EXPERIMENT 1 – INTRODUCTION TO GENERAL MOLECULAR BIOLOGY TECHNIQUES

STRATEGY

I. MICROPIPETTING EXERCISE

II. SERIAL DILUTION EXPERIMENT

III. POLYMERASE CHAIN REACTION (PCR)

IV. PURIFICATION OF PCR PRODUCTS

V. SEQUENCING REACTION WITH BIG DYE V. 3

VI. RETRIEVING AND ANALYZING DNA SEQUENCES

I. MICROPIPETTING EXERCISE

Purpose: To learn how to use micropipettors

Taken From: DNA Science: A First Course, Second Edition
Laboratory 1: Measurements, Micropipetting, and Sterile Techniques
pp. 327-328

Solutions Needed:

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

Apparatus Needed:

- Pipetman (P-10, P-20, P-200 & P-1000)
- Microcentrifuge tubes
- Rack for microcentrifuge tubes
- Pipet Tips (Regular = Not Filtered tips for PCR)

I. MICROPIPETTING EXERCISES

Taken From: DNA Science: A First Course, Second Edition
Laboratory 1: Measurements, Micropipetting, and Sterile Techniques
pp. 327-328

A. Small Volume Micropipettor Exercise

This exercise simulates setting up a reaction, using a micropipettor with a range of 0.5-10 µL or 1-20 µL.

1. Use a permanent marker (sharpie) to label three 1.5-mL tubes **A**, **B**, and **C**.
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
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 micropipettor to **4 µL** and add **Solution I** to each reaction tube.
4. Use a *fresh tip* to add 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 microfuge and apply a short, few-second pulse. Make sure that the **reaction tubes are placed** in a **balanced configuration** in the microfuge

rotor. *Spinning tubes in an unbalanced position will damage the microfuge motor*.

8. A **total** of **10 µL** of reagents was added to each reaction tube. To check that the previous pipetting measurements were accurate, set the pipette to 10 µL and very carefully withdraw 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 fluid, is an air space left in the tip end? What does this suggest? (The air can be displaced and actual volume determined simply by rotating volume adjustment to push fluid to very end of tip. Then, read the volume directly.)

9. How can you tell if the measurements were inaccurate? If several measurements were inaccurate, repeat this exercise to obtain a near-perfect result.

B. Large-Volume Micropipettor Exercise

This exercise simulates a bacterial transformation or plasmid preparation, for which a 100-1000-μL micropipettor is used. It is far easier to mismeasure when using a large-volume micropipettor. If the plunger is not released slowly, an air bubble may form or solution may be drawn into piston.

1. Use a permanent marker to label two 1.5-mL tubes **D** and **E**.
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 micropipettor to add appropriate volume of Solutions I-IV to reaction tubes **D** and **E**. Follow the same procedure as for the small-volume micropipettor add **Solutions I-IV** to each reaction tube.
4. A **total** of **1000 μL** of reactants was added to each tube. To check that the measurements were accurate, set the pipette to 1000 μL and very carefully withdraw 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 fluid, is an air space left in the tip end? (The air can be displaced and actual volume determined simply by rotating volume adjustment to push fluid to very end of tip. Then, read the volume directly.)
5. If several measurements were inaccurate, repeat this exercise to obtain a near-perfect result.

II. SERIAL DILUTION EXPERIMENT

Purpose: To test accuracy and precision of pipetting

Reference: Sambrook et al. (1989). Molecular Cloning Manual.

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 techniques as determined by gel electrophoresis and spectrophotometer readings.

Solutions Needed:

- DNA Stock (Known Concentration)
- Diluted 1Kb DNA ladder solution
- TE Buffer
- Agarose solution
- 1X TAE buffer
- 10 mg/mL Ethidium Bromide (EtBr)
- 6X Loading Dye containing xylene cyanol and bromophenol blue dyes

Apparatus Needed:

- Microcentrifuge tubes
- Nanodrop Spectrophotometer
- Kimwipes
- P-10, P-20 Pipetman
- Gel casts
- Gel box
- Cables
- Gel combs
- Plastic (Saran) wrap
- Gel Document system (Bio-Rad)
- Plastic container for carrying the gel
- Pipet Tips (Regular = Not Filtered tips for PCR)

A. Serial Dilution of a DNA Stock

1. Label **3 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** pipetman)
3. Pipet **5 µL** of your DNA stock solution into the **Dil.#1** microcentrifuge tube. (Use the **P-10** or **P-20 pipetman**)
4. Vortex the content of the tube for 5 seconds. Then, spin the tube for **10 seconds** to ensure that all of your solution is on the bottom of the tube.
5. Pipet **5 µL** of DNA solution from the **Dil. #1** tube into the **Dil. #2** tube.
6. Vortex the content of the tube for 5 seconds. Then, spin the tube for **10 seconds** to ensure that all of your solution is on the bottom of the tube.
7. Pipet **5 µL** of DNA solution from the **Dil. #2** tube into the **Dil. #3** tube.
8. Vortex the content of the tube for **5 seconds**. Then, spin the tube for **10 seconds** to ensure that all of your solution is on the bottom of the tube.

