
2. Thaw **12 µM primers** and **5x sequencing buffer** on a microcentrifuge tube rack at room temperature for 5 minutes. After the tubes have thawed, vortex briefly and centrifuge at full speed for 5 seconds. Place tubes on ice.
3. Label the top or sides of **TWO 0.2 mL PCR tubes** with **your initials** and **"T-DNA"** or **"Gene"**. Set the tubes on a PCR tube rack sitting on ice.
4. Label a **1.5 mL microcentrifuge tube** as "**Mmix**" and **your initials**. Set the tube on ice.
5. Prepare a **master mix (Mmix)** for **3 reactions** (2 reactions + 1 extra) by pipetting the following components into the **Mmix tube** as shown in the table below. *<u>Note:</u> Use the information on the previous page to fill in the volume of DNA solution to be added and calculate the volume of water to be added to the Mmix tube for 3 reactions.*

Master Mix (Mmix) for Sequencing Reactions:

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

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

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

		either	***or***
Components	**LBb1.3 primer**	**Gene-specific Salk <u>Forward</u> primer**	**Gene-specific Salk <u>Reverse</u> primer**
Mmix	**9 µL**	**9 µL**	**9 µL**
12 µM LB1 primer	**1 µL**	-	-
12 µM Gene-specific Salk Forward primer	-	**1 µL**	-
12 µM Gene-specific Salk Reverse primer	-	-	**1 µL**
Total volume	**10 µL**	**10 µL**	**10 µL**

7. Spin PCR tubes in the minicentrifuge for PCR tubes for **5 seconds** to bring the liquid to the bottom of the tubes.
8. Carry out cycling reaction using either **Applied Biosystems GeneAmp 9700**

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

   or **Bio-Rad MyCycler** with a **Big Dye** protocol with the same profile as above.
9. After the cycling reaction is finished, clean up the sequencing reactions using the DyeEx 2.0 Spin Columns (stored in the refrigerator drawer) as following:
   a. Resuspend the resin by flicking or gently vortexing upside down.

b. Loosen the cap of the column a **quarter turn**. *This is necessary to avoid a vacuum inside the spin column.*
c. Snap off the bottom closure of the spin column and discard it. Place the spin column in a 2 mL collection tube.
d. Centrifuge at **3,000 rpm** for **3 minutes** at room temperature.
e. Meanwhile, clearly label a new set of 1.5 mL microcentrifuge tubes with your initials and the primer name.
f. Carefully transfer the spin columns to the new tubes.
g. **Slowly** apply the sequencing reactions to the gel beds of the appropriate columns.

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

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

C. Retrieving and Analyzing DNA Sequences

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

1. Log into WebSeq at http://www.genetics.ucla.edu/webseq/

   a. Enter Username: **goldberg_r**
   b. Enter Password: **embryo**
   c. Click "LOGIN."
2. Find your sequence files by looking up the ***assigned file number*** and the name of the gene you are working on.

   Example: The ***assigned file number*** is **106203**, and the gene of interest is **At5g09250**. You would see the following files:
   106203GoldR At5g09250Fw A12.ab1
   106203GoldR At5g09250Rv B12.ab1
   What are the annotations?
   106203 = assigned file number; **GoldR** = user name; **At5g09250Fw** = name of sequence obtained with the Forward sequencing primer, **A12** = capillary position used in loading sequencing sample in the Sequencer (Biosystems 3730 Capillary DNA Analyzer), ab1 = ABI file format.
3. Check the boxes next to the sequences to be downloaded, and click "Download selected." Alternatively, click on each filename that you want to download.
4. Open the ab1 files in the "Downloads" window using a sequence viewer program (CHROMAS on Windows, or 4PEAKS on Mac).
5. Copy DNA sequences to a Microsoft Word file. Note: *Name the files according to the name of your gene of interest (for example, At5g09250).*
6. Determine the identity of your gene that has been knockout out by the T-DNA and the exact location of the T-DNA insertion by "BLASTN" and "BLASTX" searches. See Appendix 2. Note: *Blast search may take a few minutes or longer to complete depending on how busy the NCBI server in Washington D.C. is.*
7. Print out the Blast results as hard-copy records for your lab notebook.
8. Save the Blast results in the **pdf** format so that you can upload them to your webbook.

EXPERIMENT 3 - IDENTIFYING FEATURES OF MUTANT SEEDS USING NOMARSKI MICROSCOPY (GENE ONE)

STRATEGY

I. **OBSERVATION OF SEEDS USING LIGHT MICROSCOPY AND FIXING SEEDS FOR OBSERVATION WITH NOMARSKI OPTICS**

II. **OBSERVATION OF SEEDS AND EMBRYOS USING NOMARSKI OPTICS**

III. **OBSERVATION OF THE MATURE PLANT PHENOTYPE**

I. Observation of Seeds Using Light Microscopy and Fixing Seeds for Observation with Nomarski Optics

<u>Purpose:</u> To introduce the **D**ifferential **I**nterference **C**ontrast (**DIC**) or **N**omarski **I**nterference **C**ontrast (**NIC**) microscopy technique as a tool to identify features of defective embryos in knockout mutants.

<u>Reference:</u> The protocol was written by Dr. Miguel Aguilar in Professor Robert L. Fischer's laboratory at University of California, Berkeley.

Materials Needed:

- Siliques containing seeds with a wide range of embryo stages (globular to mature green) from *Arabidopsis*
    a. wild type
    b. homozygote or heterozygote mutant
- 100% ethanol
- Acetic acid
- Sterile water
- Chloral Hydrate (Cat. #C8383, Sigma-Aldrich; should be fresh)
- Glycerol (Invitrogen)
- Double-distilled water

Materials Needed:

- Pipettes
- Pipette tips (regular, non-filter)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Rulers with METRIC scale (mm)
- Plant layout chart
- Phenotype observation record
- Fine point forceps
- 30-gauge hypodermic needles
- Fine-point scissors or razor blades
- Coverslips
- Microscope Slides
- Double-sided tape
- Dissecting microscopes (borrowed from Dr. Pei Yun Lee)
- A microscope equipped with Nomarski optical parameter (Leica CTR5000)
- Microscope camera

PROCEDURE

Each student collects the following from wild type and his/her homozygous or heterozygous mutant:

a) 5 siliques containing seeds with embryo stages of globular to torpedo.

b) 2 siliques containing seeds with mature green embryos.

Note: *Be sure to collect a wide* **range** *of stages. Do not collect yellow or brown siliques; these contain dry seeds.*

1. Prepare 5 mL of a fixative solution of ethanol: acetic acid (9:1, v/v) in a 14 mL centrifuge tube using disposable 5 mL pipets.

FIXATIVE SOLUTION	
100% ethanol	4.5 mL
Acetic acid	0.5 mL
Total volume	5.0 mL

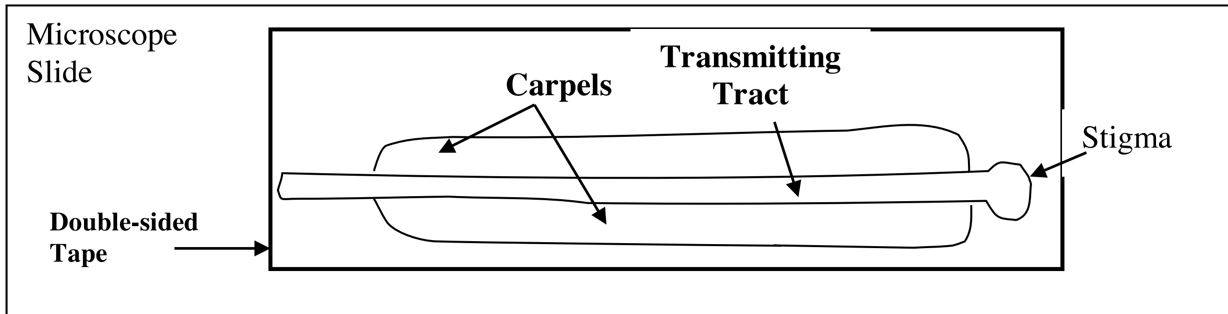
   Tightly snap the cap on the tube. Make sure the cap clicks. **Invert** the tube to mix the contents.

2. Pipet **1 mL** of the **fixative solution** into **FOUR** 1.5 mL microcentrifuge tubes sitting on a microcentrifuge tube rack. Label each tube in step 2 with your **initials**, the **plant #** and the **plant genotype**. *These tubes will be used in step 5i.*
3. Bring the following materials to the Plant Growth Center (PGC).
   - Bucket of ice
   - FOURTEEN 1.5 mL microcentrifuge tubes
   - Microcentrifuge tube rack
   - Black ultra-fine sharpie
   - Ruler with METRIC scale (mm)
   - A pair of fine point forceps
   - Plant layout chart with information about plant number and the genotype of those plants
   - This protocol
   - Bruincard with access to the PGC
   - Key to growth chambers in the PGC

4. Measure and collect siliques according to the chart below. Place each silique in a 1.5 mL microcentrifuge tube. Write your **initials**, the **plant #**, the **plant genotype** and the **length** on the tube. Keep the tube on **ice**. *Note: Collect the **same length** of siliques for wild type and homozygous/heterozygous mutant so that you can compare them.*

Plant Genotype	Seed Stages Collected	Length of Siliques Collected	Number of Siliques Collected
Wild type	globular to torpedo	*0.5 - 1.0 cm*	5
Wild type	mature green	*1.0 - 1.9 cm*	2
Heterozygous or homozygous mutant	globular to torpedo	*0.5 - 1.0 cm*	5
Heterozygous or homozygous mutant	mature green	*1.0 - 1.9 cm*	2

5. Go back to the lab. **Dissect** the siliques and **observe** the seed phenotype using a dissecting microscope. *Note: Work quickly so the seeds don't dry out. You may also place a drop of water on the silique.*
   a. **Place** a **piece of double-sided tape** on a **microscope slide**. Label the microscope slide with a small piece of white tape with your **initials**, the **plant #**, the **plant genotype** and the **length**.
   b. Carefully, use **fine-point forceps** to place a silique on the tape.
   c. Under a dissecting microscope, use **fine-point forceps** to carefully **arrange** the silique such that the **transmitting tract** is facing you (see diagram below, NOT drawn to scale).



d. With your left hand, use forceps to hold the silique on the side closest to the stem.
e. With your right hand, use a **28G** or **30G hypodermic needle attached to a 1 cc syringe** to **slice** the **carpels along each side of the transmitting tract.**
f. **Gently** peel back the carpels and stick them to the tape to reveal the seeds.
g. Observe the phenotype. Note any phenotypes that you observe on your **Screening Seeds Using Light Microscopy** chart.

In what stage of development are the seeds?

How many seeds are in the silique?

How many are green?

How many are white?

How many are brown?

What is the expected ratio of wild type seeds to mutant seeds if the mutation is seed lethal?

What is the observed ratio of wild type seeds to mutant seeds?

Are the observed results significantly different from the expected results? Use a Chi-Square test.

$$\chi^2 = \Sigma \frac{(observed - expected)^2}{expected}$$

Probability that the deviation is due to chance alone

Degrees of Freedom	0.5	0.1	0.05	0.02	0.01	0.001
1	0.455	2.706	3.841	5.412	6.635	10.827
2	1.386	4.605	5.991	7.824	9.210	13.815
3	2.366	6.251	7.815	9.837	11.345	16.268
4	3.357	7.779	9.488	11.668	13.277	18.465
5	4.351	9.235	11.070	13.388	15.086	20.517

What is your null hypothesis?

How many degrees of freedom are there?

(The degrees of freedom is one less than the number of different phenotypes possible.)

What is your chi-square value?

(The chi-square statistic is a probability that indicates the chance that, in repeated experiments, deviations from the expected would be as large or larger than the ones observed in this experiment)

What is the probability that the deviation of the observed values from the expected values was a chance occurrence?

(Look up your degrees of freedom in the table. Find where your chi-square value falls in that row.)

Can you reject the null hypothesis?

If the probability is less than 0.05 (5%), reject your null hypothesis.

If the probability is 0.05 (5%) or greater, then you cannot reject your null hypothesis.

h. Ask your TA to take pictures of the seeds within the siliques.

i. **Before the seeds dry out**, use the fine-point forceps to transfer the cut silique into the tube with fixative solution from **step 2**.

j. Repeat steps a-i for the other siliques. *Note: You collected an excess of siliques so that you would have some to practice dissecting and to have a*

range of developmental stages for each genotype. However, you only need to fix FOUR siliques.

  i. *Wild type, early development*
  ii. *Heterozygous (or homozygous), early development*
  iii. *Wild type, late development (mature green stage)*
  iv. *Heterozygous (or homozygous), late development (mature green stage)*

6. Fix seeds and siliques in the fixative solution for at least 2 hours. Note: *It is recommended to fix the siliques* ***overnight*** *to ensure that the fixative solution penetrates the seeds and their embryos. It is okay to leave siliques in the fixative solution for up to 3 days.*
7. **Carefully**, pipet off **900 µL** of the fixative solution using a **P-1000** pipette and discard into a beaker labeled "acetic acid waste." Then remove the **remaining volume** with a **P-200** pipette. Note: *Do not let the seeds and siliques dry out, and do not pipet up your seeds.*
8. **Immediately**, pipet **1 mL** of **90% ethanol** solution into the tube using a P-1000 pipette. Note: *The 90% ethanol solution will remove chlorophyll from the embryos.*

90% ETHANOL SOLUTION

Absolute ethanol	4.5 mL
Double-distilled water	0.5 mL
Total volume	5.0 mL

9. Incubate seeds and siliques in the 90% ethanol solution for **0.5 - 1 hour**. Note: *It is safe to store the materials in the ethanol indefinitely.*
10. Replace the 90% ethanol solution with **70% ethanol** as in steps 7 & 8.

70% ETHANOL SOLUTION

Absolute ethanol	3.5 mL
Double-distilled water	1.5 mL
Total volume	5.0 mL

11. Incubate seeds and siliques in the ethanol solution for **0.5 - 1 hour**. <u>Note:</u> *It is safe to store the materials in the ethanol indefinitely*.

II. Observation of Seeds and Embryos Using Nomarski Optics

<u>Note:</u>

- ***Before observation of the seeds and their embryos, seeds must be submerged in the clearing solution. For young seeds, clearing is usually fast (~5 minutes). The older the silique, the longer it takes to clear (~ 1 hour). Seeds are ready for observation after they sink in the clearing solution.***
- ***Tissues CANNOT be stored in the CLEARING solution for more than TWO days because they will lose their structures quickly***.

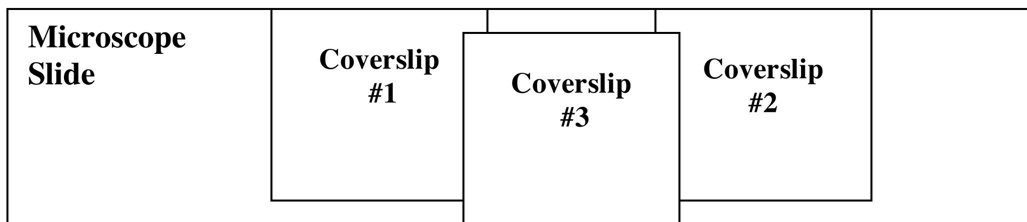
1. Prepare a ***fresh*** clearing solution of chloral hydrate/glycerol/water (8:1:2, w/v/v) in a 14 mL centrifuge tube. <u>Note:</u> *The TA will prepare this solution before the lab class begins*.

<u>CLEARING SOLUTION</u>	
Chloral hydrate	8 g
Glycerol	1 mL
Water	2 mL
Total volume	~7 mL

2. **Carefully**, pipet off **900 µL** of the 70% ethanol solution using a **P-1000** pipette and discard into a beaker labeled "ethanol waste." Then remove the **remaining volume** with a **P-200** pipette. <u>Note:</u> *Do not let the seeds and siliques dry out, and do not pipet up your seeds*.
3. Replace the 70% ethanol solution with **100 µL of clearing solution**.
4. Incubate seeds and siliques in the clearing solution for **5 min - 1 hour**. Wait until the seeds **sink** to the bottom of the tubes. You may lay the tube on its side so that the

silique is immersed in the clearing solution. <u>*Note:*</u> *Tissues CANNOT be stored in the CLEARING solution*.

5. Set a new glass microscope slide on the bench. Label it with your **initials**, the **plant #**, the **plant genotype** and **silique length**.
6. Use forceps to remove a silique from the clearing solution and place it on the labeled glass slide.
7. Pipet the remaining clearing solution and seeds onto the slide with the silique.
8. Carefully, place two square coverslips, one on each side of the solution. Then, place a third coverslip over the clearing solution. Avoid trapping bubbles in the solution (see diagram below).



9. Observe the seeds under Nomarski optics using the Leica CTR5000 microscope.
10. Take pictures of the embryos.

    In what stage of development are the seeds?

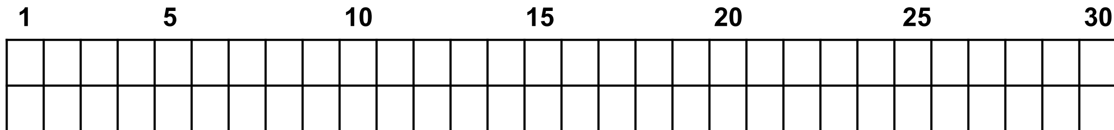
11. Repeat steps 2-10 for the remaining 3 fixed siliques.

