A Gene Discovery Lab Manual For Undergraduates:

Searching For Genes Required To Make A Seed

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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 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,

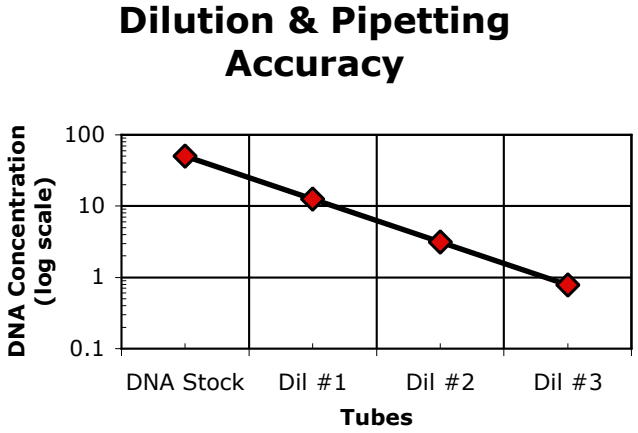
   $\mathbf{V_i}$ = **initial volume** (the volume of original DNA solution is **5 µL**)

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