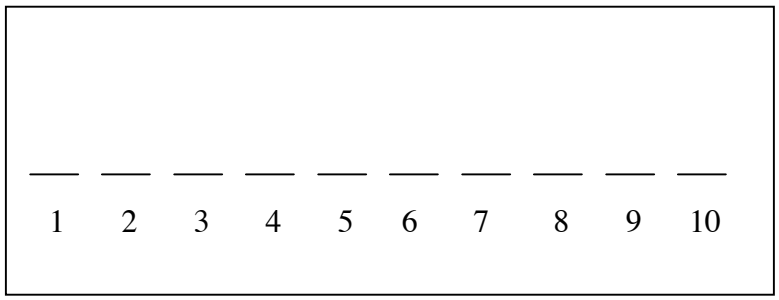
B. Determination of Pipetting Accuracy by Gel Electrophoresis

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

from	**Dil. #1**	**Dil. #2**	**Dil. #3**
to	Tube **A**	Tube **B**	Tube **C**

3. Pipet **2 µL** of Loading Dye into tubes **A**, **B**, **C**. Mix by pipetting up and down for 5 times. The total volume for each solution is **12 µL**.

4. Load **10 µL** of diluted **1-kb DNA ladder solution** (50 ng of DNA/µL; see **Appendix 1C**) into the **first well** of a **1% agarose gel** (see **Appendix** below for Preparation of the Agarose Gel).
5. Load the contents in tubes A, B, and C into the wells next to the 1-kb ladder solution.
6. Add **10 µL** of **10 mg/mL of EtBr** to the running buffer at the **cathode** (positively charged) of the gel box. (The **cathode** is the **opposite** side from the wells)
   Note: Ethidium bromide is positively charged. Therefore, it migrates towards the negative end of the gel box from cathode to anode. (Opposite direction from DNA migration). Remember that DNA is negatively charged; so, it migrates to the positive end of the gel. (DNA migrates from anode to cathode).
7. Put the lid of the gel box on the gel box and connect the electrodes to the power supply (**RED** to **RED** and **BLACK** to **BLACK**).
8. Record the **pattern** of **samples loaded** on the gel:



9. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue or BPB) has migrated one-half or two-thirds of the gel length. *Note: Use **~130 volts** for **TWO gels** connected to the **same power supply**.*
   Time power supply turned ON:
   Time power supply turned OFF:
   How long was the gel run? _____ minutes or _____ hour(s)

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 2828. ***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 to a different room (2828) for taking a picture of the gel.*
12. Take a picture of the gel using the BioRad Gel Document System in room 2828.
13. Label the picture using a text program of the Gel Document System (your TA will show you how).
14. Print out the picture.
15. (Optional) Label the picture by:
    a. Putting a piece of white tape (on the picture) at a position immediately above the wells
    b. Marking samples corresponding to all wells
16. Store the labeled picture a glassine envelope (obtain the envelopes from your TAs) that is pasted on a sheet of your note.

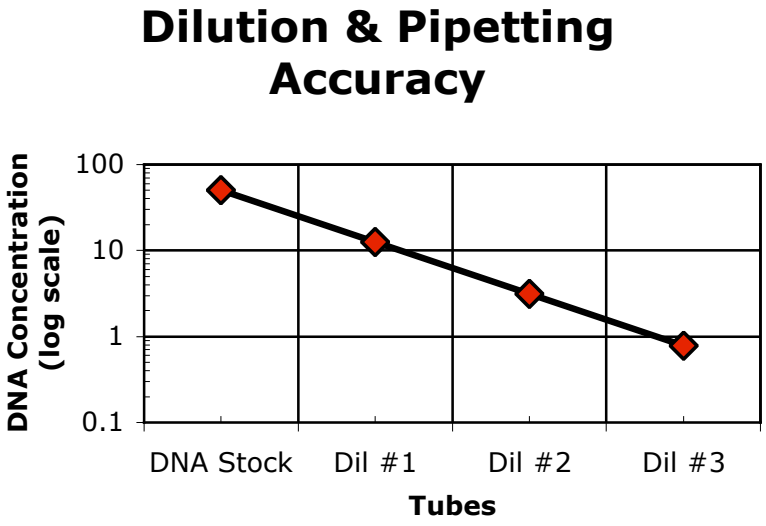
C. Determination of Pipetting Accuracy Using a Spectrophotometer