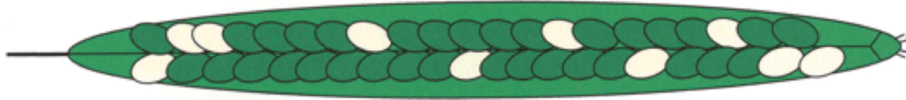
Screening Seeds Using Light Microscopy

AGI# ________________ SALK # ________________ Plant #______Genotype ____________

Silique # _____ Length of Silique (cm) _____ Total Seeds ______ Total Mutant Seeds ______

<u>Instructions:</u> The grid represents the layout of the silique. Put a number in each square that corresponds to a mutant seed. Describe the seed phenotypes in the chart below. The base of the silique is defined as the region closest to the pedicel and main stem, which is at the left of the grid.





Seed	Seed Coat Color	Embryo Color	Notes
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

III. Observation of the Mature Plant Phenotype

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

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

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

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

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

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

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

STRATEGY

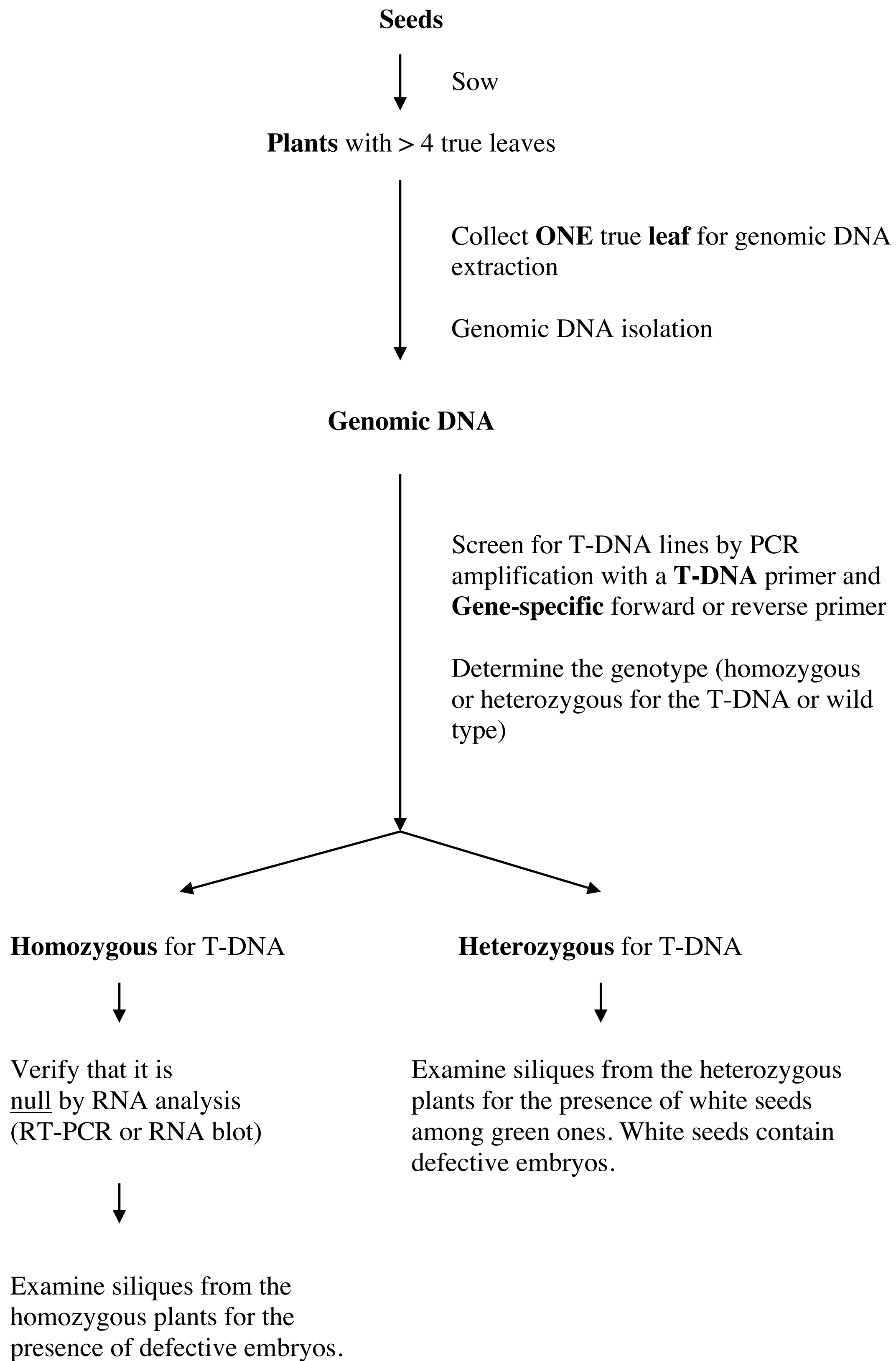
I. **SOWING SEEDS AND GROWING PLANTS**

II. **ISOLATING GENOMIC DNA FROM LEAVES OF AT LEAST 2-3 WEEK-OLD SEEDLINGS**

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

IV. **DETERMING THE T-DNA INSERTION SITE**

STRATEGY



I. SOWING SEEDS AND GROWING PLANTS

Purpose: To generate seedlings for genomic DNA extraction.

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

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

Materials Needed:

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

PROCEDURE

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

1. Obtain **tubes of seeds** to be grown from the cold room and put them on a microcentrifuge tube rack. *For example, S_112701 (Salk line for gene At5g11240) and wild type seeds (Columbia-0).*
2. Bring the **materials** to the **Plant Growth Center** (**PGC**).
3. At the PGC, put all of these items on **the bench** that runs along the **east wall**. This bench does NOT have any soil on it. *Note: Do NOT put tubes of seeds or plants full of mature siliques near the bench of soil because the prepared soil will be*

contaminated with these seeds, which could in turn, result in false mutant phenotypes for other people's work.

4. In the PGC, prepare ONE flat with **12 pots** of soil for every TWO knockout lines being planted.
    a. Assemble each flat as follows:
        i. Obtain a black plastic tray without holes.
        ii. Obtain a sheet of 12 rectangular plastic pots.
        iii. Obtain a clear plastic cover.
        iv. Set the sheet of 12 pots in the black plastic tray.
        v. Fill the pots loosely with soil, without compressing. Use the sifter to add a layer of fine soil. *(Soil prepared by PGC staff, Mr. Weimin Deng).*
        vi. Flatten the surface of the soil by scraping off excess soil with a metal plate.
    b. Remove **one pot** from the corner of the flat and put the soil back into the mound of soil. So, there are **only 11 pots**. *The empty space will make it easier to put the water in.*
    c. Bring the flat to the bench near the sink.
    d. Use the hand brush to clean up the soil bench.
    e. Make sure that the water hose is attached to the water pipeline labeled "**fertilizer-supplemented**."
    f. Fill each flat **2/3 of the way up** the tray with "fertilizer-supplemented" water.
    g. Cover the flat with a **clear plastic cover** to prevent the growth of air-borne molds and to protect the soil from **stray *Arabidopsis* seeds**.
    h. Wait **20 minutes or until the surface of the soil appears darker** due to water seeping up from the bottom of the pots.
5. Label **11 plastic tags** with a black sharpie.
    a. For **knockout lines**: **Gene name**
Salk line #
Date
Pot # 1-10
    b. For **wild type**: **Columbia-0**

Date
Pot # 11

6. Bring the flat over to the bench where the seeds and planting tools are located (or any other bench removed from the soil).
7. Fold each **quarter sheet** of white paper **in half**, lengthwise.
8. Gently pour out the **knockout seeds** from the microcentrifuge tube onto one of the folded pieces of paper.
9. Bring the folded paper with seeds close to the soil of each of the first 5 pots. Lower one end of the paper near the soil surface. Use the **forceps** to guide one seed off of the paper to a precise location in the pot without dumping all of the seeds from the paper.
10. Visually divide the pot into 4 quadrants, and sow a seed in each quadrant. Sow **4 seeds** per pot, for the first 5 pots. *Note: Planted seeds should not be covered with additional soil because Arabidopsis seeds need light for germination.*
11. Put the labeled tags for the **knockout line** into **each** of the **5 pots** containing knockout seeds.
12. Put the seeds that were not used back into the **appropriate knockout seed** microcentrifuge tube.
13. Repeat steps 7-12 with the seeds for the next knockout line and pots #6-10. Use a **new** folded piece of white paper for each line.
14. For pot **#11**, pour out wild type seeds onto a **new** folded piece of white paper. Visually divide the pot into 4 quadrants, and sow a wild type seed in each quadrant. **Four wild type seeds** should be sown in **pot #11.**
15. Put a **wild type labeled tag** into pot #11.
16. Cover the flat with the **clear plastic cover**. Put the flat aside.
17. After all of the lines are sown, put the flats on a metal cart and take the elevator to the lower level.
18. Put the flats on the wire racks in the cold room (the first room on the right after entering the double doors across from the elevator).
    CAUTION: Make sure the clear covers completely cover the flats so that no air-borne molds in the cold-room get in the soil. Note: Turn the lights off when you leave the cold room.

19. Leave the flats in the cold room for **2-5 days** to **vernalize seeds.** _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."

PLANT LAYOUT CHART

GENOTYPING ARABIDOPSIS PLANTS

Gene ID: **At__ g _________ Salk line#: ____________ Date: ________**
Primers for PCR: _______________________________
Size of PCR product: __________

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

II. ISOLATING GENOMIC DNA FROM LEAVES OF AT LEAST 2-3 WEEK-OLD SEEDLINGS

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

Recommendation:

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

Materials Needed:

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

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

PROCEDURE

<u>Attention:</u> *You will need to* ***assess*** *the* ***quality*** *of* ***isolated genomic DNA*** *later (at* ***step 36****). If you plan to do this on the same day as the genomic DNA isolation, use time efficiently by preparing a 0.7% agarose gel before you start the isolation of genomic DNA (see* ***Appendix 1A****). While the agarose mixture cools in the 55-60°C water bath for at least 10 minutes, go to the Plant Growth Center to collect leaves. When you come back to the lab, add 10,000x SYBR Safe DNA gel stain to the agarose mixture, swirl to mix, pour the gel, and let the agarose mixture solidify in the dark.*

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

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

   Tube #**6**: **1** Seedling from **wild type** (Columbia-0)
3. Pipet **100 μL** of **Extraction Buffer** into each tube. Keep the tubes on ice. *<u>Note:</u> It is* ***not*** *necessary to keep tubes of* ***Extraction Buffer on ice*** *during collection of the leaf samples if genomic DNA will be isolated from samples within one hour.*
4. Gather together the following items on a plastic tray or container:
   - ➢ Bucket of ice
   - ➢ 1.5 mL microcentrifuge tubes containing 100 μL of Extraction Buffer
   - ➢ One or two pairs of latex gloves
   - ➢ Two pairs of pointed-end forceps
   - ➢ Squirt bottle of 100% ethanol solution
   - ➢ Kimwipes
   - ➢ Black sharpie (ultra-fine or fine)
   - ➢ Pen
   - ➢ Plant layout chart
   - ➢ This protocol
   - ➢ Digital camera
   - ➢ Key to the Plant Growth Center
   - ➢ BruinCard with access to PGC
5. Go to the **Plant Growth Center (PGC)** and locate your flat with plants.

6. Take pictures of the plants to document the phenotype. Take pictures of the tags to identify the plants in the pictures.
7. Use the **Plant Layout Chart** to mark the **locations of the plants** and to indicate the plants that you will collect samples from by numbering them. Also make a note of any interesting phenotypes. (For example, some plants may be smaller than others.) <u>Note:</u> *NOT all of the seeds will have germinated.*
8. Use a piece of Kimwipes to clean the forceps with ethanol. <u>*Note:*</u> *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.
    <u>*Note:*</u> *MAKE SURE TO CLEAN THE FORCEPS BETWEEN LEAF SAMPLES!*
12. Go back to the lab.
13. Homogenize or macerate the collected leaf in **tube #1** by crushing it with a **blue micropestle** until no more chunks of plant tissue are observed in the mixture. <u>*Note:*</u> *Do NOT dispose of the micropestle.*
14. Rinse the **micropestle** with **300 µL** of Extraction Buffer into the microcentrifuge tube. Put the used micropestles in a beaker labeled "used micropestles" so that they can be washed. The **total volume** of Extraction Buffer in the microcentrifuge tube is now **400 µL**.
15. Vortex the **homogenate** for 5 seconds. Set the tube on ice.
16. Repeat steps 13-15 for the other tubes.
17. Centrifuge the tubes of homogenates at **room temperature** for **5 minutes** at **FULL speed**. <u>*Note:*</u> *Position the tubes in the centrifuge so that the hinge of the microcentrifuge tubes faces the outside of the microcentrifuge. This way after centrifugation you know to look for your pellet on the side of the microcentrifuge tube that has the hinge.*
18. Meanwhile, **label** a set of **microcentrifuge tubes** with **Gene Name, plant #,** "**gDNA**," **your initials** and the **date.**
19. Pipet **350 µL** of **isopropanol** to each of the new labeled tubes.

20. After centrifugation, transfer the tubes from the microcentrifuge onto a microcentrifuge tube rack. Organize tubes on the rack such that the **numbers** on the lids of the **NEW tubes match** with the **numbers** on the lids of the **tubes containing homogenates**.
21. Pipet **350 µL** of **supernatant** (**homogenate**) from the centrifuged tubes to the corresponding tubes containing isopropanol. Close the lids of the tubes. *Note: Use your pipet to draw off liquid from the side of the tube opposite to the side where the plant material is pelleted. Start at the top and move downward as the liquid level drops. AVOID disturbing the plant debris on the bottom of the tubes as much as possible. However, it is okay if you accidentally transfer some plant debris into the isopropanol tube.*
22. Mix the isopropanol and homogenate by inverting the tubes **5-10 times**.
23. Incubate the mixture at **room temperature** for **5 minutes** to precipitate **nucleic acids** *(both genomic DNA and total RNA)*.
24. Centrifuge the tubes at **room temperature** for **10 minutes** at **FULL** speed.
25. Pour or pipet each isopropanol supernatant into a waste container. *Note: Be extremely careful when pouring off the isopropanol because the pellets are sometimes loose. DNA is now in your white or clear pellet along with RNA.*
26. Add **1 mL** of **80% ethanol** solution to each pellet. Close the lid of the tube and invert **5 times**. *This step is to wash off any residual salt (from the extraction buffer) and isopropanol.*
27. Centrifuge the tubes at **room temperature** for **5 minutes**.
28. Pour or pipet each ethanol supernatant into a waste container. Dab the tubes upside down on Kimwipes to remove as much ethanol as possible. *Note: Be extremely careful when pouring off the ethanol solution because the pellets are sometimes loose.*
29. Put the tubes on a microcentrifuge tube rack with their lids open, allowing the ethanol to evaporate. *Note: You may use a P-200 pipette to carefully draw off excess ethanol from the side of the tube opposite that against which the nucleic acid is pelleted. Be very careful not to pipet the pellet.*
30. Dry pellets either in a **SpeedVac** at room temperature for **5-10 minutes** (Your instructor will operate the SpeedVac) or by leaving on the **bench at room temperature for up to 60 minutes**.

31. After drying the pellets, resuspend each pellet by adding **200 µL** of **TE** buffer, closing the lids of the tubes, and **raking** the tubes over a microcentrifuge tube rack **10 times** 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 the liquid and any contaminants to the bottom of the tubes.
33. Store DNA solutions at **4°C** (on ice or in refrigerator) until use.
    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 that can degrade DNA.

    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 SIX microcentrifuge tubes with **#1-6** and **your initials**, and set tubes on a microcentrifuge tube rack.
    c. Pipet **20 µL** of **isolated genomic DNA** solution into each of the labeled tubes.
    d. Add **2 µL** of **6x loading dye solution** to each tube and mix the contents by pipetting up and down 5 times.
    e. Load **10 µL** of **1 Kb Plus DNA ladder solution** into the first well.
    f. Load **20 µL** of each sample-dye mixture prepared in step d using a P-20 pipette.
    g. Record the identity of the sample in each well.

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

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

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

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

Starting time:

Ending time:

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

What do you observe on the gel?

What is the size of genomic DNA?

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

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

*<u>Note:</u> First, spin the tubes of DNA solutions in a microcentrifuge at **room temperature** for **2 minutes** at **FULL** speed to bring down water condensation on the lids as well as any contaminating plant debris and/or carbohydrates in the solutions to the bottom of the tubes.*

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

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

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

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

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

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

STRATEGY

A. Polymerase Chain Reaction (PCR)

B. Gel Electrophoresis Analysis of PCR Product

C. Label T-DNA-Tagged Plants

Solutions Needed:

- Ex Taq DNA polymerase (Takara)
- 10x Ex Taq buffer (Takara)
- dNTP mix (Takara)
- Sterile water
- 12 μM Gene-specific Salk Forward primer
- 12 μM Gene-specific Salk Reverse primer
- 12 μM LBb1.3 primer (anneals to the Left Border (LB) region of the T-DNA)
- Genomic DNA extracted from the plants to be genotyped (including WT)
- Genomic DNA extracted from wild type seedlings by TA
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain
- 6x Loading dye
- 50 ng/μL 1 Kb Plus DNA ladder solution

Materials Needed:

- Pipettes
- Filter pipet tips for PCR
- 0.2 mL PCR tubes in strips of 8
- 1.5 mL microcentrifuge tubes
- Rack for 0.2 mL PCR tubes
- Rack for 1.5 mL microcentrifuge tubes
- Ice bucket
- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- Microcentrifuge
- Gel electrophoresis materials (Appendix 1A)
- Key to the Plant Growth Center

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

A. Polymerase Chain Reaction (PCR)

PROCEDURE

*Note: Because you do not know the orientation of the T-DNA insertion, and therefore the direction of the LBb1.3 primer, you will need to set up **THREE** PCR reactions for **each** sample.*

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

*Reaction B: **LBb1.3** primer and **Gene-specific Salk Reverse** primer*

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

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

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

1. Get ice from the icemaker in room 4128.
2. Thaw tubes of **10x Ex Taq buffer, dNTP mix, 12 μM Gene-specific Salk Forward primer, 12 μM Gene-specific Salk Reverse primer** and **12 μM LBb1.3 primer** on a rack for 1.5 mL microcentrifuge tubes at **room temperature** for 5-10 minutes.
3. **Vortex** each tube on a setting of 2-3 for 10 seconds. **Spin** in microcentrifuge at full speed for 5 seconds. Put the tubes on **ice** until needed.

