EXPERIMENT 1 – INTRODUCTION TO GENERAL MOLECULAR BIOLOGY TECHNIQUES

STRATEGY

I. PIPETTING EXERCISE

II. SERIAL DILUTION EXPERIMENT

III. POLYMERASE CHAIN REACTION (PCR)

IV. PURIFYING PCR PRODUCTS

V. SEQUENCING REACTION WITH BIG DYE V. 3

VI. RETRIEVING AND ANALYZING DNA SEQUENCES

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. <u>Caution:</u> *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 front 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,

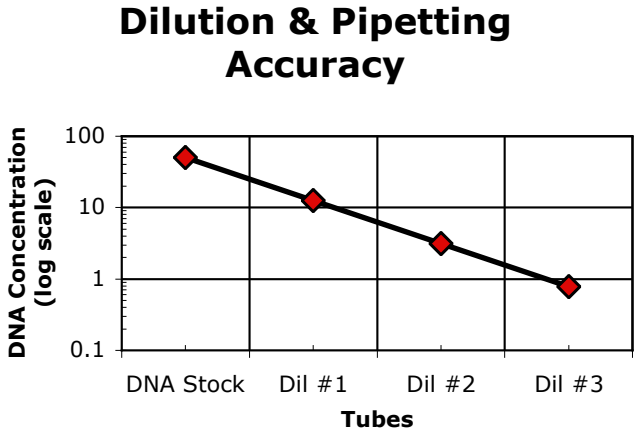
   $\mathbf{V_i}$ = **initial volume** (the volume of original DNA solution is **5 µL**)

   $\mathbf{C_i}$ = **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?*

III. POLYMERASE CHAIN REACTION (PCR)

Purpose: To obtain a large amount of DNA molecules in a short time for other purposes, such as determination of DNA sequence or cloning into a vector for gene expression or promoter analysis.

Reference: Ex Taq DNA Polymerase Manual (Takara; see Appendix 1E)

Solutions Needed:

- Ex Taq DNA polymerase (Takara)
- 10x Ex Taq buffer (Takara; comes with the Ex Taq DNA polymerase)
- dNTP mix (Takara; comes with the Ex Taq DNA polymerase)
- Sterile water
- 12 µM Gene-specific Salk Forward primer
- 12 µM Gene-specific Salk Reverse primer
- 0.2 ng/µL *Arabidopsis* Columbia-0 genomic DNA
- 2 µl gene-specific PCR positive control (gene-specific PCR product made by TAs)
- 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:

- 1.5 mL sterile microcentrifuge tubes
- 0.2 mL sterile PCR tubes
- Microcentrifuge tube rack
- PCR tube rack
- Pipettes
- Filter pipet tips
- Black ultra-fine sharpie pen
- Ice bucket, plastic container or Styrofoam box
- Microcentrifuge
- Minicentrifuge
- PCR machine
- Vortex
- Gel electrophoresis materials (Appendix 1A)

PROCEDURE

A. Polymerase Chain Reaction (PCR)

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

1. Get ice from the icemaker in room 4128.
2. Thaw tubes of **10x Ex Taq buffer** and **dNTP mix** on a rack for 1.5 mL microcentrifuge tubes at **room temperature** for 5-10 minutes. Once the solutions are thawed, put the tubes on **ice** until needed.
3. Thaw **12 µM Gene-specific Salk Forward primer** and **12 µM Gene-specific Salk Reverse primer** (corresponding to the gene to be knocked out) at room temperature as in step 2.
4. Place 0.2 ng/µL *Arabidopsis* Columbia-0 **genomic DNA** on ice.
5. Thaw 5 µL of positive control on ice.
6. Obtain **THREE** 0.2 mL sterile PCR tubes and set them on a rack for PCR tubes.
7. Write on the **lids** of the tubes the numbers **1**, **2** and **3**. Label the **sides** of the tubes with the **sample identity**, **your initials** and the **date** as follows: (*your TA will show you how to write on the tubes*)

   Tube #1: **Name of the gene**

   Tube #2: **Pos.** (Positive control containing gene-specific PCR product made by TAs)

   Tube #3: **Neg.** (Negative control containing same components as in tube #1, but **NO** genomic DNA)

   Keep the labeled PCR tubes in their rack on **ice**.
8. Obtain **ONE** 1.5 mL microcentrifuge tube and set it on a rack for 1.5 mL microcentrifuge tubes.
9. Write "**Mmix**" (for Master mix) on the lid of the tube with a black ultra-fine sharpie. Keep the tube on **ice**.
10. Briefly **vortex** each reagent. **Centrifuge** at full speed for 10 seconds. Place on **ice**.