While running the gel, determine the **concentration** of DNA solutions in tubes labeled "DNA Stock", "Dil #1", "Dil #2", and "Dil #3" by using the **Nanodrop Spectrophotometer** (TAs will show how to use the instrument).
What is a spectrophotometer? (see Appendix 1B)

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

D. Question and Summary

1. What did you expect to see on your gel?
2. How is your pipetting accuracy determined by gel electrophoresis?
3. Is the gel result showing 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, what is the expected DNA concentration in tubes "Dil #1", "Dil #2", and "Dil #3"?
6. Make a plot on log graph paper of the expected DNA concentration in question five (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 expected DNA concentration



7. Add the DNA concentration reading you obtained from the spectrophotometer for each tube.
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 by sequencing analysis, cloning into an appropriate vector for gene expression or promoter analysis.

Reagents and Apparatus Needed:

- 10x Ex-Taq buffer (Takara Mirus Bio) came with the Ex-Taq DNA polymerase
- dNTP mix (Takara Mirus Bio) came with the Ex-Taq DNA polymerase
- Ex-Taq DNA polymerase (Takara Mirus Bio)
- Sterile water
- 12 μM Gene-specific Forward primer
- 12 μM Gene-specific Reverse primer
- 0.2 ng/μL *Arabidopsis* Columbia-0 genomic DNA
- 1.5 mL sterile microcentrifuge tubes
- 0.2 mL sterile microcentrifuge (or PCR) tubes
- P-10, P-20, P-200 pipetman
- Filtered Pipet tips (0.1-10 μL, 1-20 μL and 1-200 μL) for PCR
- Rack for 1.5 mL microcentrifuge tubes
- PCR Rack for 0.2 mL microcentrifuge tubes
- Gloves
- Black Ultra-fine sharpie pen
- Ice bucket or plastic container
- Microcentrifuge
- PCR machine (MyCycler, BioRad)

PROCEDURE

Note: ALWAYS wear gloves and use PCR filter tips when performing PCR preparation to prevent contamination.

1. Get ice from an icemaker in room 2911 or 3906.
2. Thaw out tubes of **10x Ex-Taq buffer** and **dNTP mix** on a microcentrifuge rack for 1.5 mL microcentrifuge tubes at **room temperature** for 5-10 minutes. Once the solutions are thawed out, put the tubes on ice until needed.
3. Thaw out **Forward** and **Reverse primer solutions** corresponding to a gene to be knocked out at room temperature as in step 2.
4. Obtain **THREE** 0.2-mL sterile PCR tubes and set them on a PCR rack for 0.2 mL microcentrifuge tubes.
5. Write on the **lids** of the tubes the number **1**, **2**, **3** and on the **side** of the tubes **Name of the gene**, **your Initial** and **Date** as follows: (*your TA will show you how to write on the tubes*)

   Tube #1: **Name of the gene** (same name as primer's gene)

   Tube #2: **Pos.** (Positive control serving as the positive control for PCR amplification)

   Tube #3: **Neg.**(Negative control containing same components as in tube #1, but **NO** genomic DNA)
6. Obtain **ONE** 1.5 mL microcentrifuge tube and set it on a rack for 1.5 mL microcentrifuge tubes.
7. Write on the lid of the tube with black ultra-fine sharpie as "**Mmix**" (for Master mix). Keep the tube on ice.

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

Master Mix:

	Mmix for ONE reaction	**Mmix (for 4 rxns)**
Sterile water	36.6 µL	146.4 µL
10x Ex-Taq buffer	5.0 µL	20.0 µL
dNTP mix	4.0 µL	16.0 µL
12 µM Gene-specific **Forward** primer	1.0 µL	4.0 µL
12 µM Gene-specific **Reverse** primer	1.0 µL	4.0 µL
Ex-Taq DNA polymerase (**5 Units/µL**)	0.4 µL	1.6 µL
Total volume	**48.0 µL**	**192.0 µL**

a. Pipet into the **Mmix** tube the reagents with order from top down (example: water, 10x Ex-Taq buffer, dNTP mix, etc.)

b. After pipetting all reagents into the **Mmix** tube, close the lid of the tube. Mix the contents by vortexing on the **vortex mixer** at **setting of 3-4** for **5 seconds**. Spin the tube in a microcentrifuge at FULL speed (13,200 rpm) for 10 seconds. Put the tube back **on ice**.