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

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

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

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

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

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

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

72°C 4 min
4°C ∞

19. Press "**F5**" for "**DONE.**" The "CHOOSE OPERATION" menu will appear. Press "**ENTER**" to **run the protocol**.
20. Enter the **volume** of the PCR reaction (50 μL). Press "**F5**" to "**Begin Run**." *Note: It will take about 3 hours for the PCR amplification to be completed.*
21. Once the PCR amplification is complete, remove the PCR tubes from the PCR machine and store them on ice or in the **refrigerator** until gel electrophoresis or leave them in the PCR machine at 4°C until you have a chance to put them away later.

B. Gel Electrophoresis Analysis of PCR Product

PROCEDURE

1. Prepare a **1% agarose gel** in 1x TAE buffer with a **30-tooth** comb (or 20-tooth comb depending on how many samples you have).
2. Record the identity of the sample in each lane. Use the table below as a guide.
3. Label **24 1.5 mL microcentrifuge** tubes (one for each sample) and set them on a microcentrifuge tube rack.
4. Add **3 μL** of **6x loading dye** to each tube.
5. Pipet **25 μL** of **PCR solution** to each tube. Spin briefly. *Note: Store remaining PCR product in the PCR tubes in the refrigerator.*
6. Slowly pipet **10 μL** of **100 bp DNA ladder** in the first well. *Note: Avoid pipetting bubbles into the well.*
7. Mix the contents of your first sample-dye mixture by pipetting up and down 5 times. Slowly pipet **~28 μL** of sample-dye mixture into the next well using a P-20 pipette *(i.e. load ~15 μL first, then then remaining ~15 μL into the same well)*. Repeat for the remaining sample-dye mixtures.
8. Slowly pipet **10 μL** of **1 Kb Plus DNA ladder** in the last well.

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

9. Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode (near the bottom of the gel).
10. Run the gel at **105 volts** for **1-2 hours** or until the lower dye (bromophenol blue) travels two-thirds of the gel.
11. Stop the gel electrophoresis.
12. Take a picture of the gel and annotate it.
13. Analyze the data.
    Do you observe PCR fragments?
    What are the sizes of these fragments?
    Do the sizes agree with expected sizes for the gene of interest and T-DNA insertion?
    What is the orientation of the T-DNA relative to the gene?
    What are the genotypes of the 6 plants?

Note:

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

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

C. Label T-DNA-Tagged Plants

PROCEDURE

1. After determining the genotypes of the plants, make labels for the plants containing a T-DNA insert by putting a small piece of tape on a wooden stick. Write the **number** that corresponds to the **plant #** on the Plant Layout Chart and either "**homozygous for the T-DNA**" or "**heterozygous for the T-DNA**." You can also note the genotype of the plants on the Plant Layout Chart.
2. Go to the Plant Growth Center. Put the labeled wooden sticks next to the identified T-DNA tagged plants.

IV. DETERMING THE T-DNA INSERTION SITE

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

STRATEGY

A. **Purifying PCR Products**

B. **Sequencing Reaction with Big Dye v. 3**

C. **Retrieving and Analyzing DNA Sequences**

A. Purifying PCR Products

QIAquick PCR Purification Procedure

Note: This procedure is used when you run 25 μL of PCR product on the gel and observe only one band.

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

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

Solutions Needed:

- QIAquick PCR Purification Kit (Qiagen, Cat. # 28104)
- Remaining PCR solutions (~25 μL)

Materials Needed:

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

- Kimwipes

PROCEDURE

1. Write "**T-DNA**" and **your initials** on a 1.5 mL microcentrifuge tube.
2. Pipet **~25 µL** of the **PCR product solution** from one of the PCR tubes containing the T-DNA fragment into the labeled 1.5 mL microcentrifuge tube.
3. Measure the exact volume of solution.
4. Add **125 µL** of **Buffer PB** (or **5 volumes** of **Buffer PB** to **1 volume** of the PCR product solution) to the tube in step 2. Mix by vortexing for 5 seconds.
5. Spin the tube in the microcentrifuge at **FULL speed** for **10 seconds** to bring all the solution down to the bottom of the tube. Set the tube back on the microcentrifuge tube rack.
6. Place a **QIAquick spin column** (lilac) in the provided **2 mL collection tube**. Label the lid of the column "**T-DNA**" and **your initials**.
7. Pipet the sample mixture in step 4 to the QIAquick spin column. Spin the column and collection tube in the microcentrifuge at **FULL speed** for **1 minute**. *This step allows the binding of DNA to the membrane.*
8. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube.
9. Add **750 µL** of **Buffer PE** to the QIAquick spin column and spin at **FULL speed** for **1 minute**.
10. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube.
11. Spin the column and collection tube at FULL speed for an **additional 1 minute** to get rid of residual ethanol in Buffer PE. *Caution: Residual ethanol from Buffer PE will NOT be completely removed unless the flow-through solution is discarded before this additional spin. If the residual ethanol is not removed from the column after spinning for 1 min, then spin for an additional 1-2 min.*

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

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

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

QIAquick Gel Extraction Procedure

Note: This procedure is used when you run 25 µL of PCR product on the gel and observe more than a single band.

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

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

Solutions Needed:

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

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

Materials Needed:

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

PROCEDURE

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

<u>Note:</u> *If the desired band is faint, you many collect all the T-DNA bands on the gel and pool them together. Alternatively, run the remaining 25 μL of the desired PCR solutions on a new gel and excise those bands as well.*

a. *Prepare a **1% agarose** gel with a **20-tooth comb** (see Appendix 1A).*
b. *Add **3 µL** of **6x loading dye** to each tube of **~25 µL** PCR solutions containing the "T-DNA fragment."*
c. *Slowly pipet **10 µL of 1 Kb Plus DNA ladder** into the first well. <u>Note:</u> Avoid pipetting bubbles into the well.*
d. *Mix the contents of your first sample-dye mixture by pipetting up and down 5 times. Slowly pipet **~28 µL** of sample-dye mixture into the next well using a P-20 pipette (i.e. load ~15 µL first, then then remaining ~15 µL into the same well).*
e. *Record the identity of the sample in each well.*

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

f. *Add **10 µL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.*
g. *Run the gel at **105 volts** for **1-2 hours** or until the lower dye (bromophenol blue) travels two-thirds of the gel.*
h. *Take a picture of the gel.*
i. *Verify the presence of the **expected size PCR product**.*
j. *Place a NEW piece of plastic wrap on an ultraviolet (UV) light box. Then place your gel on the plastic wrap.*
k. *Put on a UV shield to protect your eyes and face.*
l. *Turn **on** the **UV box**. <u>Note:</u> Turn **off** the UV box as soon as you are done excising the DNA band.*
m. *Excise the desired DNA fragments from the gel using a razor blade. <u>Note:</u> Trim off excess agarose surrounding the DNA band as much as possible. Your TA will demonstrate.*

n. *Place the agarose slices in the 1.5 mL microcentrifuge tube from step 6 labeled "**T-DNA**" and **your initials**.*
o. *Take a picture of the gel **after removing the agarose slices**. This step serves as a record of the DNA fragment being collected.*

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

8. Estimate the **gel volume** in the microcentrifuge tube using a **scale**. Use an empty 1.5 mL microcentrifuge tube as a blank. Write the **gel volume** on the side of the tube. *Note: **0.1 g** of **agarose gel** is **equivalent to 100 μL**.*
9. Add **3 gel volumes** of **Buffer QG** to the tube containing the gel slice. *For example, if the **weight** of **the agarose slice** is **0.15 g**, then its **gel volume** is **150 μL**. Therefore, add **450 μL** of **Buffer QG** to the tube.*
10. Incubate the tube at **50°C** for **10 minutes** or until the gel slice has **completely** dissolved. *Do **not** incubate at 50°C for more than 10 minutes! To help dissolve the gel, you may vortex the tube for 5 seconds every 2-3 min during the incubation. This step solubilizes the agarose completely. Make sure the color of the mixture is yellow.*
11. Add **1 gel volume** of **isopropanol** to the mixture and mix by **vortexing** for **5 seconds** or **inverting** the tubes **5-10 times**. *This increases the yield of DNA fragments. Note: Do not centrifuge the samples at this stage.*
12. Place a **QIAquick spin column** (lilac) in a provided **2 mL collection tube**. Label the lid of the spin column with "**T-DNA**" and **your initials**. *Note: If the gel bands were faint, use one column for multiple gel slices. This will increase the final concentration of purified DNA.*
13. Pipet the **mixture** from **step 12** into the **spin column**. *Do NOT pipet **more than 800 μL** of the mixture into the column. If the total volume is **more than 800 μL**, repeat **steps 13-15**.*
14. Centrifuge the spin column in the collection tube for **1 minute**. *This step allows DNA binding to the membrane.*
15. Discard the **flow-through solution** in the collection tube by pouring it into a waste container. Blot the collection tube on Kimwipes. Put the QIAquick column back into the same collection tube.

16. Add **500 µL** of **Buffer QG** to the **spin column** and centrifuge at FULL speed for **1 minute**. Discard the flow-through solution. *This step removes all traces of agarose.*
17. Add **750 µL** of **Buffer PE** to the column and let the tube stand for **2-5 minutes**. Centrifuge the tube at FULL speed for **1 minute**. Discard the flow-through solution. *This step washes the column.*
18. Repeat step 17 **two more times**.
19. Discard the flow-through solution and centrifuge for an **additional minute** to remove all the ethanol from the column. *Note: If any ethanol remains on the column, centrifuge for an additional 1-2 minutes.*
20. While spinning the tube, label a new 1.5 mL microcentrifuge tube with "**Gel Purified T-DNA**", **your initials** and **the date.**
21. Transfer the **QIAquick column** to the NEWLY labeled microcentrifuge tube. Discard the flow-through solution and the collection tube.
22. Add **30 µL** of **Buffer EB** to the **center of the column membrane**. Let the column stand for **1 minute**, and then centrifuge for **1 minute**. *This step elutes the DNA from the membrane. **DNA** is in the **microcentrifuge tube**. Note: If some liquid remains on the column after centrifugation, remove it with a P10 pipette and dispense the liquid back onto the center of the column membrane. Centrifuge again at FULL speed for 1 minute.*
23. Determine the DNA concentration using a NanoDrop spectrophotometer.

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

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

B. Sequencing Reaction with Big Dye v. 3

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

References: Applied Biosystems

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

Solutions Needed:

- Applied Biosystems Big Dye version 3 (Obtained from UCLA Sequencing Facility)
- 5x Sequencing Buffer (Obtained from UCLA Sequencing Facility or Sigma Cat. #S3938)
- 12 μM LBb1.3 primer
- 12 μM Gene-specific Salk Forward primer OR Gene-specific Salk Reverse primer
- Sterile water

Materials Needed:

- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- 0.2 mL PCR tubes or strips of 8 tubes
- PCR tube rack
- Filter pipet tips
- Sequencing reaction purification columns (Qiagen DyeEx 2.0 Spin Kit)

Overview:

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

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

General Components of One Reaction:

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

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

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

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

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

Note: If the DNA concentration is too low, you may not be able to add the recommended amount of DNA. In this case, just add 6 μL. Alternatively: You may use the speed vac to concentrate your DNA.

Table: Amount of DNA to Use in a Sequencing Reaction

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

For this exercise, there is **ONE DNA template** (the purified PCR product of the T-DNA fragment), but there are **TWO primers**: LBb1.3 (T-DNA) primer and gene-specific salk primer. The gene-specific salk primer will be either forward or reverse depending on the orientation of the T-DNA Left Border (LB) relative to the gene of interest. The orientation of the T-DNA relative to the gene of interest was determined by your analysis of the genotyping PCR results. The sequencing reaction with the gene-specific primer serves as a control for the master mix of Big Dye and 5x Sequencing buffer. Therefore, it is best to prepare a **master mix** with **all components except** the **primers**, which will be added to individual reaction tubes.

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

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

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

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

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. Thaw **12 µM primers** and **5x sequencing buffer** on a microcentrifuge tube rack at room temperature for 5 minutes. After the tubes have thawed, vortex briefly and centrifuge at full speed for 5 seconds. Place tubes on ice.
3. Label the top or sides of **TWO 0.2 mL PCR tubes** with **your initials** and **"T-DNA"** or **"Gene"**. Set the tubes on a PCR tube rack sitting on ice.
4. Label a **1.5 mL microcentrifuge tube** as "**Mmix**" and **your initials**. Set the tube on ice.
5. Prepare a **master mix (Mmix)** for **3 reactions** (2 reactions + 1 extra) by pipetting the following components into the **Mmix tube** as shown in the table below. *Note: Use the information on the previous page to fill in the volume of DNA solution to be added and calculate the volume of water to be added to the Mmix tube for 3 reactions.*

Master Mix (Mmix) for Sequencing Reactions:

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

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

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

either ***or***

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

7. Spin PCR tubes in the minicentrifuge for PCR tubes for **5 seconds** to bring the liquid to the bottom of the tubes.
8. Carry out cycling reaction using either **Applied Biosystems GeneAmp 9700**

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

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

9. After the cycling reaction is finished, clean up the sequencing reactions using the DyeEx 2.0 Spin Columns (stored in the refrigerator drawer) as following:
   a. Resuspend the resin by flicking or gently vortexing upside down.

b. Loosen the cap of the column a **quarter turn**. *This is necessary to avoid a vacuum inside the spin column.*
c. Snap off the bottom closure of the spin column and discard it. Place the spin column in a 2 mL collection tube.
d. Centrifuge at **3,000 rpm** for **3 minutes** at room temperature.
e. Meanwhile, clearly label a new set of 1.5 mL microcentrifuge tubes with your initials and the primer name.
f. Carefully transfer the spin columns to the new tubes.
g. **Slowly** apply the sequencing reactions to the gel beds of the appropriate columns.

   <u>Note:</u> Pipet the sequencing reaction directly onto the center of the slanted gel-bed surface. Do not allow the reaction mixture or the pipet tip to touch the sides of the column. The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel bed surface with the pipet tip.
h. Centrifuge at **3,000 rpm** for **3 minutes** at room temperature.
i. Discard the spin columns. *The eluate in the microcentrifuge tubes contains the purified DNA.*

10. Keep samples on ice or in the refrigerator. Take the purified sequencing reactions to the UCLA Sequencing Facility. *<u>Note:</u> Use your initials and the primer name as the name of your sequence. Make sure to copy down the* ***assigned file number*** *(example,* #106203*), which is automatically given by the computer after you enter the samples.*
11. After one to two days, retrieve your sequences from the Sequencing Facility webpage.

C. Retrieving and Analyzing DNA Sequences

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

1. Log into WebSeq at http://www.genetics.ucla.edu/webseq/

a. Enter Username: **goldberg_r**
b. Enter Password: **embryo**
c. Click "LOGIN."

2. Find your sequence files by looking up the ***assigned file number*** and the name of the gene you are working on.

   Example: The ***assigned file number*** is **106203**, and the gene of interest is **At5g09250**. You would see the following files:
   106203GoldR At5g09250Fw A12.ab1
   106203GoldR At5g09250Rv B12.ab1
   What are the annotations?
   106203 = assigned file number; **GoldR** = user name; **At5g09250Fw** = name of sequence obtained with the Forward sequencing primer, **A12** = capillary position used in loading sequencing sample in the Sequencer (Biosystems 3730 Capillary DNA Analyzer), ab1 = ABI file format.

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

EXPERIMENT 5 - IDENTIFYING FEATURES OF MUTANT SEEDS USING NOMARSKI MICROSCOPY (GENE TWO)

STRATEGY

I. **OBSERVATION OF SEEDS USING LIGHT MICROSCOPY AND FIXING SEEDS FOR OBSERVATION WITH NOMARSKI OPTICS**

II. **OBSERVATION OF SEEDS AND EMBRYOS USING NOMARSKI OPTICS**

III. **OBSERVATION OF THE MATURE PLANT PHENOTYPE**

I. Observation of Seeds Using Light Microscopy and Fixing Seeds for Observation with Nomarski Optics

Purpose: To introduce the **D**ifferential **I**nterference **C**ontrast (**DIC**) or **N**omarski **I**nterference **C**ontrast (**NIC**) microscopy technique as a tool to identify features of defective embryos in knockout mutants.

Reference: The protocol was written by Dr. Miguel Aguilar in Professor Robert L. Fischer's laboratory at University of California, Berkeley.