11. Prepare Master Mix (**Mmix**) solution for **4 reactions** (**3 samples + 1 extra**) as follows:

Master Mix:

	Mmix for ONE reaction	**Mmix for 4 reactions**
Sterile water	36.75 µL	147.0 µL
10x Ex Taq buffer	5.0 µL	20.0 µL
dNTP mix	4.0 µL	16.0 µL
12 µM Gene-specific Salk Forward primer	1.0 µL	4.0 µL
12 µM Gene-specific Salk Reverse primer	1.0 µL	4.0 µL
Ex Taq DNA polymerase (5 Units/µL)	0.25 µL	1.0 µL
Total volume	**48.0 µL**	**192.0 µL**

a. Pipet the reagents in order from top down (example: water, 10x Ex Taq buffer, dNTP mix, etc.) into the **Mmix** tube.
b. After pipetting all reagents into the **Mmix** tube, close the lid of the tube. Mix the contents by vortexing at a **setting of 2-3** for **5 seconds**. Spin the tube in a microcentrifuge at FULL speed (13,200 rpm) for **10 seconds**. Put the tube back **on ice**. *Note: Do not vortex enzyme vigorously*.

12. Prepare the PCR reactions.
    a. Pipet **48 µL** of the **Mmix** solution into each PCR tube.
    b. Pipet 1-2 µL of DNA or water to tubes #1-3. (see table below)
    c. Immediately, mix the contents by **pipetting up and down at least five times.**
    d. Briefly spin tubes in the mini microcentrifuge for PCR tubes.

PCR reactions:

Components	**Tube #1 (Gene Name)**	**Tube #2 (Positive)**	**Tube #3 (Negative)**
Mmix	48 μL	48 μL	48 μL
Arabidopsis Col-0 ecotype genomic DNA (0.2 ng/μL)	2 μL	-	-
Control DNA template (Positive control)	-	1 μL	-
Sterile water (negative control)	-	1 μL	2 μL
Total volume	**50 μL**	**50 μL**	**50 μL**

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

    94°C 3 min
    36 cycles of 94°C 15 sec
    62°C 30 sec
    72°C 2 min
    72°C 4 min
    4°C ∞
18. Press "**F5**" for "**DONE.**" The "CHOOSE OPERATION" menu will appear. Press "**ENTER**" to **run the protocol**.
19. Enter the **volume** of the PCR reaction. Press "**F5**" to "**Begin Run**." *Note: It will take about 3 hours for the PCR amplification to be completed.*
20. Once the PCR amplification is complete, remove the PCR tubes from the PCR machine and store them in the **refrigerator** until gel electrophoresis or leave them in the PCR machine at 4°C until you have a chance to put them away later.

B. Gel Electrophoresis Analysis of PCR Product

1. Write the numbers **1, 2** and **3** on the lids of THREE 1.5 mL microcentrifuge tubes.
2. Arrange your THREE PCR tubes to match the order of the THREE 1.5 mL microcentrifuge tubes.
3. Pipet into each 1.5 mL microcentrifuge tube the following:

	Tube #1	**Tube #2**	**Tube #3**
6x Loading dye	3 μL	3 μL	3 μL
PCR Solution #1	25 μL	-	-
PCR Solution #2	-	25 μL	-
PCR Solution #3	-	-	25 μL

4. Mix the contents by pipetting up and down at least 5 times, or vortex and spin.
5. Load **10 μL** of **1 Kb Plus DNA ladder** in the first well of the 1% agarose gel.
6. Load **28 μL** of the sample-dye mixtures using a P-20 pipette.
7. Record the identity of the sample in each lane. Calculate the expected size of the PCR product.