9. Prepare PCR-reaction tubes by
    a. pipetting **48 μL** of the **Mmix** solution into PCR tubes (see table below)
    b. pipetting **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**

PCR reactions:

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

10. Turn **ON** the PCR machine (MyCycler) by pressing and holding the "Standby" button for **1-2 seconds**. Wait for one minute for the machine to initializing.
11. Put the PCR tubes in the wells of the 96-well hot plate of the MyCycler.
12. Select the "**Protocol Library**" by pressing "**F1**" button.
13. Select "**Knockout**" protocol by pressing **yellow arrowheads** surrounding the "**ENTER**" button. Once it is selected, the "**Knockout**" protocol is highlighted. Press the "**ENTER**" button.

    The **PCR profile** of the Knockout as following:

    94°C, 3 min;

    36 cycles of 94°C, 15 sec → 60°C, 30 sec → 72°C, 2 min;

    72°C, 4 min;

    4°C, ∝.

14. Under the "**Choose Operation**" window, "**Run Protocol**" is highlighted. Press the "**ENTER**" button to run the protocol.

15. Press "**F5**" button to "**Begin Run**" the protocol. *Note: it would take about 3 hours for the PCR amplification to be completed under the above specified PCR profile.*
16. Once the PCR amplification is completed, remove PCR tubes from the PCR machine and store them in the **refrigerator** until gel electrophoresis or leave them in the PCR machine until you have a chance to put them away later.

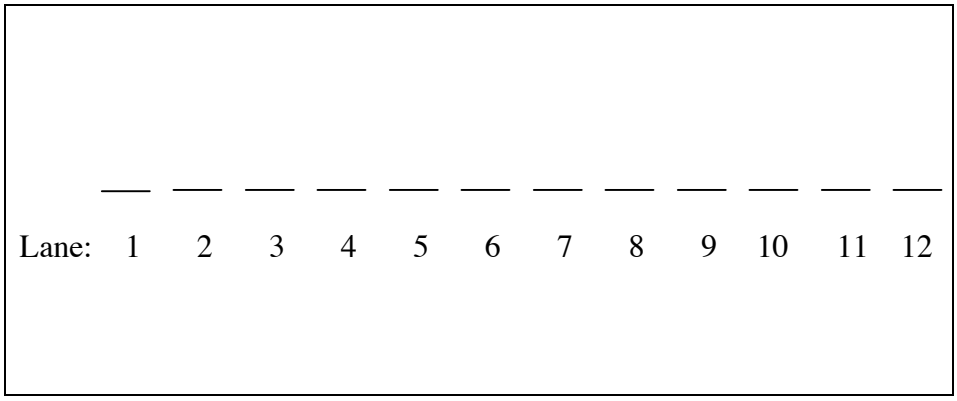
GEL ELECTROPHORESIS ANALYSIS OF PCR PRODUCT

1. Write the number (**1, 2, 3**) on the lids of **THREE 1.5-mL microcentrifuge tubes**.
2. Arrange THREE PCR tubes corresponding to THREE 1.5-mL microcentrifuge tubes.
3. Pipet into each of THREE 1.5-mL microcentrifuge tubes following:

	Tube #1	**Tube #2**	**Tube #3**
6x Loading dye	2 μL	2 μL	2 μL
PCR Solution #1	10 μL	0 μL	0 μL
PCR Solution #2	0 μL	10 μL	0 μL
PCR Solution #3	0 μL	0 μL	10 μL

Mix the contents by pipetting up and down for at least 5 times.