Materials Needed:

- Siliques containing seeds with a wide range of embryo stages (globular to mature green) from *Arabidopsis*
    a. wild type
    b. homozygote or heterozygote mutant
- 100% ethanol
- Acetic acid
- Sterile water
- Chloral Hydrate (Cat. #C8383, Sigma-Aldrich; should be fresh)
- Glycerol (Invitrogen)
- Double-distilled water

Materials Needed:

- Pipettes
- Pipette tips (regular, non-filter)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Rulers with METRIC scale (mm)
- Plant layout chart
- Phenotype observation record
- Fine point forceps
- 30-gauge hypodermic needles
- Fine-point scissors or razor blades
- Coverslips
- Microscope Slides
- Double-sided tape
- Dissecting microscopes (borrowed from Dr. Pei Yun Lee)
- A microscope equipped with Nomarski optical parameter (Leica CTR5000)
- Microscope camera

PROCEDURE

Each student collects the following from wild type and his/her homozygous or heterozygous mutant:

a) 5 siliques containing seeds with embryo stages of globular to torpedo.

b) 2 siliques containing seeds with mature green embryos.

Note: *Be sure to collect a wide* **range** *of stages. Do not collect yellow or brown siliques; these contain dry seeds.*

1. Prepare 5 mL of a fixative solution of ethanol: acetic acid (9:1, v/v) in a 14 mL centrifuge tube using disposable 5 mL pipets.

FIXATIVE SOLUTION	
100% ethanol	4.5 mL
Acetic acid	0.5 mL
Total volume	5.0 mL

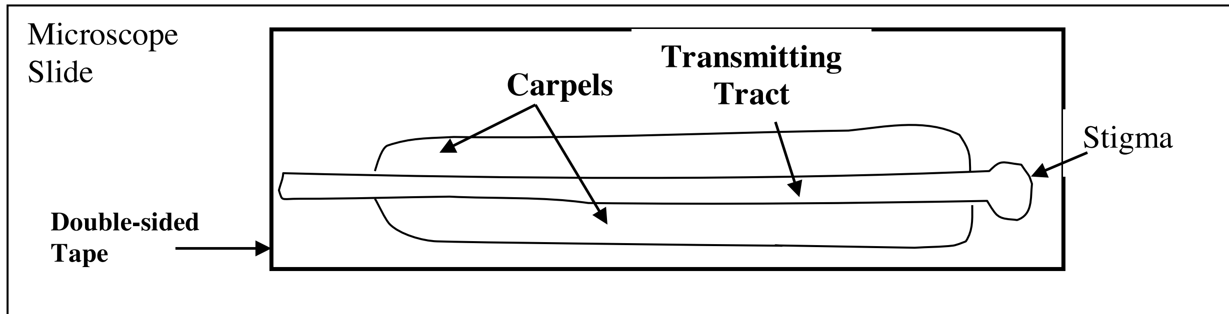
   Tightly snap the cap on the tube. Make sure the cap clicks. **Invert** the tube to mix the contents.

2. Pipet **1 mL** of the **fixative solution** into **FOUR** 1.5 mL microcentrifuge tubes sitting on a microcentrifuge tube rack. Label each tube in step 2 with your **initials**, the **plant #** and the **plant genotype**. *These tubes will be used in step 5i.*
3. Bring the following materials to the Plant Growth Center (PGC).
   - Bucket of ice
   - FOURTEEN 1.5 mL microcentrifuge tubes
   - Microcentrifuge tube rack
   - Black ultra-fine sharpie
   - Ruler with METRIC scale (mm)
   - A pair of fine point forceps
   - Plant layout chart with information about plant number and the genotype of those plants
   - This protocol
   - Bruincard with access to the PGC
   - Key to growth chambers in the PGC

4. Measure and collect siliques according to the chart below. Place each silique in a 1.5 mL microcentrifuge tube. Write your **initials**, the **plant #**, the **plant genotype** and the **length** on the tube. Keep the tube on **ice**. *Note: Collect the **same length** of siliques for wild type and homozygous/heterozygous mutant so that you can compare them.*

Plant Genotype	**Seed Stages Collected**	**Length of Siliques Collected**	**Number of Siliques Collected**
Wild type	globular to torpedo	*0.5 - 1.0 cm*	5
Wild type	mature green	*1.0 - 1.9 cm*	2
Heterozygous or homozygous mutant	globular to torpedo	*0.5 - 1.0 cm*	5
Heterozygous or homozygous mutant	mature green	*1.0 - 1.9 cm*	2

5. Go back to the lab. **Dissect** the siliques and **observe** the seed phenotype using a dissecting microscope. *Note: Work quickly so the seeds don't dry out. You may also place a drop of water on the silique.*
   a. **Place** a **piece of double-sided tape** on a **microscope slide**. Label the microscope slide with a small piece of white tape with your **initials**, the **plant #**, the **plant genotype** and the **length**.
   b. Carefully, use **fine-point forceps** to place a silique on the tape.
   c. Under a dissecting microscope, use **fine-point forceps** to carefully **arrange** the silique such that the **transmitting tract** is facing you (see diagram below, NOT drawn to scale).



d. With your left hand, use forceps to hold the silique on the side closest to the stem.

e. With your right hand, use a **28G** or **30G hypodermic needle attached to a 1 cc syringe** to **slice** the **carpels along each side of the transmitting tract.**

f. **Gently** peel back the carpels and stick them to the tape to reveal the seeds.

g. Observe the phenotype. Note any phenotypes that you observe on your **Screening Seeds Using Light Microscopy** chart.

In what stage of development are the seeds?

How many seeds are in the silique?

How many are green?

How many are white?

How many are brown?

What is the expected ratio of wild type seeds to mutant seeds if the mutation is seed lethal?

What is the observed ratio of wild type seeds to mutant seeds?

Are the observed results significantly different from the expected results? Use a Chi-Square test.

$$\chi^2 = \Sigma \frac{(observed - expected)^2}{expected}$$

Probability that the deviation is due to chance alone

Degrees of Freedom	0.5	0.1	0.05	0.02	0.01	0.001
1	0.455	2.706	3.841	5.412	6.635	10.827
2	1.386	4.605	5.991	7.824	9.210	13.815
3	2.366	6.251	7.815	9.837	11.345	16.268
4	3.357	7.779	9.488	11.668	13.277	18.465
5	4.351	9.235	11.070	13.388	15.086	20.517

What is your null hypothesis?

How many degrees of freedom are there?

(The degrees of freedom is one less than the number of different phenotypes possible.)

What is your chi-square value?

(The chi-square statistic is a probability that indicates the chance that, in repeated experiments, deviations from the expected would be as large or larger than the ones observed in this experiment)

What is the probability that the deviation of the observed values from the expected values was a chance occurrence?

(Look up your degrees of freedom in the table. Find where your chi-square value falls in that row.)

Can you reject the null hypothesis?

If the probability is less than 0.05 (5%), reject your null hypothesis.

If the probability is 0.05 (5%) or greater, then you cannot reject your null hypothesis.

h. Ask your TA to take pictures of the seeds within the siliques.

i. **Before the seeds dry out**, use the fine-point forceps to transfer the cut silique into the tube with fixative solution from **step 2**.

j. Repeat steps a-i for the other siliques. <u>*Note:*</u> *You collected an excess of siliques so that you would have some to practice dissecting and to have a*

range of developmental stages for each genotype. However, you only need to fix FOUR siliques.

i. *Wild type, early development*
ii. *Heterozygous (or homozygous), early development*
iii. *Wild type, late development (mature green stage)*
iv. *Heterozygous (or homozygous), late development (mature green stage)*

6. Fix seeds and siliques in the fixative solution for at least 2 hours. *Note: It is recommended to fix the siliques* ***overnight*** *to ensure that the fixative solution penetrates the seeds and their embryos. It is okay to leave siliques in the fixative solution for up to 3 days.*
7. **Carefully**, pipet off **900 µL** of the fixative solution using a **P-1000** pipette and discard into a beaker labeled "acetic acid waste." Then remove the **remaining volume** with a **P-200** pipette. *Note: Do not let the seeds and siliques dry out, and do not pipet up your seeds.*
8. **Immediately**, pipet **1 mL** of **90% ethanol** solution into the tube using a P-1000 pipette. *Note: The 90% ethanol solution will remove chlorophyll from the embryos.*

90% ETHANOL SOLUTION

Absolute ethanol	4.5 mL
Double-distilled water	0.5 mL
Total volume	5.0 mL

9. Incubate seeds and siliques in the 90% ethanol solution for **0.5 - 1 hour**. *Note: It is safe to store the materials in the ethanol indefinitely.*
10. Replace the 90% ethanol solution with **70% ethanol** as in steps 7 & 8.

70% ETHANOL SOLUTION

Absolute ethanol	3.5 mL
Double-distilled water	1.5 mL
Total volume	5.0 mL

11. Incubate seeds and siliques in the ethanol solution for **0.5 - 1 hour**. <u>Note:</u> *It is safe to store the materials in the ethanol indefinitely*.

II. Observation of Seeds and Embryos Using Nomarski Optics

<u>Note:</u>

- ***Before observation of the seeds and their embryos, seeds must be submerged in the clearing solution. For young seeds, clearing is usually fast (~5 minutes). The older the silique, the longer it takes to clear (~ 1 hour). Seeds are ready for observation after they sink in the clearing solution.***
- ***Tissues CANNOT be stored in the CLEARING solution for more than TWO days because they will lose their structures quickly***.

1. Prepare a ***fresh*** clearing solution of chloral hydrate/glycerol/water (8:1:2, w/v/v) in a 14 mL centrifuge tube. <u>Note:</u> *The TA will prepare this solution before the lab class begins*.

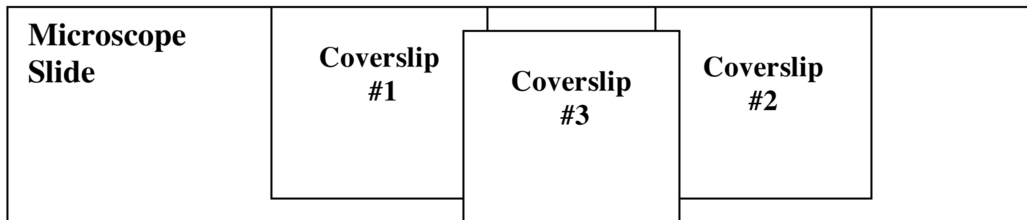
<u>CLEARING SOLUTION</u>

Chloral hydrate	8 g
Glycerol	1 mL
Water	2 mL
Total volume	~7 mL

2. **Carefully**, pipet off **900 µL** of the 70% ethanol solution using a **P-1000** pipette and discard into a beaker labeled "ethanol waste." Then remove the **remaining volume** with a **P-200** pipette. <u>Note:</u> *Do not let the seeds and siliques dry out, and do not pipet up your seeds*.
3. Replace the 70% ethanol solution with **100 µL of clearing solution**.
4. Incubate seeds and siliques in the clearing solution for **5 min - 1 hour**. Wait until the seeds **sink** to the bottom of the tubes. You may lay the tube on its side so that the

silique is immersed in the clearing solution. *Note: Tissues CANNOT be stored in the CLEARING solution.*

5. Set a new glass microscope slide on the bench. Label it with your **initials**, the **plant #**, the **plant genotype** and **silique length**.
6. Use forceps to remove a silique from the clearing solution and place it on the labeled glass slide.
7. Pipet the remaining clearing solution and seeds onto the slide with the silique.
8. Carefully, place two square coverslips, one on each side of the solution. Then, place a third coverslip over the clearing solution. Avoid trapping bubbles in the solution (see diagram below).



9. Observe the seeds under Nomarski optics using the Leica CTR5000 microscope.
10. Take pictures of the embryos.

    In what stage of development are the seeds?

11. Repeat steps 2-10 for the remaining 3 fixed siliques.

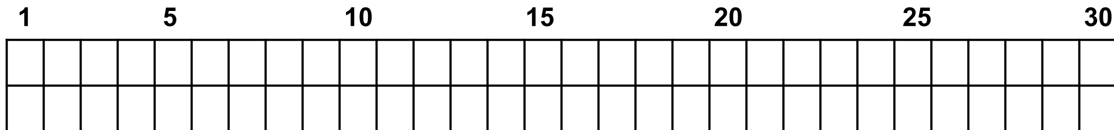
Screening Seeds Using Light Microscopy

AGI# _______________ SALK # _______________ Plant #_____Genotype ___________

Silique # _____ Length of Silique (cm) _____ Total Seeds ______ Total Mutant Seeds ______

Instructions: **The grid represents the layout of the silique. Put a number in each square that corresponds to a mutant seed. Describe the seed phenotypes in the chart below. The base of the silique is defined as the region closest to the pedicel and main stem, which is at the left of the grid.**

1 5 10 15 20 25 30



Seed	Seed Coat Color	Embryo Color	Notes
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

III. Observation of the Mature Plant Phenotype

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

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

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

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

APPENDICIES
Appendix 1A

Preparation of an Agarose Gel for Gel Electrophoresis

Solutions Needed:

- DNA samples
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain (Invitrogen)
- 50 ng/µL DNA ladder with loading dye (Invitrogen)
- 6x Loading dye containing xylene cyanol and/or bromophenol blue dyes

Materials Needed:

- Pipettes (P-20)
- Pipet tips (regular, non-filter tips)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- 250 mL Erlenmeyer flask
- 25 mL Erlenmeyer flask
- Saran wrap
- Scale
- Microwave
- 55°C water bath
- Hot hand protector
- Gel cast
- Gel comb
- Round bubble level
- Gel box
- Cables
- Electrophoresis power supply
- Plastic container for carrying the gel
- Gel document system (Bio-Rad)

PROCEDURE

Note: SYBR Safe gel stain is unstable in UV or bright room light. If possible, keep the gel in the ***dark*** *by either turning off the lights, covering the gel with a cardboard box or aluminum foil, or run the gel inside of a drawer. Realistically, hours of constant UV or bright room light exposure are required to cause any significant loss of signal.*

1. For a **1% agarose gel**, weigh out **1 gram** of agarose (powder) into a weigh boat on the scale in room 4128A2. <u>Note:</u> *The* ***percentage*** *of agarose in the gel reflects the amount of agarose (in* ***grams)*** *in* ***100 mL*** *of 1x TAE buffer.*
   <u>Example:</u> *If you want to make a 0.7% agarose gel (0.7 g/100 mL, w/v), weigh out 0.7 g of agarose for 100 mL of 1x TAE buffer.*
2. Carefully, pour the agarose into a 250 mL Erlenmeyer flask.
3. Measure **100 mL** of **1x TAE** buffer using a graduated cylinder, and add it to the flask in step 2.
4. Cover the flask with an inverted 25-mL Erlenmeyer flask or saran wrap. Swirl the solution to break up any lumps of agarose. <u>Note:</u> *The inverted flask will collect condensation from the steam produced during microwaving.*
5. Microwave the solution for **1-2 minutes** or until the agarose granules have completely melted and the solution looks **clear**. Gently **swirl** the solution every 15 sec during microwaving to help melt the agarose evenly. <u>Caution:</u> *The solution gets* ***very hot****. Use a hot hand protector.* <u>Note:</u> *Constantly watch over the solution because if it starts boiling, it might overflow.*
6. Cool down the agarose solution for **10 min** in a **55ºC water bath** until it is cool enough to handle.
7. While the agarose solution is cooling, prepare the gel cast with the appropriate comb. Use the round bubble level to make the gel cast level.
   <u>Note:</u> ***The choice of comb depends on the number of samples to be loaded on the gel****. For example, if there are ≤ 18 samples, then use a 20-tooth comb; but, if there are ≥ 19 samples, then use a 30-tooth comb.* <u>Note:</u> *Remember that at least* ***two wells*** *will be for loading* ***DNA ladder*** *(on left and right sides of loaded samples).*
8. After the agarose solution has cooled down, add **10 µL** of **10,000x SYBR Safe DNA gel stain** to 100 mL of agarose solution and **swirl** the flask **GENTLY** to mix. <u>Note:</u> *Do NOT swirl vigorously to avoid generating bubbles.* <u>Note:</u> *Add 5 µL of 10,000x SYBR Safe DNA gel stain for a small (50 mL) gel.*
9. Pour the **agarose/gel stain** solution into the gel cast with the appropriate gel comb. Wait **30 min** for the agarose solution to solidify. <u>Note:</u> *IMMEDIATELY after pouring the agarose solution, inspect the agarose solution's surface for the present of*

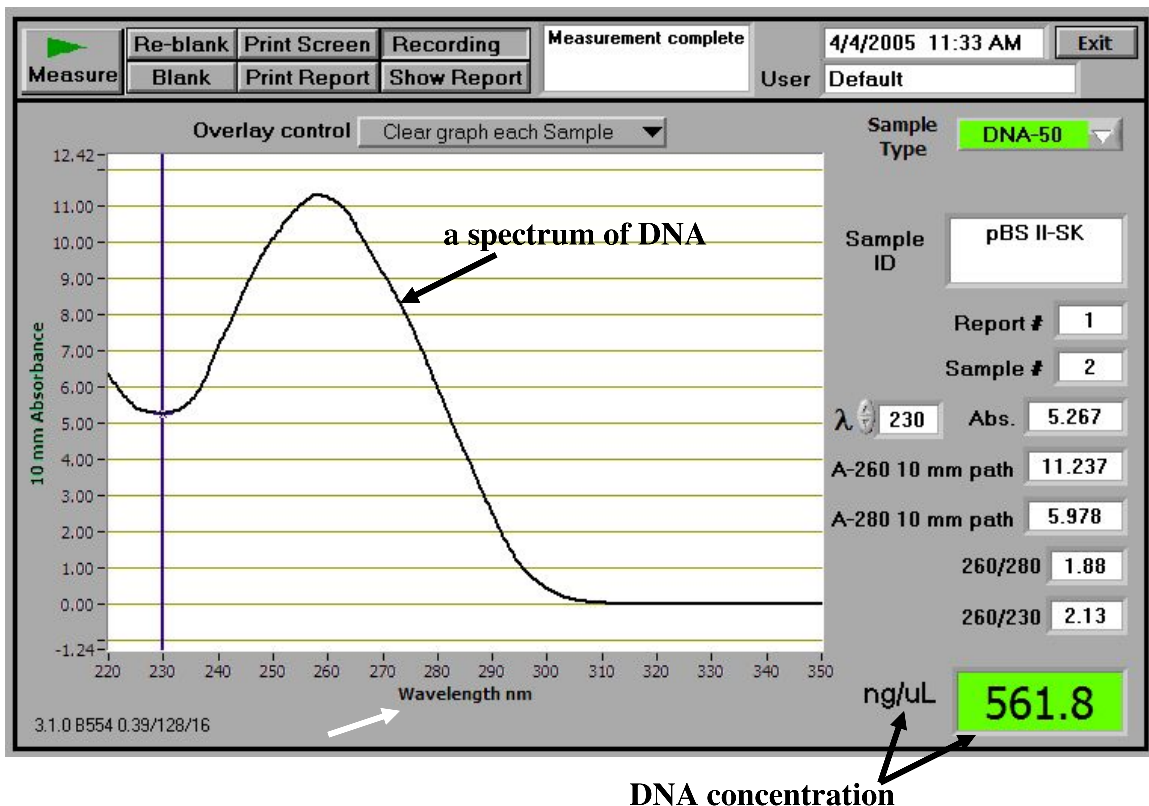
bubbles. If there are bubbles floating on the surface of the gel solution, use a pipette tip to pop them or move them to the sides of the gel before the gel has completely solidified.