Lane	**Sample**	**Expected Size (bp)**
1	1 Kb Plus DNA ladder	-
2	PCR Solution #1	
3	PCR Solution #2	
4	PCR Solution #3	-

8. Add **10 μL** of **10,000x SYBR Safe DNA gel stain** to the running buffer at the anode.
9. Run the gel at **105 volts** for 1-2 hours.

   Time power supply turned ON:

   Time power supply turned OFF:

   How long was the gel run? _____ hour(s) and _____ minutes
10. 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.*

9. Take a picture of the gel using the Bio-Rad Gel Document System.
10. Label the picture using the text program of the Gel Document System.
11. Print out the picture. Store the labeled picture in your lab notebook.
12. Analyze the size of the PCR product on the picture. *If the expected size is observed, proceed to Purifying PCR Products.*

What is the expected size (in bp) of the PCR product? _____ bp

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

_____ bp

Do you see a single band corresponding to your gene-specific PCR product?

Or

Do you see more than one band?

***Depending on the PCR results**, you can use one of the following procedures to purify the gene-specific PCR product.*

a. *If a lane on the gel contains only a **single band** corresponding to the gene-specific PCR product, then the DNA can be purified directly from the PCR solution following the **QIAquick PCR Purification Procedure** below.*

b. *If you observe more than a single band (due to contamination or mispriming), then the gene-specific PCR product must be purified from an agarose gel slice. Follow the **QIAquick Gel Extraction Procedure** below.*

IV. 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 solution (~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 **your initials** on a 1.5 mL microcentrifuge tube.
2. Pipet ~**25 μL** of the **PCR product solution** from the PCR tube containing the gene-specific DNA fragment into the 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 sample) to the tube in step 2. Mix by vortexing the tube 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 lilac **QIAquick spin column** (lilac) in a provided **2 mL collection tube**. Label the lid of the spin column with your **initials**.
7. Pipet the sample mixture in step 4 to the QIAquick spin column. Spin the column in the 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 set at **FULL speed** for an **additional 1 minute** to get rid of residual ethanol in Buffer PE. *Caution: Residual ethanol from Buffer PE will NOT be completely removed unless the flow-through solution is discarded before this additional spin. If the residual ethanol is not removed from the column after spinning for 1 min, then spin for an additional 1-2 min*.
12. While spinning, label a new **1.5 mL microcentrifuge** tube "**Purified PCR product**," **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 column sit for **1 minute**, and then centrifuge at **FULL speed** for **1 minute**. *This step elutes the DNA from the QIAquick membrane. **DNA** is in the **microcentrifuge tube**. Note: If some liquid remains on the column after centrifugation, remove it with a P10 pipette and dispense the liquid back onto the center of the QIAquick membrane. Centrifuge again at FULL speed for 1 minute*.

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:

- Remaining PCR solutions (~25 μL)
- QIAquick Gel Extraction Kit (Qiagen, Cat. #28704)
- Isopropanol
- Agarose
- 1x TAE buffer
- 10,000x SYBR Safe DNA gel stain (Invitrogen)
- 50 ng/μL 1 Kb Plus DNA ladder (Invitrogen)
- 6x Loading Dye containing xylene cyanol and bromophenol blue dyes

Materials Needed:

- Pipettes
- Filter pipet tips
- Black ultra-fine sharpie pen
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Gel electrophoresis materials (Appendix 1A)
- Razor blade
- Metal waste container for sharp objects
- Saran wrap
- UV light box
- Scale
- Microcentrifuge

- Vortex
- 50°C water bath or heat block
- 65°C water bath or heat block
- Timer

PROCEDURE

1. Label the **lid** of a 1.5 mL microcentrifuge tube with **your initials**.
2. Place a NEW piece of plastic wrap on an ultraviolet (UV) light box. Then place your gel on the plastic wrap.
3. Put on a UV shield to protect your eyes and face.
4. Turn **on** the **UV box**. *Note: Turn **off** the UV box as soon as you are done excising the DNA band.*
5. Excise the desired DNA fragment from the gel using a razor blade. *Note: Trim off excess agarose surrounding the DNA band as much as possible. Your TAs will demonstrate.*
6. Place the agarose 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 was faint, run the remaining 25 µL of the desired PCR solution on a new gel and excise that band as well.