4. Load the samples along with diluted 1-kb DNA ladder on the 1% agarose gel.
5. Record the loading pattern of samples.



6. 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? _____ minutes or _____ hour(s)

7. Take a picture of the gel using the Gel Document system.
8. Analyze the size of the PCR product on the picture. *If the expected size is observed, proceed to purification of PCR product.*

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

IV. PURIFICATION OF PCR PRODUCTS

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

Reference: QIAquick Gel Extraction protocol (Qiagen)

Solutions Needed:

- QIAquick PCR Purification Kit (Qiagen, Cat. # 28104)
- PCR solutions

Materials Needed:

- 1.5 mL microcentrifuge tubes
- Microcentrifuge
- Nanodrop spectrophotometer

PROCEDURE

1. Write on the **lid** and **side** of a 1.5-mL microcentrifuge tube **your initial**.
2. Pipet **15 µL** of the **PCR product solution** from the PCR tube containing the gene-specific DNA fragment into the 1.5-mL microcentrifuge tube.
3. Add **75 µL** of **Buffer PB** (or **5 volumes** of **Buffer PB** to **1 volume** of the PCR sample) to the tube in step 2. Mix by vortexing the tube for 5 seconds. Spin the tube in the microcentrifuge at **FULL speed** for **10 seconds** to bring all the solution down to the bottom of the tube. Set the tube back on the microcentrifuge rack.
4. Place a **QIAquick spin column** in a provided **2-mL collection tube**.
5. Apply the sample mixture in step 3 to the QIAquick column. Spin the column set in the microcentrifuge at **FULL speed** for **1 minute**. *This step allows the binding of DNA to the membrane*
6. Discard the **flow-through solution** in the collection tube. Put the QIAquick column back into the same collection tube.
7. Add **750 µL** of **Buffer PE** to the QIAquick spin column and spin at **FULL speed** for **1 minute**.
8. Discard the flow-through solution in the collection tube. Put the QIAquick column back into the same collection tube.
9. Spin the column set at FULL speed for an **additional 1 minute** to get rid of residual ethanol in Buffer PE. *Caution: Residual ethanol from Buffer PE will NOT be completely removed unless the flow-through solution is discarded before this additional spin.*
10. While spinning, label on the **lid** and **side** of a **1.5-mL microcentrifuge** tube "**Purified PCR product**", **your initial**, and **date**.
11. Transfer the appropriate **QIAquick columns** in the NEWLY labeled microcentrifuge tubes. Discard the flow-through solutions and the collection tubes.
12. Pipet **30 µL** of **Buffer EB** to the center of the QIAquick membrane. Let the column sit for **1 minute**, and then centrifuge at **FULL speed** for **1 minute**. *This step elutes the DNA from the QIAquick membrane.*
13. Determine DNA concentration using the Nanodrop spectrophotometer (measuring nucleic acids) or a DNA Fluorometer (measuring only DNA).

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

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

V. SEQUENCING REACTION WITH BIG DYE V. 3

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

.

Reference: Perkin Elmer/Applied Biosystems

Solutions Needed:

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

Materials Needed:

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

Overview:

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

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

General Components of One Reaction:

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

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

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

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

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

  Table: Amount of DNA Used in Sequencing Reactions Depending

on **Size of PCR Fragment**

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

For this exercise, there is **ONE DNA template**, i.e. the purified PCR product of the gene of interest; but, there are **TWO primers**, gene-specific forward and gene-specific reverse primers. Therefore, it is best to prepare a **master mix** with **all components**, **except** the **primers**, which will be added to individual reaction tubes.

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

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

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

Sample calculations:

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

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

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

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

PROCEDURE

1. Get ice from the icemaker in room 2911 or 3906.
2. Label on the **side** of **TWO 0.2-mL PCR tubes** with **your initial** and **primer name**. Set the tube on a PCR rack sitting on ice.
3. Label on the **lid** and **side** of a **1.5-mL microcentrifuge tube** as "**Mmix**" and **your initial**. 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 information on the previous page to fill in the volume of DNA solution to be added and calculate the volume of water to be added to the Mmix tube for 3 reactions.*

Master Mixes (Mmix) of Sequencing Reactions:

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

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

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

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

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

   <u>or</u> **BioRad MyCycler** with a **Big Dye** protocol with the same profile as above.
6. After the cycling reaction is finished, clean up sequencing reactions using Edge Biosystems spin columns (stored in the cold room) as following:
   a. Spin the pre-packed columns in a microcentrifuge at 3,000 rpm for 2 minutes at room temperature.
   b. Meanwhile, label a new set of 1.5 mL microcentrifuge tubes according to your reactions.
   c. Transfer the columns to new tubes.
   d. Pipet 20 μL of sequencing reaction to appropriate columns.
   e. Spin the columns as in step a.
   f. Discard the columns.

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

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

VI. RETRIEVING AND ANALYZING DNA SEQUENCES

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

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

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

   You would see the following files:

   5677 GOLDR_At5g09250Fw_080.ab1

   5677 GOLDR_At5g09250Rv_081.ab1

   What are the annotations?

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

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