10. Pour ~600 mL of **1x TAE** buffer into the gel box.
11. After the agarose has solidified into a gel, take out the comb by **gently** pulling it side to side and out of the gel. Put the gel in its cast into the gel box containing the 1x TAE running buffer.
12. Add **6x loading dye** to your samples. *Note: Do not use a loading dye that will travel to the same place as your DNA on the gel because the dye will obscure the DNA band. Xylene cyanol runs at ~3-4 kb, and bromophenol blue runs at ~400 bp on a 1% agarose gel.*
13. Load **10 μL** of **50 ng/μL DNA ladder with loading dye**.
14. Load samples and record the identity of the sample in each lane.
15. Add **10 μL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode (red) side of the gel box (near the bottom of the gel). Pipet up and down to mix. *Note: Add 5 μL of 10,000x SYBR Safe DNA gel stain for a small (50 mL) gel.*
16. Put the lid on the gel box and firmly connect the electrodes to the power supply (**RED** to **RED** and **BLACK** to **BLACK**).
17. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) has migrated one-half or two-thirds of the gel length.
18. After 1-2 hours of running the gel, turn **off** the power supply.
19. Remove the lid of the gel box. Put the gel in its gel cast into a small plastic container and bring the container to room 4128A2. ***Caution:*** *It is a **MUST** to put the gel into a plastic container so that the gel cannot slide off the gel cast, fall on the floor and be broken into pieces while walking.*
20. Take a picture of the gel using the Bio-Rad Gel Document System. Label the picture using the text program of the Gel Document System. (*Your TA will show you how.*) *Alternatively: Print out the picture. Tape it to a piece of paper by putting a piece of white tape at a position immediately above the wells. Label the wells with the sample names.*
21. Print out the picture. Store the labeled picture in your lab notebook.

Appendix 1B

What is a **spectrophotometer**?

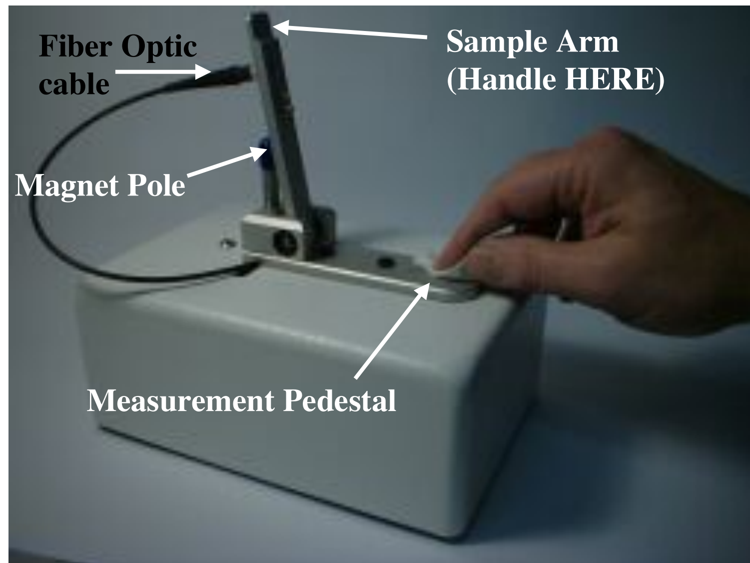
It is an **instrument** that **measures** the **amount** of **molecules absorbing a given wavelength of energy**. In this exercise, we measure the amount of DNA molecules in a given volume in the **ultraviolet wavelengths** of **200 – 280 nm** (**nm** stands for **nanometer**, which is **1 billionth** of a **meter**). The bases of DNA absorb at a wavelength of 254 nm. The absorbance of DNA molecules over the wavelength range of 220 - 350 nm is represented as a **spectrum** with a **peak** at **near 260 nm** as illustrated by the **NanoDrop spectrophotometer readout** below:



OPERATION OF NANODROP SPECTROPHOTOMETER ND-1000

<u>Note:</u> *The NanoDrop is powered by the laptop via the USB port.*

1. **Open** the NanoDrop program by clicking the "**ND-1000 v3.7.1**" icon on the computer desktop. Wait for a few seconds for the program to open. You see the NanoDrop 1000 3.7.1 menu panel with **user field** set to **default.**
2. To read the concentration of DNA or RNA solutions, **click** on the "**Nucleic Acid**" button on the top left column.
   <u>Note:</u> *You see the following message: "Ensure sample pedestals are clean and then load a water sample. After loading water sample, click OK to initialize instrument."*
3. **Clean** the **measurement pedestals** as following:
   a. **Raise** the **sample arm** by holding its end.
   ***<u>Caution:</u> NEVER** hold the fiber optic cable when lifting and lowering the sample arm because the cord is fragile, and it is very expensive to replace it.*
   b. **Blot BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes slightly wetted with distilled water.



c. **Pipet 1.5 - 2 µL** of water onto the **measurement pedestal**.

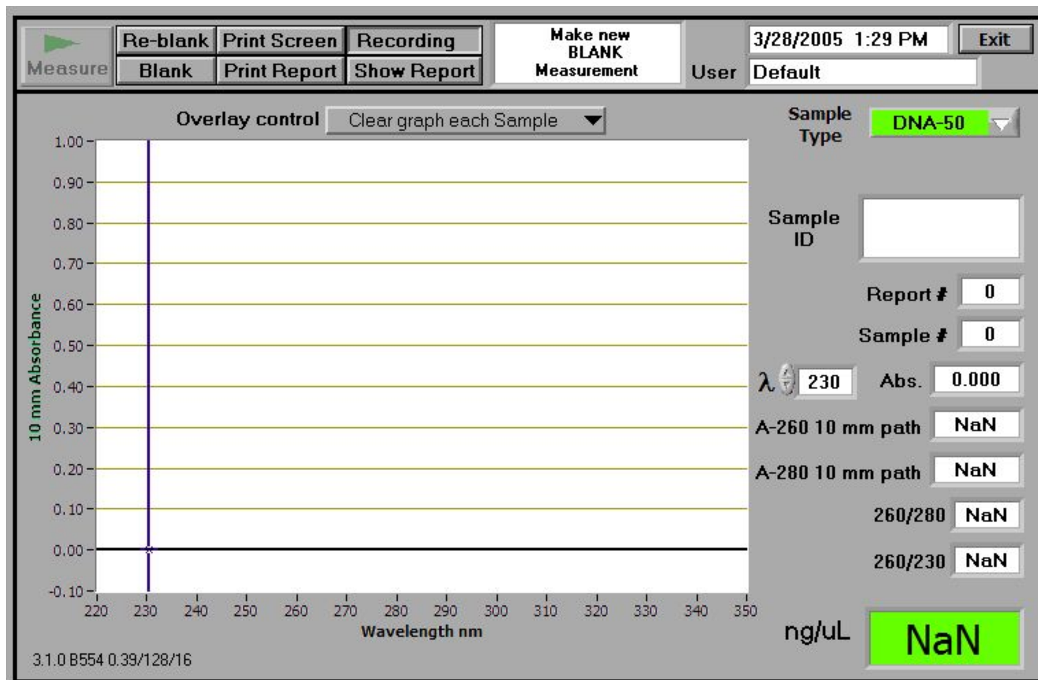
Note: *Even though NanoDrop Inc. claims that the NanoDrop can read as low as 1 µL, the concentration reading is NOT consistent at this volume. Therefore, the* ***minimal*** *volume for the concentration reading is* ***1.5 µL.***

d. **Slowly lower** the sample arm to its horizontal position.

Caution: *NEVER let the arm fall freely.*

e. **Click** the **OK** button.

Note: *Now the NanoDrop is* ***INITIALIZED.*** *You will see the* ***dialog panel*** *as shown below.*



f. Raise the sample arm to the vertical position.

g. **Wipe off** the **liquid** from **BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes.

4. **Change SAMPLE TYPE** (if necessary) from **DNA-50** (default, for DNA) to **RNA-40** (for RNA) or **ssDNA-33** (for oligonucleotides), depending on your sample.
5. (Optional) **Change** the **OVERLAY CONTROL** field from the default setting of "CLEAR GRAPH EACH SAMPLE" to "CLEAR GRAPH ON NEW REPORT" or "ACCUMULATE UNTIL CLEAR" or "CLEAR GRAPH NOW."
6. Make a **NEW BLANK measurement.**
   a. Pipet 1.5 - 2.0 μL of either double-distilled water, TE or EB (depending on what solution your sample is dissolved in) on the measurement pedestal.
   b. Lower the sample arm to the horizontal position.
   c. **Click** the **BLANK** button. *The blank was made*.
   d. After the reading is done, raise the sample arm to the vertical position.
   e. **Blot off** the **liquid** from **BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes.
7. **Confirm** that the blank was made.
   a. Pipet 1.5 - 2.0 μL of either double-distilled water, TE or EB (depending on what solution your sample is dissolved in) on the measurement pedestal.
   b. Lower the sample arm to the horizontal position.
   c. In the **SAMPLE ID** field, **type** the **identity** of the solution that you are measuring (either ddH_2O, TE or EB).
   d. **Click** the **MEASURE** button.

      *Note: After the reading is done, a **concentration** (in **ng/μL**) and a **spectrum** of the absorbance, along with other information, are shown. The reading should be **less than 1 ng/μL**. If it is not, make a new blank measurement by repeating step 6.*
   e. Raise the sample arm to the vertical position.
   f. **Blot off** the **liquid** from **BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes.
8. Measure the **samples**.

a. In the **SAMPLE ID** field, **type** the **identity** of the sample solution.

b. Pipet 1.5 - 2.0 µL of SAMPLE on the measurement pedestal.

c. Lower the sample arm to the horizontal position.

d. **Click** the **MEASURE** button to determine concentration of your sample.

e. After the reading is done, a **sample concentration** (in **ng/µL**) and a **spectrum** of the sample, along with other information, are shown. You can either:

   i. **Save** the **window** of measured sample.

      1. Click on FILE.
      2. Choose SAVE WINDOW.
      3. Select an existing folder or create a new folder (give a name for the new folder).
      4. Type in a file name in the FILE NAME field.
      5. Click the SAVE button to save the file.

   ii. **Or**, **print** the **window** by **clicking** the **PRINT SCREEN** button.
   Note: *To print the spectrum of the current sample, you MUST print it before reading the next sample. Otherwise, you need to repeat the sample reading.*

f. Raise the sample arm to the vertical position.

g. **Blot off** the **liquid** from **BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes.

9. **Repeat** step 8 for other samples.
10. After reading the **last sample**, **click** the **PRINT REPORT** button to print the concentrations of all samples.
11. If you are done with the NanoDrop, **click** the **EXIT** buttons.
12. **Blot BOTH** the **measurement pedestal** and the **sample arm pedestal** with a piece of Kimwipes slightly wetted with distilled water.

Appendix 1C
100 bp DNA Ladder

100 bp DNA Ladder

Cat. No. 15628-019 **Size: 50 µg**
Concentration: 1 µg/µl **Store at -20°C.**

Description:

The 100 bp DNA Ladder consists of 15 blunt-ended fragments between 100 and 1500 bp in multiples of 100 bp and an additional fragment at 2072 bp. The 600 bp band is approximately 2 to 3 times brighter than the other ladder bands to provide internal orientation. This ladder is not designed for quantitation.

Storage Buffer:

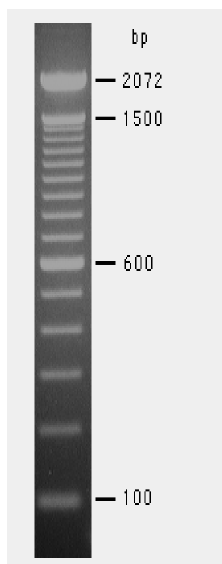
10 mM Tris-HC1 (pH 7.5)
1 mM EDTA

Recommended Procedure:

A final concentration of 20 mM NaCl is recommended for gel electrophoresis. Apply approximately 0.1 µg of ladder per mm lane width. **Do not heat** before loading.

Quality Control:

Agarose gel analysis shows that the bands between 100 to 1500 bp are distinguishable. The 600 bp band must be more intense than any other band except the band at 2072 bp.



100 bp DNA Ladder
0.5 µg/lane
2% agarose gel stained with ethidium bromide.

Note:

During 2% agarose gel electrophoresis with tris-acetate (pH 7.6) as the running buffer, bromophenol blue migrates near the 100-bp fragment. The 100-bp band migrates behind the bromophenol blue marker on 6% polyacrylamide gels with tris-borate (pH 8.0) as the running buffer.

Part of the 600-bp band may migrate anomalously slowly in polyacrylamide gels (1,2,3). This band may appear as an extra band near or on top of the 700-bp band.

References:

1. Hsieh, C., et al. (1991) *Mol. Gen. Genet. 225*, 25.
2. Stellwagen, N.C. (1983) *Biochemistry 22*, 6186.
3. Jordan, H. and Hartley, J. (1997) *Focus® 19*, 9.

Cat. No. 15628-019

Appendix 1D
1 Kb Plus DNA Ladder

invitrogen™
by life technologies™

1 Kb Plus DNA Ladder

Cat. no. 10787-018 **Size** 250 µg at 1 µg/µL **Store at –30°C to –10°C**

Doc. Part no. 10787018.pps **Pub. no.** MAN0000898 **Rev.** 2.0

Description

Use the 1 Kb Plus DNA Ladder is to size linear double-stranded DNA fragments from 100 bp to 12 kb. The ladder contains a total of twenty bands: twelve bands ranging in size from 1000 bp to 12,000 bp in 1000-bp increments and eight bands ranging in size from 100 to 1650 bp. The 1650-bp band contains approximately 8% of the mass applied to the gel.

Storage Buffer

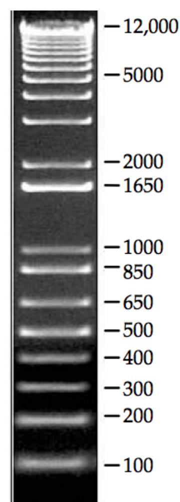
- 10 mM Tris-HCl (pH 7.5)
- 1 mM EDTA
- 50 mM NaCl

Recommended Procedure

We recommend using 10X BlueJuice™ Gel Loading Buffer (Cat. no. 10816-015) at a concentration of 2X for the electrophoresis of DNA standards on agarose gels. Alternately, you may dilute the DNA standard so that the final concentration of NaCl is 20 mM. Apply approximately 0.1 µg of ladder per mm lane width. *Do not heat* before loading.

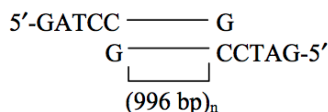
Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.



0.9 µL/lane
0.9% agarose gel stained with ethidium bromide

Structure of Fragments in 1-Kb Increments



Notes

During 1% agarose gel electrophoresis with Tris-acetate (pH 7.5) as the running buffer, bromophenol blue migrates with the 500 bp band.

The 1650 bp band is generated from pUC. The bands smaller than 1000 bp are derived from lambda DNA.

Appendix 1E
Ex Taq DNA Polymerase Manual

10XPCR Buffer,dNTP Mixture for PCR

TaKaRa Ex Taq™

Code No. RR001A
Size: 250 units

Shipping at -20˚C
Stored at -20˚C

Supplied Reagents : 10X *Ex Taq*™ Buffer
dNTP Mixture

Lot No.
Conc. : units/μl
Volume : μl
Expiry Date :

Storage Buffer:

20 mM	Tris-HCl (pH8.0)
100 mM	KCl
0.1 mM	EDTA
1 mM	DTT
0.5%	Tween®20
0.5%	Nonidet P-40®
50%	Glycerol

Unit definition: One unit is the amount of the enzyme that will incorporate10 nmol of dNTP into acid-insoluble products in 30 minutes at 74˚C with activated salmon sperm DNA as the template-primer.