   a. *Prepare a **1% agarose** gel with a **20-tooth comb** (see Appendix 1A).*
   b. *Add **3 µL** of **6x loading dye** the tube of **~25 µL** PCR solution containing the gene-specific fragment.*
   c. *Load **10 µL of 1 Kb Plus DNA ladder** into the first well.*
   d. *Very slowly load the **~28 µL** sample-dye mixtures on the gel using a P-20 pipette (i.e. load ~15 µL first, then then remaining ~15 µL into the same well). Note: If you load the sample into the well too fast, the sample will be forced out of the well. Pipet slowly so that the sample sinks into the well.*
   e. *Record the identity of the sample in each lane.*

Lane	*Sample*	*Expected Size (bp)*
1	*1 Kb Plus DNA ladder*	-
2	*PCR Solution #1*	

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

g. *Run the gel at **105 volts** for 1.5 - 2 hours in the dark.*

h. *Take a picture of the gel.*

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

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

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

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

m. *Excise the desired DNA fragment from the gel using a razor blade. Note: Trim off excess agarose surrounding the DNA band as much as possible. Your TAs will demonstrate.*

n. *Place the agarose slice in the 1.5 mL microcentrifuge tube from step 6.*

o. *Take a picture of the gel **after removing the agarose slice**. This step serves as a record of the DNA fragment being collected.*

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

8. Pipet **Elution Buffer** into a 1.5 mL microcentrifuge tube labeled with "**EB**" and **your initials**. Pipet **30 µL** x (number of samples +1). Warm the 1.5 mL microcentrifuge tube with elution buffer to **65°C** in a heat block. *This will be used in step 25 to remove the DNA from the membrane.*
9. Centrifuge the gel fragment at full speed for **1 minute**.
10. 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**.*

11. 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.*
12. Incubate the tube at **50°C** for **10 minutes** or until the gel slice has **completely** dissolved. *To help dissolve the gel, you may vortex the tube for 5 seconds every 2-3 min during the incubation. This step solubilizes the agarose completely. Make sure the color of the mixture is yellow.*
13. Add **1 gel volume** of **isopropanol** to the mixture and mix by **vortexing** for **5 seconds** or **inverting** the tube **5-10 times**. *This increases the yield of DNA fragments. Note: Do not centrifuge the sample at this stage.*
14. Place a **QIAquick spin column** (lilac) in a provided **2 mL collection tube**. Label the lid of the spin column with **your initials.** *Note: If the gel bands were faint, use one column for both gel slices. This will increase the final concentration of purified DNA.*
15. 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 15-17**.*
16. Centrifuge the tube for **1 minute**. *This step allows DNA binding to the membrane.*
17. 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. *Keep collection tube for use in steps 17-19.*
18. 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.*
19. Add **750** of **µL 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.*
20. r **two more times**.
21. 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.*
22. While spinning the tube, label a new 1.5 mL microcentrifuge tube with "**T-DNA gel**" and **your initials.**

23. Transfer the **QIAquick column** to the NEWLY labeled microcentrifuge tube. Discard the flow-through solution and the collection tube.
24. Add **30 µL** of **warm Buffer EB** (from step 8) 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.*
25. 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

V. SEQUENCING REACTION WITH BIG DYE V. 3

Purpose: To determine the sequence of a desired DNA fragment, such as a PCR product of the gene of interest.

References: Applied Biosystems

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

Solutions Needed:

- Applied Biosystems Big Dye version 3 (obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)
- 5x Sequencing Buffer (Obtained from UCLA Sequencing Facility, 5th floor, Gonda Building; or Sigma Cat. #S3938)
- 20 μM Gene-specific Salk Forward primer
- 20 μM Gene-specific Salk Reverse primer
- Sterile water

Materials Needed:

- Pipettes
- Filter pipet tips
- 0.2 mL PCR tubes or strips of 8 tubes/strip
- PCR tube rack
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Microcentrifuge
- Vortex
- PCR machine (Applied Biosystems GeneAmp 9700 or Bio-Rad MyCycler)
- Sequencing reaction purification columns (Qiagen DyeEx 2.0 Spin Kit; obtained from UCLA Sequencing Facility, 5th floor, Gonda Building)

Overview:

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

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

General Components of One Reaction:

	ONE Reaction
DNA template *	**x** μL
Sterile water	**y** μL
20 μM Sequencing 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 the better reads.*
- For **PCR product**, use the amount of DNA according to the table below. (Taken from UCLA WebSeq website. Also, see Perkin-Elmer Big Dye Protocol).