Reaction mixture for unit definition:

25 mM	TAPS (pH 9.3 at 25˚C)
50 mM	KCl
2 mM	$MgCl_2$
1 mM	2-mercaptoethanol
200 μM	each dATP,dGTP,dTTP
100 μM	[α-^{32}P]-dCTP
0.25 mg/ml	activated salmon sperm DNA

Purity: Nicking activity, endonuclease and exonuclease activity were not detected after the incubation of 0.6 μg of supercoiled pBR322 DNA, 0.6 μg of λ DNA or 0.6 μg of λ-*Hin*d III digest with 10 units of this enzyme for 1 hour at 74˚C.

Applications:
For DNA amplification by Polymerase Chain Reaction (PCR).

PCR products : As most PCR products amplified with *TaKaRa Ex Taq* ™ have one A added at 3'-termini, the obtained PCR product can be directly used for cloning into T-vector. Also it is possible to clone the product in blunt-end vectors after blunting and phosphorylation of the end.

PCR test : Good performance of DNA amplification by Polymerase Chain Reaction (PCR) was confirmed by using λ DNA as the template (amplified fragment : 20 kbp).
Good performance of DNA amplification of β-globin gene by PCR was also confirmed by using human genomic DNA as the template (amplified fragment : 17.5 kbp).

PCR condition (an example)
When amplifying 1 kbp DNA fragment

98°C	10 sec	30 cycles	or	98 °C	10 sec	30 cycles
55°C	30 sec			68 °C	1 min	
72°C	1 min					

Note:Denaturation condition varies depending on an used thermal cycler and tube. It is recommended for 10-30 sec. at 94°C, or 1-10 sec. at 98°C.

General reaction mixture for PCR (total 50 μl)

TaKaRa Ex Taq ™ (5 units/μl)	0.25 μl
10X *Ex Taq* Buffer	5 μl
dNTP Mixture (2.5 mM each)	4 μl
Template	< 500 ng
Primer 1	0.2 ~ 1.0 μM (final conc.)
Primer 2	0.2 ~ 1.0 μM (final conc.)
Sterilized distilled water	up to 50 μl

Supplied 10X *Ex Taq* Buffer
Supplied Size : 1 ml/vial
Mg^{2+} concentration (10X): 20 mM
Storage : –20˚C

Supplied dNTP Mixture
Mixture of dNTP, ready for use in Polymerase Chain Reaction (PCR) without dilution.

Supplied Size	: 800 μl/vial
Concentration	: 2.5 mM of each dNTP
pH	: pH 7 ~ 9
Form	: Solved in water (sodium salts)
Purity	: ≥ 98% for each dNTP
Storage	: –20˚C

< **Cool Start Method** >
"Cool Start Method' , enables to minimize the amplification of non-specific band in PCR and achieves more accurate amplification. This is a simpler method without need for special enzyme nor additional reagents.+ Higher reaction specificity can be achieved by combining Hot Start PCR techniques with Taq Antibody (Code.9002A) and Cool Start method.

Protocol of Cool Start Method
1) Keep all reagents on ice until use.
2) Prepare the reaction mixture on ice.*,**
  *The adding order of reagents dose not influence on results.
  **The result will not be affected even when the mixture is left on ice 30 min. before thermal cycling.
3) Set a thermal cycler ready to start with the designated program.***
  ***No need to change PCR conditions especially for Cool Start.
4) Set the tubes in a thermal cycler and start thermal cycling immediately.

+ JAPAN Patent 2576741 for Cool Start Method is owned by SHIMADZU CORPORATION

Note
For research use only. Not for use in diagnostic or therapeutic procedures.

U.S. Patent 5 ,436,149 for LA Technology is owned by TAKARA BIO INC.

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Produced by TAKARA BIOTECHNOLOGY (DALIAN) CO.,LTD.

V2005.06

March 2008

QIAquick® Spin Handbook

QIAquick PCR Purification Kit
For purification of PCR products, 100 bp to 10 kb

QIAquick Nucleotide Removal Kit
For oligonucleotide (17-40mers) and DNA
(40 bp to 10 kb) cleanup from enzymatic reactions

QIAquick Gel Extraction Kit
For gel extraction or cleanup of DNA
(70 bp to 10 kb) from enzymatic reactions

Sample & Assay Technologies

Kit Contents

QIAquick PCR Purification Kits Catalog no.	(50) 28104	(250) 28106
QIAquick Spin Columns	50	250
Buffer PB*	30 ml	150 ml
Buffer PE (concentrate)	2 x 6 ml	55 ml
Buffer EB	15 ml	55 ml
pH Indicator I	800 µl	800 µl
Collection Tubes (2 ml)	50	250
Loading Dye	110 µl	550 µl
Handbook	1	1

QIAquick Nucleotide Removal Kits Catalog no.	(50) 28304	(250) 28306
QIAquick Spin Columns	50	250
Buffer PN*	30 ml	140 ml
Buffer PE (concentrate)	2 x 6 ml	55 ml
Buffer EB	15 ml	55 ml
Collection Tubes (2 ml)	100	500
Loading Dye	110 µl	550 µl
Handbook	1	1

QIAquick Gel Extraction Kits Catalog no.	(50) 28704	(250) 28706
QIAquick Spin Columns	50	250
Buffer QG*	2 x 50 ml	2 x 250 ml
Buffer PE (concentrate)	2 x 10 ml	2 x 50 ml
Buffer EB	15 ml	2 x 15 ml
Collection Tubes (2 ml)	50	250
Loading Dye	110 µl	550 µl
Handbook	1	1

* Buffers PB, PN, and QG contain chaotropic salts which are irritants. Take appropriate laboratory safety measures and wear gloves when handling.

Storage

QIAquick Spin Kits should be stored dry at room temperature (15–25°C). Under these conditions, QIAquick Spin Kits can be stored for up to 12 months without showing any reduction in performance and quality. Check buffers for precipitate before use and redissolve at 37°C if necessary. The entire kit can be stored at 2–8°C, but in this case the buffers should be redissolved before use. Make sure that all buffers and spin colums are at room temperature when used.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffer PB contains guanidine hydrochloride, which can form highly reactive compounds when combined with bleach.

In case liquid containing this buffer is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

The following risk and safety phrases apply to the components of the QIAquick system.

Buffer PB

Contains guanidine hydrochloride and isopropanol: harmful, irritant, flammable. Risk and safety phrases*: R10-22-36/38. S23-26-36/37/39-46

Buffer PN

Contains sodium perchlorate and isopropanol: harmful, highly flammable. Risk and safety phrases*: R11-22. S13-16-23-26-36-46

Buffer QG

Contains guanidine thiocyanate: harmful. Risk and safety phrases*: R20/21/22-32. S13-26-36-46

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany
Tel: +49-6131-19240

* R10: Flammable. R11: Highly Flammable. R22: Harmful if swallowed. R20/21/22: Harmful by inhalation, in contact with skin and if swallowed. R32: Contact with acids liberates very toxic gas. R36/38: Irritating to eyes and skin. S13: Keep away from food, drink and animal feedingstuffs. S16: Explosive when mixed with oxidizing substances. S23: Do not breathe vapour/spray. S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. S36: Wear suitable protective clothing. S36/37/39: Wear suitable protective clothing, gloves and eye/face protection. S46: If swallowed, seek medical advice immediately and show the container or label.

Product Specifications

	QIAquick PCR Purification Kit	QIAquick Nucleotide Removal Kit	QIAquick Gel Extraction Kit
Maximum binding capacity	10 μg	10 μg	10 μg
Maximum weight of gel slice	—	—	400 mg
Minimum elution volume	30 μl	30 μl	30 μl
Capacity of column reservoir	800 μl	800 μl	800 μl
Typical recoveries			
Recovery of DNA	90–95% (100 bp – 10 kb)	80–95% (40 bp – 10 kb)	70–80% (70 bp – 10 kb)
Recovery of oligonucleotides (17–40mers)	0	60–80%	10–20%
Recovered			
Oligonucleotides	—	17–40mers	—
dsDNA	100 bp – 10 kb	40 bp – 10 kb	70 bp – 10 kb
Removed			
<10mers	**YES**	**YES**	**YES**
17–40mers	**YES**	no	no

Introduction

The QIAquick system, designed for rapid DNA cleanup, includes:

- **QIAquick PCR Purification Kits** for direct purification of double- or single-stranded PCR products (100 bp – 10 kb) from amplification reactions and DNA cleanup from other enzymatic reactions.
- **QIAquick Nucleotide Removal Kits** for general cleanup of oligonucleotides and DNA up to 10 kb from enzymatic reactions (e.g., labeling, dephosphorylation, restriction, and tailing).
- **QIAquick Gel Extraction Kits** for extraction of DNA fragments (70 bp – 10 kb) from standard, or low-melt agarose gels in TAE (Tris·acetate/EDTA) or TBE (Tris·borate/EDTA) buffer and DNA cleanup from enzymatic reactions.

QIAquick PCR Kits are also available in multiwell format for preparation of 8 to 96 samples (see page 37 for ordering information).

Enzymatic reaction cleanup using QIAquick Kits

The QIAquick system is suitable for fast cleanup of up to 10 µg of DNA fragments from enzymatic reactions and agarose gels (Table 1). Enzyme contamination of DNA samples can interfere with subsequent downstream applications. QIAquick Spin Kits can be used for highly efficient removal of a broad spectrum of enzymes widely used in molecular biology. In addition, QIAGEN offers the MinElute® Reaction Cleanup Kit, which is specially designed for fast and easy DNA cleanup from all enzymatic reactions. Using proven microspin technology, the MinElute Reaction Cleanup Kit delivers highly concentrated purified DNA by using an elution volume of only 10 µl (see ordering information, page 37).

Table 1. QIAquick DNA Cleanup Guide

	From solutions			From gels
	QIAquick PCR Purification Kit	QIAquick Nucleotide Removal Kit	QIAquick Gel Extraction Kit	QIAquick Gel Extraction Kit
Alkaline phosphatase	**YES**	**YES**	**YES**	**YES**
cDNA synthesis	**YES**	no	no	**YES**
DNase, nuclease digestion	**YES**	**YES**	**YES**	**YES**
Kinase:				
DNA fragments	**YES**	**YES**	**YES**	**YES**
Oligonucleotides	no	**YES**	no	no
Ligation	**YES**	**YES**	**YES**	**YES**
Nick translation	**YES**	**YES**	**YES**	**YES**
PCR	**YES**	no	no	**YES**
Random priming	**YES**	**YES**	**YES**	**YES**
Restriction digestion	**YES**	**YES**	**YES**	**YES**
Tailing:				
DNA fragments	**YES**	**YES**	**YES**	**YES**
Oligonucleotides	no	**YES**	no	no

QIAquick Kits provide high yields of pure nucleic acids, for direct use in applications such as:

- Fluorescent and radioactive sequencing
- Restriction
- Labeling
- Hybridization
- Ligation and transformation
- Amplification
- In vitro transcription
- Microinjection

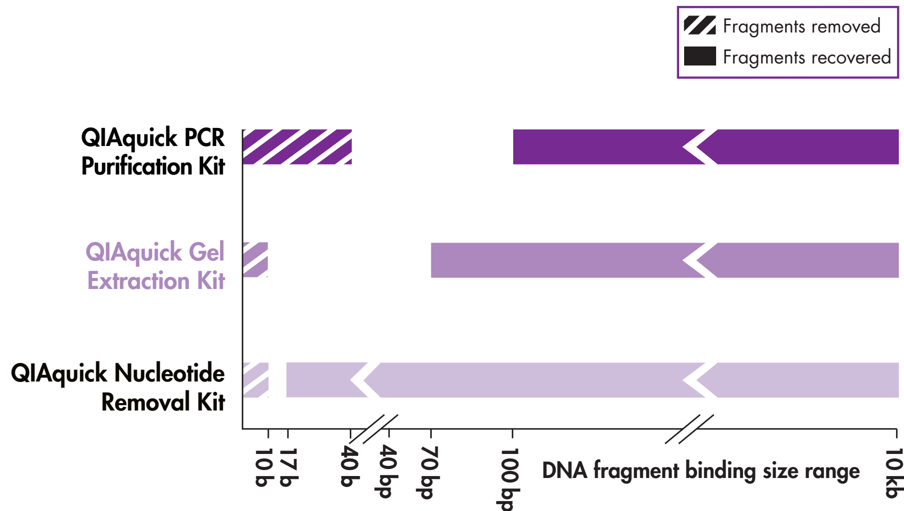


Figure 1. DNA fragment binding-size range. Recoveries of DNA fragments in the size range between "removed" and "recovered" are not defined.

Automated DNA cleanup

The QIAquick PCR Purification Kit and QIAquick Gel Extraction Kit can be fully automated on the QIAcube. The innovative QIAcube uses advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using the QIAcube follows the same steps as the manual procedure (i.e., bind, wash, and elute) enabling purification of high-quality DNA.

The QIAcube is preinstalled with protocols for purification of plasmid DNA, genomic DNA, RNA, viral nucleic acids, and proteins, plus DNA and RNA cleanup. The range of protocols available is continually expanding, and additional QIAGEN protocols can be downloaded free of charge at www.qiagen.com/MyQIAcube .

A detailed protocol for using QIAquick spin columns on the QIAcube is provided with the QIAcube.

Note: It is not necessary to add pH indicator I to Buffer PB when using the QIAcube.

The QIAquick Principle

The QIAquick system combines the convenience of spin-column technology with the selective binding properties of a uniquely designed silica membrane. Special buffers provided with each kit are optimized for efficient recovery of DNA and removal of contaminants in each specific application. DNA adsorbs to the silica membrane in the presence of high concentrations of salt while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is eluted with Tris buffer or water (see page 17). QIAquick spin columns offer 3 handling options — as an alternative to processing the spin columns in a microcentrifuge, they can now also be used on any commercial vacuum manifold with luer connectors (e.g., QIAvac 6S or QIAvac 24 Plus with QIAvac Luer Adapters) or automated on the QIAcube.

Adsorption to QIAquick membrane — salt and pH dependence

The QIAquick silica membrane is uniquely adapted to purify DNA from both aqueous solutions and agarose gels, and up to 10 µg DNA can bind to each QIAquick column. The binding buffers in QIAquick Spin Kits provide the correct salt concentration and pH for adsorption of DNA to the QIAquick membrane. The adsorption of nucleic acids to silica surfaces occurs only in the presence of a high concentration of chaotropic salts (1), which modify the structure of water (2).

Adsorption of DNA to silica also depends on pH. Adsorption is typically 95% if the pH is ≤7.5, and is reduced drastically at higher pH (Figure 1). If the loading mixture pH is >7.5, the optimal pH for DNA binding can be obtained by adding a small volume of 3 M sodium acetate, pH 5.0.

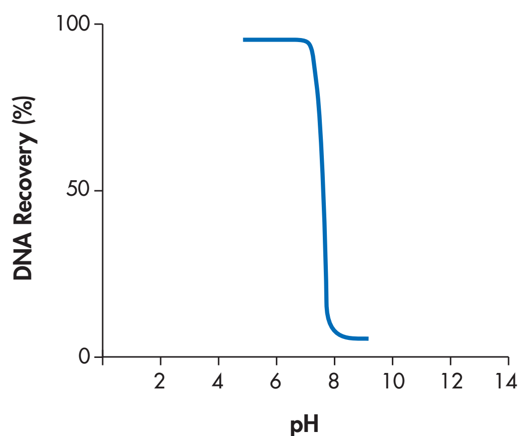


Figure 2. pH dependence of DNA adsorption to QIAquick membranes. 1 µg of a 2.9 kb DNA fragment was adsorbed at different pHs and eluted with Buffer EB (10 mM Tris·Cl, pH 8.5). The graph shows the percentage of DNA recovery, reflecting the relative adsorption efficiency, versus pH of adsorption.

Optimized binding buffers for every DNA cleanup task

All QIAquick Spin Kits contain identical QIAquick spin columns but different binding buffers optimized for each specific application:

- Buffer PB in the QIAquick PCR Purification Kit allows the efficient binding of single- or double-stranded PCR products as small as 100 bp and the quantitative (99.5%) removal of primers up to 40 nucleotides. This kit can therefore be used to remove oligo-dT primers after cDNA synthesis or to remove unwanted linkers in cloning experiments.
- Buffer PN in the QIAquick Nucleotide Removal Kit promotes the adsorption of both oligonucleotides ≥17 bases and DNA fragments up to 10 kb to the membrane.
- Buffer QG in the QIAquick Gel Extraction Kit solubilizes the agarose gel slice and provides the appropriate conditions for binding of DNA to the silica membrane.

All of these buffers are available separately (see ordering information, page 37).

pH indicator

Binding buffer PB and binding and solubilization buffer QG are specially optimized for use with the QIAquick silica membrane. Buffer QG contains an integrated pH indicator, while an optional pH indicator can be added to Buffer PB allowing easy determination of the optimal pH for DNA binding. DNA adsorption requires a pH ≤7.5, and the pH indicator in the buffers will appear yellow in this range. If the pH is >7.5, which can occur if during agarose gel electrophoresis, the electrophoresis buffer had been used repeatedly or incorrectly prepared, or if the buffer used in an enzymatic reaction is strongly basic and has a high buffering capacity, the binding mixture turns orange or violet (Figure 2). This means that the pH of the sample exceeds the buffering capacity of Buffer PB or QG and DNA adsorption will be inefficient. In these cases, the pH of the binding mixture can easily be corrected by addition of a small volume of 3 M sodium acetate*, pH 5.0, before proceeding with the protocol. In addition, in the QIAquick Gel

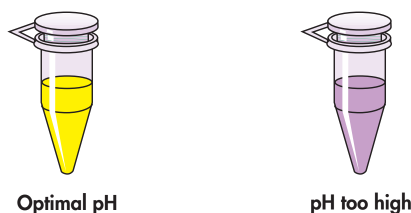


Figure 3. Indicator enables easy checking of the optimal pH. Indicator dye in solubilization and binding Buffers QG and PB identifies optimal pH for DNA binding.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs) available from the product supplier.

Extraction Kit procedure, the color of the binding mixture allows easy visualization of any unsolubilized agarose, ensuring complete solubilization and maximum yields. The indicator dye does not interfere with DNA binding and is completely removed during the cleanup procedure. Buffers PB and QG do not contain sodium iodide (NaI). Residual NaI may be difficult to remove from DNA samples, and reduces the efficiency of subsequent enzymatic reactions such as blunt-end ligation.

Washing

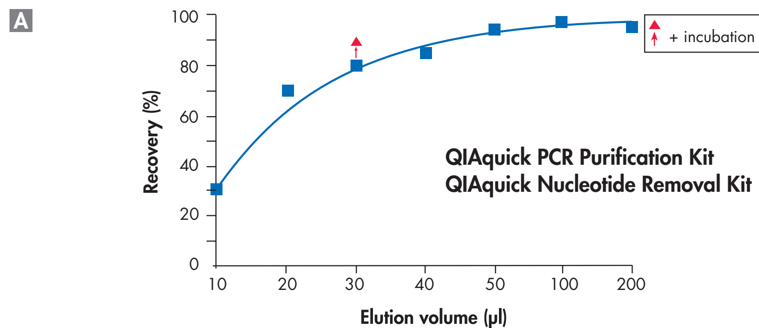
During the DNA adsorption step, unwanted primers and impurities, such as salts, enzymes, unincorporated nucleotides, agarose, dyes, ethidium bromide, oils, and detergents (e.g., DMSO, Tween® 20) do not bind to the silica membrane but flow through the column. Salts are quantitatively washed away by the ethanol-containing Buffer PE. Any residual Buffer PE, which may interfere with subsequent enzymatic reactions, is removed by an additional centrifugation step.

Elution in low-salt solutions

Elution efficiency is strongly dependent on the salt concentration and pH of the elution buffer. Contrary to adsorption, elution is most efficient under basic conditions and low salt concentrations. DNA is eluted with 50 or 30 µl of the provided Buffer EB (10 mM Tris·Cl, pH 8.5), or water. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water to elute, make sure that the pH is within this range. In addition, DNA must be stored at –20°C when eluted with water since DNA may degrade in the absence of a buffering agent. Elution with TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) is possible, but not recommended because EDTA may inhibit subsequent enzymatic reactions.

DNA yield and concentration

DNA yield depends on the following three factors: the volume of elution buffer, how the buffer is applied to the column, and the incubation time of the buffer on the column. 100–200 µl of elution buffer completely covers the QIAquick membrane, ensuring maximum yield, even when not applied directly to the center of the membrane. Elution with ≤50 µl requires the buffer to be added directly to the center of the membrane, and if elution is done with the minimum recommended volume of 30 µl, an additional 1 minute incubation is required for optimal yield. DNA will be up to 1.7 times more concentrated if the QIAquick column is incubated for 1 minute with 30 µl of elution buffer, than if it is eluted in 50 µl without incubation (Figure 4, page 14).



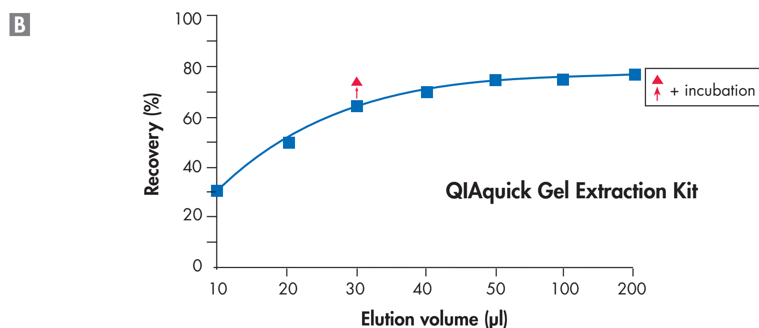


Figure 4. Highly concentrated DNA. Effect of elution buffer volume on DNA yield for A the QIAquick PCR Purification and QIAquick Nucleotide Removal Kit; B the QIAquick Gel Extraction Kit. 5 µg of a 2.9 kb DNA fragment were purified and eluted with the indicated volumes of Buffer EB. 30 µl plus 1 minute incubation on the QIAquick column gives DNA yields similar to 50 µl without incubation, but at a concentration 1.7 times greater.

Loading dye

Loading dye is provided for analysis of purified DNA samples using electrophoresis. It contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type. Loading dye is supplied as a 5x concentrate; thus 1 volume of loading dye should be added to 5 volumes of purified DNA.

Table 2. Migration Distance of Gel Tracking Dyes

%TAE (TBE) agarose gel	Xylene cyanol (light blue)	Bromophenol blue (dark blue)	Orange G (orange)
0.8	5000 bp (3000 bp)	800 bp (400 bp)	150 bp (<100 bp)
1.0	3000 bp (2000 bp)	400 bp (250 bp)	<100 bp (<100 bp)
1.5	1800 bp (1100 bp)	250 bp (100 bp)	<100 bp (<100 bp)
2.0	1000 bp (600 bp)	200 bp (<100 bp)	<100 bp (<100 bp)
2.5	700 bp (400 bp)	100 bp (<50 bp)	<50 bp (<50 bp)

Agarose gel analysis of yield

Yields of DNA following cleanup can be determined by agarose gel analysis. Table 3 shows the total yield obtained following extraction of 1 µg or 0.5 µg starting DNA from an agarose gel with a recovery of 80% or 60% using the QIAquick Gel Extraction Kit. The corresponding amount of DNA in a 1 µl aliquot from 50 µl eluate is indicated. Quantities of DNA fragment corresponding to these 1 µl aliquots are shown on the agarose gel in Figure 4.

Table 3. Amount of DNA in 1 µl aliquots of a 50 µl eluate following QIAquick purification

Starting DNA	Recovery	Total yield (50 µl eluate)	Amount of DNA in 1 µl
1 µg	80%	0.8 µg	16 ng
	60%	0.6 µg	12 ng
0.5 µg	80%	0.4 µg	8 ng
	60%	0.3 µg	6 ng

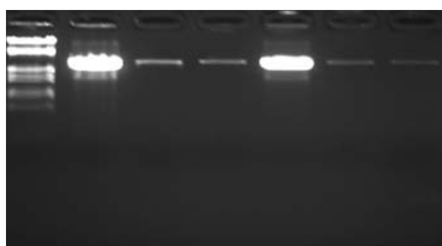


Figure 5. High DNA recovery. Quantities of purified 2.7 kb DNA fragment corresponding to 1/50 of the DNA obtained following purification from 1 µg or 0.5 µg starting DNA with a recovery of 80% or 60% (see Table 1). Samples were run on a 1% TAE agarose gel. **M**: lambda–EcoRI-HindIII markers.

Quantification of DNA fragments

DNA fragments can be quantified by running a sample alongside standards containing known quantities of the same-sized DNA fragment. The amount of sample DNA loaded can be estimated by visual comparison of the band intensity with that of the standards (Figure 5).

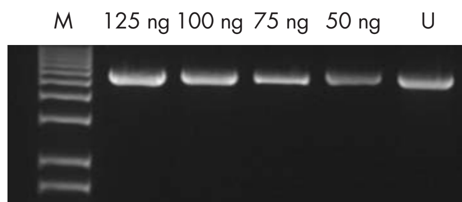
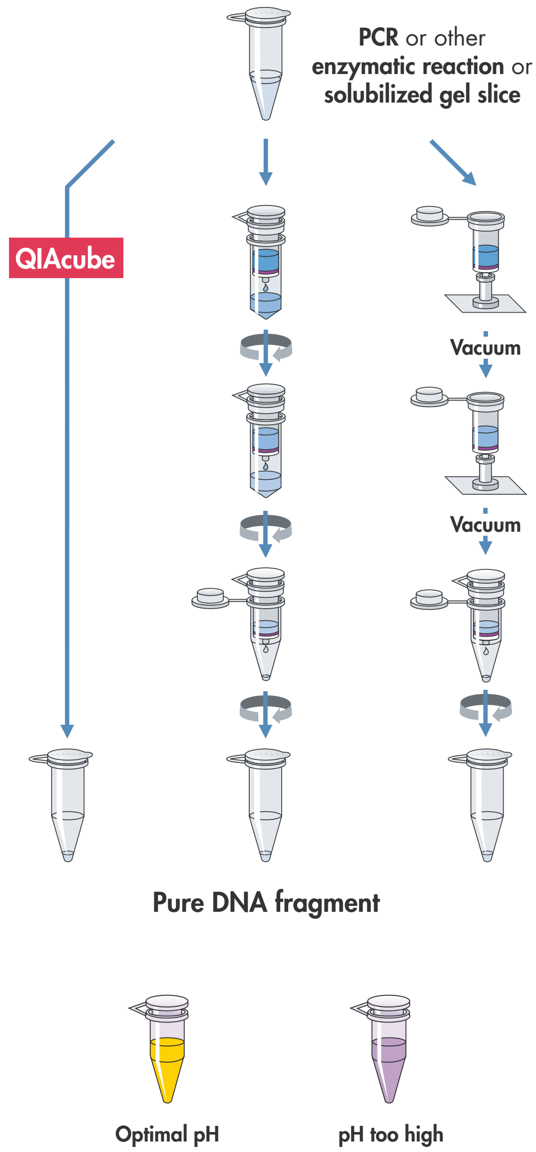


Figure 6. Agarose gel analysis. An unknown amount of a 5.5 kb DNA fragment (**U**) was run alongside known quantities (as indicated in ng) of the same DNA fragment. The unknown sample contained 75–100 ng DNA, as estimated by visual comparison with the standards. **M**: 1 kb DNA ladder.

Applications using QIAquick purified DNA

DNA purified with QIAquick is suitable for any subsequent application, such as restriction, labeling, hybridization, PCR, ligation and transformation, radioactive and fluorescent sequencing, in vitro transcription, or microinjection.

The QIAquick Procedure



Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs) available from the product supplier.

For all protocols

- Ethanol (96–100%)*
- Microcentrifuge
- 1.5 or 2 ml microcentrifuge tubes
- 3 M sodium acetate, pH 5.0, may be necessary for PCR purification and gel extraction protocols.
- Optional: Distilled water or TE buffer (10 mM Tris·Cl. 1 mM EDTA, pH 8) for elution of DNA.

Vacuum protocols

- Vacuum manifold (e.g., QIAvac 24 Plus or QIAvac 6S)
- Vacuum pump (e.g., QIAGEN Vacuum Pump, see ordering information).

Gel extraction protocols

- Isopropanol (100%)
- Heating block or water bath set at 50°C

* Do not use denaturated alcohol, which contains other substances such as methanol or methylethylketone.

QIAquick PCR Purification Kit Protocol

using a microcentrifuge

PCR Purification Spin Protocol

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5.
- Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

1. **Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.**

   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. **If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow.**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.
3. **Place a QIAquick spin column in a provided 2 ml collection tube.**
4. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**
5. **Discard flow-through. Place the QIAquick column back into the same tube.**

   Collection tubes are re-used to reduce plastic waste.
6. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**
7. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.**

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

PCR Purification Spin Protocol

8. **Place QIAquick column in a clean 1.5 ml microcentrifuge tube.**
9. **To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.**

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
10. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

QIAquick Gel Extraction Kit Protocol

using a microcentrifuge

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. This kit can also be used for DNA cleanup from enzymatic reactions (see page 8). For DNA cleanup from enzymatic reactions using this protocol, add 3 volumes of Buffer QG and 1 volume of isopropanol to the reaction, mix, and proceed with step 6 of the protocol. Alternatively, use the MinElute Reaction Cleanup Kit.

Important points before starting

- The yellow color of Buffer QG indicates a pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.

Procedure

1. **Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.**

   Minimize the size of the gel slice by removing extra agarose.

2. **Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).**

   For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. **Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.**

   IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. **After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

   The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. **Add 1 gel volume of isopropanol to the sample and mix.**

   For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

6. **Place a QIAquick spin column in a provided 2 ml collection tube.**
7. **To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.**

   The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
8. **Discard flow-through and place QIAquick column back in the same collection tube.**

   Collection tubes are reused to reduce plastic waste.
9. **Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.**

   This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription, or microinjection.
10. **To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.**

    Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.
11. **Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x *g* (13,000 rpm).**

    IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. **Place QIAquick column into a clean 1.5 ml microcentrifuge tube.**
13. **To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.**

    IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl.

    Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
14. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx . The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information or protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

		Comments and Suggestions
Low or no recovery		
a)	Buffer PE did not contain ethanol	Ethanol must be added to Buffer PE (concentrate) before use. Repeat procedure with correctly prepared Buffer PE.
b)	Inappropriate elution buffer	DNA will only be eluted efficiently in the presence of low-salt buffer (e.g., Buffer EB: 10 mM Tris·Cl, pH 8.5) or water. See "Elution in low-salt solutions", page 13.
c)	Elution buffer incorrectly dispensed	Add elution buffer to the center of the QIAquick membrane to ensure that the buffer completely covers the membrane. This is particularly important when using small elution volumes (30 µl).
Gel		
d)	Gel slice incom-pletely solubilized	After addition of Buffer QG to the gel slice, mix by vortexing the tube every 2–3 min during the 50°C incubation. DNA will remain in any undissolved agarose.
e)	pH of electro-phoresis buffer too high (binding mixture turns orange or violet)	The electrophoresis buffer has been repeatedly used or incor-rectly prepared, resulting in a sample pH that exceeds the buffering capacity of Buffer QG and leads to inefficient DNA binding. Add 10 µl of 3 M sodium acetate, pH 5.0, to the sample and mix. The color of the mixture will turn yellow indicating the correct pH for DNA binding. Even for binding mixtures with only small color changes (slight orange color), add the 10 µl sodium acetate.
f)	Gel slice was too large (>400 mg)	70–80% recovery can only be obtained from ≤400 mg gel slice per QIAquick column. For gel slices >400 mg, use multiple QIAquick columns.

Gel: refers to QIAquick Gel Extraction Kits only.
PCR: refers to QIAquick PCR Purification Kits only.
Other notes refer to all kits.

		Comments and Suggestions
PCR		
g)	Insufficient/no PCR product	Estimate DNA recovery by running 10% of PCR product before and after purification on an agarose gel.
PCR/Gel		
h)	Cloudy and gelatinous appearance of sample mixture after addition of isopropanol	This may be due to salt precipitation, and will disappear upon mixing the sample. Alternatively, the gel slice may not be completely solubilized. In this case, apply the mixture to the QIAquick column, centrifuge, and then add 0.5 ml Buffer QG to the column. Let stand for 1 min at room temperature, and then centrifuge and continue with the procedure. This additional wash will solubilize remaining agarose.
i)	Binding mixture turns orange or violet	The pH in the sample exceeds the buffer capacity of Buffer QG or PB respectively. Add 20 µl of 3 M sodium acetate, pH 5.0, to the sample and mix. The color of the mixture will turn yellow indicating the correct pH for DNA binding. Even for samples with slight color changes (orange color), add 10 µl sodium acetate.
DNA does not perform well (e.g., in ligation reactions)		
a)	Salt concentration in eluate too high	Modify the wash step by incubating the column for 5 min at room temperature after adding 750 µl of Buffer PE, then centrifuge.
b)	Eluate contains residual ethanol	Ensure that the wash flow-through is drained from the collection tube and that the QIAquick column is then centrifuged at 17,900 x *g* (13,000 rpm) for an additional 1 min.
Gel		
c)	Eluate contaminated with agarose	The gel slice is incompletely solubilized or weighs >400 mg. Repeat procedure, including the optional Buffer QG column-wash step.
PCR		
d)	Eluate contains primer-dimers	Primer-dimers formed are >20 bp and are not completely removed. After the binding step, wash the QIAquick column with 750 µl of a 35% guanidine hydrochloride aqueous solution (35 g in 100 ml). Continue with the Buffer PE wash step and the elution step as in the protocol.

		Comments and Suggestions
e)	Eluate contains denatured ssDNA, which appears as smaller smeared band on an analytical gel	Use the eluted DNA to prepare the subsequent enzymatic reaction but omit the enzyme. To reanneal the ssDNA, incubate the reaction mixture at 95°C for 2 min, and allow the tube to cool slowly to room temperature. Add the enzyme and proceed as usual. Alternatively, the DNA can be eluted in 10 mM Tris buffer containing 10 mM NaCl. The salt and buffering agent promote the renaturation of DNA strands. However the salt concentration of the eluate must then be considered for subsequent applications.

References

1. Vogelstein, B. and Gillespie, D. (1979) Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA ***76***, 615.
2. Hamaguchi, K. and Geiduschek, E.P. (1962) The effect of electrolytes on the stability of deoxyribonucleate helix. J. Am. Chem. Soc. ***84***, 1329.

HC70AL Spring 2004

An Introduction to Bioinformatics -- Part I

By

Brandon Le

April 6, 2004

What are the Characteristics of a Gene?

- An ordered sequence of nucleotides
- A unique position/location in the genome
- Polarity (5' to 3')
- Exons and Introns

What are the Anatomical Features of Genes?

- Discrete beginning and discrete end
- Two strands of DNA
- Double helical
- Strand one (5' to 3')
- Strand two (3' to 5')
- Sense strand (5' to 3')
  - specifies the trait
- Nonsense strand (3' to 5')
  - template for transcription

Sense Strand

```
5' - ACGTCAGTCGATGCATGCTAGCTAGC - 3'
3' - TGCAGTCAGCTACGTACGATCGATCG - 5'
```

Nonsense Strand

Genes Have a Unique Position in the Genome!