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

Table: Amount of DNA to Use in a Sequencing Reaction

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 gene of interest), but there are **TWO primers**: gene-specific salk forward primer and gene-specific salk reverse primer. Therefore, it is best to prepare a **master mix** with **all components except the primers**, which will be added to the individual reaction tubes.

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

What is the size of the PCR product? _____ bp

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

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

Sample calculations:

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

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

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

PROCEDURE

1. Get ice from the icemaker in room 4128.
2. Label the **sides** of **TWO 0.2 mL PCR tubes** with **your initials** and **primer name**. Set the tubes on a PCR tube rack sitting on ice.
3. Label the **lid** and **side** of a **1.5 mL microcentrifuge tube** "**Mmix**" and **your initials**. Set the tube on ice.
4. 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 above 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**. *Note: Big Dye v. 3 contains **enzyme**, keep it on **ice** as much as possible and **do not mix vigorously**.*
b. Spin the tube for **10 seconds** to bring all the contents to the bottom of the tube.
c. Set the tube back on ice.

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

Components	**Gene-specific Salk Forward primer**	**Gene-specific Salk Reverse primer**
Mmix	9 μL	9 μL
20 μM **Gene-specific Salk Forward** primer	1 μL	-
20 μM **Gene-specific Salk Reverse** primer	-	1 μL
Total volume	**10 μL**	**10 μL**

6. Carry out the 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.

7. After the cycling reaction is finished, clean up the sequencing reactions using the DyeEx 2.0 Spin Columns (stored in the refrigerator drawer) as following:
    a. Resuspend the resin by inversion or gently vortexing.
    b. Loosen the cap of the column a **quarter turn**. *This is necessary to avoid a vacuum inside the spin column.*
    c. Snap off the bottom closure of the spin column, and place the spin column in a 2 mL collection tube.
    d. Centrifuge at **3,000 rpm** for **3 minutes** at room temperature.
    e. Meanwhile, label a new set of 1.5 mL microcentrifuge tubes according to your reactions.
    f. Carefully transfer the spin columns to the new tubes.
    g. **Slowly** apply the sequencing reactions to the gel beds of the appropriate columns.

        Note:

        - *Pipet the sequencing reaction directly onto the center of the slanted gel-bed surface. Do not allow the reaction mixture or the pipet tip to touch the sides of the column. The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel bed surface with the pipet tip.*
        - *For easier handling, more reproducible pipetting, and reduced error with small sample volumes, you may adjust the volume of your sequencing reaction to 20 μL using distilled water, before application to the gel bed.*

    h. Centrifuge the columns at **3,000 rpm** for **3 minutes** at room temperature.
    i. Remove the spin columns from the microcentrifuge tubes. *The eluate contains the purified DNA.*
8. Keep samples on ice or in the refrigerator. Take the purified sequencing reactions to the UCLA Sequencing Facility located on the 5th floor in the Gonda Building. *Note: Use the primer name as the name of your sequence. Make sure to copy down the*

assigned file number *(example,* #106203*), which is automatically given by the computer after you enter the samples*.

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

VI. RETRIEVING AND ANALYZING DNA SEQUENCES

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

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 gene of interest (for example, At5g09250).*
6. Process the DNA sequences by "BLASTN" and "BLASTX" searches. See Appendix 2. *Note: Blast search may take a few minutes or longer to complete depending on how busy the NCBI server in Washington D.C. is.*
7. Determine if the DNA sequence corresponds to the gene of interest.
8. Print out the Blast results as hard-copy records for your lab notebook.
9. Save the Blast results in the **pdf** format so that you can upload them to your webbook.