Task: Where is your gene located in the genome?

Tools: The Arabidopsis Information Resources (TAIR) (http://www.arabidopsis.org)

01 AT2G22800
02 AT2G23290
03 AT2G37120
04 AT3G09735
05 AT3G12840
06 AT3G50060
07 AT3G53370
08 AT4G37260
09 AT4G37790
10 AT5G03220
11 AT5G03500
12 AT5G19490
13 AT5G67300

Procedure:

1. Select Seqviewer
2. Enter gene number (ex. AT1G18260)
3. Submit

Results/Question:

1. What chromosome is your gene in?
2. What other genes/markers are next to your gene?
3. What is the exact position of your gene in the genome?

Genes Have a Unique Order of Nucleotides!

Task: What is the order of nucleotides for your gene?

Tools: The Arabidopsis Information Resources (TAIR) (http://www.arabidopsis.org)

Procedure: (Continue from previous slide)

1. Click on Location

Results/Question:

1. What are your neighbor genes?
2. What is the orientation of your gene?
3. How big is your gene?

Genes Have Exons and Introns!

Task: How many exons and introns does your gene have?

Tools: The Arabidopsis Information Resources (TAIR) (http://www.arabidopsis.org)

Procedure: (Continue from previous slide)

1. Click on gene information on the right

Results/Question:

1. How many exons/introns in your gene?
2. What are exons?
3. What are introns?

Gene Encodes a Protein

Task: Determine the protein encoded by gene?

Tools: The Arabidopsis Information Resources (TAIR) (http://www.arabidopsis.org)

Results/Question:

1. How large is your protein?
2. What are the anatomy of a protein?

N-terminal C-terminal

What is the identity of your gene?

Task: What does your gene code for?

Tools: NCBI BLAST Tools (http://www.ncbi.nlm.nih.gov/BLAST)

What is BLAST?

Basic Local Alignment Search Tool (BLAST)

What does BLAST do?

A family of programs that allows you to input a query sequence and compare it to DNA or protein sequences in db.

What are the steps to performing BLAST search?

Paste sequence of interest into BLAST input box
Select BLAST program
Select db
Select Optional Parameters

NCBI translating BLAST
Nucleotide Protein Translations Retrieve results for an RID

Search
Choose a translation TRANSLATED query – PROTEIN database [blastx]
Set subsequence From: To:
Choose database nr
Genetic codes Standard (1)
Now: BLAST! or Reset query Reset all

What are the different BLAST Programs?

Fastest

blastp - protein query vs protein db

blastn - DNA query vs DNA db

blastx - translated DNA query vs protein db

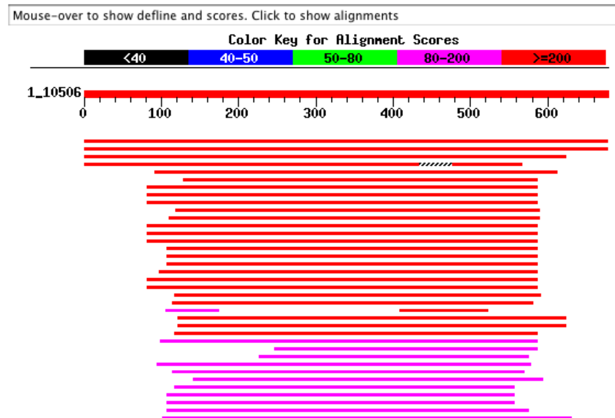
tblastx - protein query vs translated DNA db

tblastn - translated DNA query vs translated DNA db

Slowest

Anatomy of a BLAST Result -- Part I

Distribution of 339 Blast Hits on the Query Sequence



Anatomy of a BLAST Result -- Part II

```
                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

gi|14532716|gb|AAK64159.1|  unknown protein [Arabidopsis tha...  1206   0.0
gi|18394588|ref|NP_564049.1|  suppressor of lin-12-like prot...  1209   0.0
gi|15219499|ref|NP_177498.1|  suppressor of lin-12-like prot...   877   0.0
gi|11120786|gb|AAG30966.1|  hypothetical protein, 3' partial...   426   e-118
gi|41151276|ref|XP_046437.5|  chromosome 20 open reading fra...   291   3e-77  L
gi|13559241|emb|CAB65792.2|  dJ842G6.2 (novel protein imilar...   282   2e-74  L
gi|19923669|ref|NP_005056.3|  sel-1 suppressor of lin-12-lik...   268   4e-70  L
gi|6851089|gb|AAF29413.1|  SEL1L [Homo sapiens] >gi|17646138...   268   4e-70  L
gi|9967440|dbj|BAB12403.1|  SEL1L [Mesocricetus auratus]          264   4e-69
gi|31203035|ref|XP_310466.1|  ENSANGP00000019196 [Anopheles ...   263   1e-68
gi|21355295|ref|NP_651179.1|  CG10221-PA [Drosophila melanog...   263   1e-68  L
gi|20857527|ref|XP_127076.1|  Sel1 (suppressor of lin-12) 1 ...   261   4e-68  L
gi|4159995|gb|AAD05210.1|  SEL1L [Mus musculus] >gi|20073079...   259   1e-67  L
gi|29336095|ref|NP_808794.1|  Sel1 (suppressor of lin-12) 1 ...   259   2e-67  L
gi|29612522|gb|AAH49959.1|  Sel1h protein [Mus musculus]          258   4e-67  L
gi|17563256|ref|NP_506144.1|  Suppressor/Enhancer of Lin-12 ...   247   9e-64  L
gi|1255199|gb|AAC47112.1|  sel-1 gene product                     247   9e-64
```

Anatomy of a BLAST Result -- Part III

```
>gi|14532716|gb|AAK64159.1|   unknown protein [Arabidopsis thaliana]
          Length = 678

 Score = 1206 bits (3120), Expect = 0.0
 Identities = 614/678 (90%), Positives = 614/678 (90%)

Query: 1   MRILSYGIVILSLLVFSFIEFGVHARPVVLVXXXXXXXXXXXXXXVXXXXXXXXXXXXXX 60
            MRILSYGIVILSLLVFSFIEFGVHARPVVLV              V
Sbjct: 1   MRILSYGIVILSLLVFSFIEFGVHARPVVLVLSNDDLNSGGDDNGVGESSDFDEFGESEP 60

Query: 61  XXXXXLDPGSWRSIFEPDDSTVQAASPQYYSGLKKILSAASEGNFRLMEEAVDEIEAASS 120
                 LDPGSWRSIFEPDDSTVQAASPQYYSGLKKILSAASEGNFRLMEEAVDEIEAASS
Sbjct: 61  KSEEELDPGSWRSIFEPDDSTVQAASPQYYSGLKKILSAASEGNFRLMEEAVDEIEAASS 120

Query: 121 AGDPHAQSIMGFVYGIGMMREKSKSKSFLHHNFAAAGGNMQSKMALAFTYLRQDMHDKAV 180
            AGDPHAQSIMGFVYGIGMMREKSKSKSFLHHNFAAAGGNMQSKMALAFTYLRQDMHDKAV
Sbjct: 121 AGDPHAQSIMGFVYGIGMMREKSKSKSFLHHNFAAAGGNMQSKMALAFTYLRQDMHDKAV 180

Query: 181 QLYAELAETAVNSFLISKDSPVVEPTRIHSGTEENKGALRKSRGEEDEDFQILEYQAQKG 240
            QLYAELAETAVNSFLISKDSPVVEPTRIHSGTEENKGALRKSRGEEDEDFQILEYQAQKG
Sbjct: 181 QLYAELAETAVNSFLISKDSPVVEPTRIHSGTEENKGALRKSRGEEDEDFQILEYQAQKG 240

Query: 241 NANAMYKIGLFYYFGLRGLRRDHTKALHWFLKAVDKGEPRSMELLGEIYARGAGVERNYT 300
            NANAMYK GLFYYFGLRGLRRDHTKALHWFLKAVDKGEPRSMELLGEIYARGAGVERNYT
Sbjct: 241 NANAMYKNGLFYYFGLRGLRRDHTKALHWFLKAVDKGEPRSMELLGEIYARGAGVERNYT 300
```

PubMed - Endless Resources

NCBI | PubMed | National Library of Medicine NLM

Entrez | PubMed | Nucleotide | Protein | Genome | Structure | OMIM

Search PubMed for [ ] Go Clear

Limits | Preview/Index | History | Clipboard | Details

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- Enter author names as smith jc. Initials are optional.
- Enter journal titles in full or as MEDLINE abbreviations. Use the Journals Database to find journal titles.

PubMed, a service of the National Library of Medicine, includes over 14 million citations for biomedical articles back to the 1950's. These citations are from MEDLINE and additional life science journals. PubMed includes links to many sites providing full text articles and other related resources.

HC70AL Spring 2004

An Introduction to Bioinformatics -- Part II

By

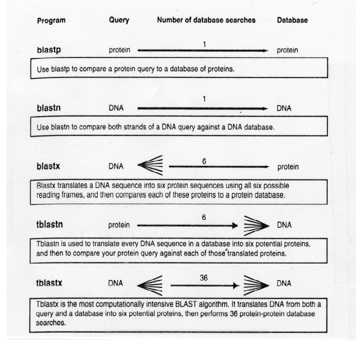
Brandon Le

April 8, 2004

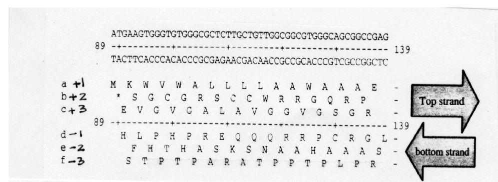
Review of BLAST Search

1. What is the purpose of running BLAST Search?
2. What are the steps to performing BLAST search?
3. What does the e-value from a blast result tell you?
4. How may BLAST program can you perform?
5. What BLAST program(s) takes the least computational time?
6. What BLAST program(s) takes the most computational time? Why?

What are the Five BLAST Search Programs?



•**How many proteins can a short DNA sequence <u>potentially</u> encode?**



Question:

You have <u>DNA</u> Sequence. You want to know which protein in the main <u>protein</u> database is most similar to some <u>protein</u> encoded by your DNA.

Which BLAST program should you use?

Suppose you have a <u>protein</u> sequence.
Which BLAST program should you use?

HOW to interpret BLAST results?

```
>gi|13559241|emb|CAB65792.2|  dJ842G6.2 (novel protein imilar to SEL1L (sel-1
          lin-12, C.elegans)-like)) [Homo sapiens]
          Length = 505

 Score =  297 bits (760), Expect = 8e-79
 Identities = 177/468 (37%), Positives = 269/468 (57%), Gaps = 9/468 (1%)
 Frame = +1

Query: 388  MGFV--YGIGMMREKSKSKSFLHHNFAAAGGNMQSKMALAFTYLRQ----DMHDKAVQLY 549
             +GF+  YGIGM  E  ++K+ +++ F +AGGNM S+M L + YL         + A+  Y
Sbjct: 2    LGFLSSYGIGM--EYDQAKALIYYTFGSAGGNMMSQMILGYRYLSGINVLQNCEVALSYY 59

Query: 550  AELAETAVNSFLISKDSPVVEPTRIHSGTEENKGALRKSRGEEDEDFQILEYQAQKGNAN 729
              ++A+    ++F  S+  PV E  R+    TE  +       S   + + +Q  ++ A++G+
Sbjct: 60   KKVADYIADTFEKSEGVPV-EKVRL---TERPENLSSNSEILDWDIYQYYKFLAERGDVQ 115

Query: 730  AMYKIGLFYYFGLRGLRRDHTKALHWFLKAVDKGEPRSMELLGEIYARG-AGVERNYTKA 906
                 +G  +  G +GL +D+ KALH+FLKA   G   +M  +G++Y  G A V +N   A
Sbjct: 116  IQVSLGQLHLIGRKGLDQDYYKALHYFLKAAKAGSANAMAFIGKMYLEGNAAVPQNNATA 175

Query: 907  LEWLTLAAKEGLYSAFNGIGYLYVKGYGVDKKNYTKAREYFEKAVDNEDPSGHYNLGVLY 1086
             ++ ++AA +G      +G+G LY   G GV    NY +A +YF+KA +    P    + LG +Y
Sbjct: 176  FKYFSMAASKGNAIGLHGLGLLYFHGKGVPL-NYAEALKYFQKAAEKGWPDAQFQLGFMY 234

Query: 1087 LKGIGVNRDVRQATKYFFVAANAGQPKAFYQLAKMFHTGVGLKKNLEMATSFYKLVAERG 1266
              G G+ +D + A KYF++A++ +GQP A Y LAKM+ TG G+ ++    A  YK V E G
Sbjct: 235  YSGSGIWKDYKLAFKYFYLASQSGQPLAIYYLAKMYATGTGVVRSCRTAVELYKGVCELG 294

Query: 1267 PWSSLSRWALEAYLKGDVGKALILYSRMAEMGYEVAQSNAAWILDKYGERSMCMGVSGFC 1446
             W+      A AY  GD+  +L+ Y+ +AEMGYEVAQSN+A+IL+          +
Sbjct: 295  HWAEKFLTAYFAYKDGDIDSSLVQYALLAEMGYEVAQSNSAFILESKKANIL-------- 346

Query: 1447 TDKER-HERAHSLWWRASEQGNEHAALLIGDAYYYGRGTERDFVRAAEAY-MHAKSQSNA 1620
             +KE+ +  A  LW RA+ QGN  A + IGD +YYG GT++D+  AA  Y + A    NA
Sbjct: 347  -EKEKMYPMALLLWNRAAIQGNAFARVKIGDYHYYGYGTKKDYQTAATHYSIAANKYHNA 405

Query: 1621 QAMFNLGYMHEHGQGLPFDLHLAKRYYDESLQSDAARLPVTLALASL 1764
             QAMFNL YM+EHG G+  D+HLA+R YD + Q+   A +PV  A+  L
Sbjct: 406  QAMFNLAYMYEHGLGITKDIHLARRLYDMAAQTSPDAHIPVLFAVMKL 453
```

Identifier of protein in database

Expect = 8e-79

probability of finding this alignment in a database by chance and chance alone.

origin of protein sequence

\# of amino acid in the protein

What does Frame mean?

exact match of amino acid

what is this?

what is query?

what is sbjct?

what does the "+" mean?

what does these number corresponds to?

positively charged amino acids { K = Lysine, R = Arginine }

4

Review of gene transcription

1. What product is made after transcription?
2. How is the product similar/different from the gene?
3. What is cDNA?
4. What important information does a cDNA tell you about a gene?
5. What are ESTs?
6. What important information does ESTs tell you about a gene?

Annotation of your gene

1. What chromosome is your gene in?
2. How "big" is your gene?
3. How many exons and introns in your gene?
4. What orientation is your gene in the genome?
5. What is the specific position of your gene in the genome?
6. What gene is "upstream" of your gene?
7. What gene is "downstream" of your gene?
8. How far are the other genes (6 & 7) from your gene?
9. What is the "structure" of your gene?
10. What is the size of the protein in your gene encodes?
11. What protein does your gene encode
12. Is your gene structure predicted by a program?

Webbook - A Virtual Lab Notebook

Webbook is a web lab notebook

Purpose/goal: To have access to experiments carried out b
Lab members, etc… from anywhere
Also serves as a repository for protocols, stocks/reagents

Created by: Harry Hahn
Brandon Le
Bob Goldberg

http//estdb.biology.ucla.edu/webbook

Using the Webboook

1. Username: email username
   Password: 9 digit student id

2. Check message board for important news/updates

3. An overview of the different sections

   - Projects - list of experiments
   - Stocks - catalog of stocks/reagent in the lab
   - Protocols - procedures carried out in the lab (pdf format)
   - Calendar - calendar to plant your experiments
   - Browse - search and look at other members experiments
   - Contact - email for help
   - Logout - will logout if idle for 30 min

Webbook Login Page

Creating Projects / Experiments

1. Title of project
2. Questions/Purpose of project
3. Summary of project (ideas)

Entering Gene Information

Genes

Create gene

Fields marked with a red asterisk (*) are REQUIRED)

Gene Name:*

Species:

Sequence:

Sequence Type:* -- Select --

Amino Acid Sequence:

Chromosome:

EST Data:

Functional Category:

Promoter:

Domains:

Hits:

Attach a file: Title:

File: Choose File no file selected

Description:

Entering Experiments Information
Part 1

Experiments

Fields marked with a red asterisk (*) are **REQUIRED**

Title:*	
Goal:*	
Background Info:*	
Approach:*	
Controls:*	
Discussion:	
Next:	

Entering Experiment Information
Part II

Materials	**Primer** * AT2G22800-FW AT2G22800-RV AT2G23290-FW AT2G23290-RV AT2G37120-FW AT2G37120-RV AT3G09735-FW AT3G09735-RV
Protocols:	**Protocols** * *Sequencing Using SPPCR Alkali Lysis Plasmid Isolation Arabidopsis Tissue Harvest For GeneChip Experiment Bacteria Chromosome Mini-Prep Bacteriophage Chromatin Immunoprecipitation with Leaves from Arabidopsis
Attach a file:	Title: File: Choose File no file selected Description: All files must have a file name extension. Images must end in .jpg, .png, or .gif. Additional files can be attached by later editing this record.

Entering References Relating to your Gene

References

Create reference record

Fields marked with a red asterisk (*) are **REQUIRED**

Author(s):*	
Title:*	
Journal:*	
Year*	
PDF File	Choose File no file selected

Create