LABORATORY 1

Measurements, Micropipetting, and Sterile Techniques

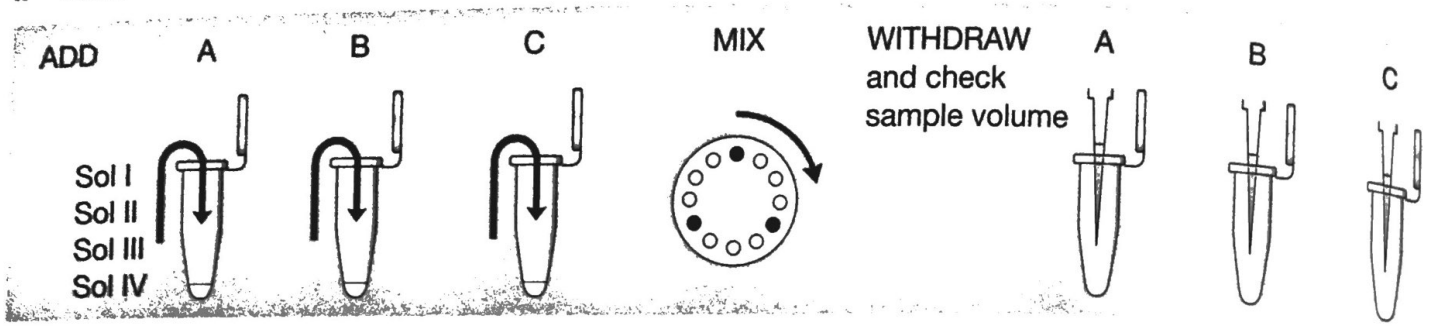
LABORATORY 1 INTRODUCES MICROPIPETTING AND STERILE PIPETTING techniques used throughout this course. Mastery of these techniques is important for good results in all of the experiments that follow. Most of the laboratories are based on *microchemical* protocols that use very small volumes of DNA and reagents. These require use of an adjustable micropipettor (or microcapillary pipette) that measures as little as one microliter (μl)—a millionth of a liter.

Many experiments require growing *Escherichia coli* in a culture medium that provides an ideal environment for other microorganisms as well. Therefore, it is important to maintain sterile conditions to minimize the chance of contaminating an experiment with foreign bacteria or fungi. *Sterile conditions* must be maintained whenever living bacterial cells are to be used in further cultures. Use sterilized materials for everything that comes in contact with a bacterial culture: nutrient media, solutions, pipettes, micropipettor tips, inoculating and spreading loops, flasks, culture tubes, and plates.

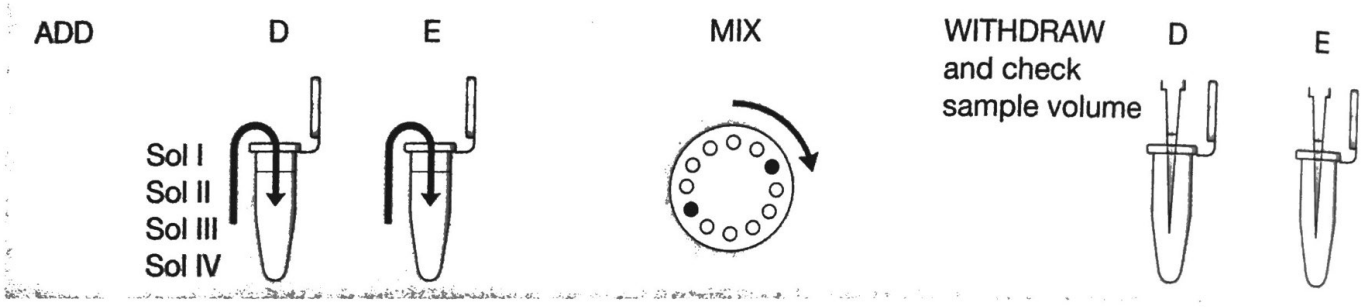
Remember this rule of thumb: Use sterile technique if live bacteria are needed at the end of a manipulation (general culturing and transformations). Sterile technique is not necessary when the bacteria are destroyed by the manipulations in the experiment or when working with solutions for DNA analysis (plasmid isolation, DNA restriction, and DNA ligation).

Equipment and materials for this laboratory are available from the Carolina Biological Supply Company (see Appendix 1).

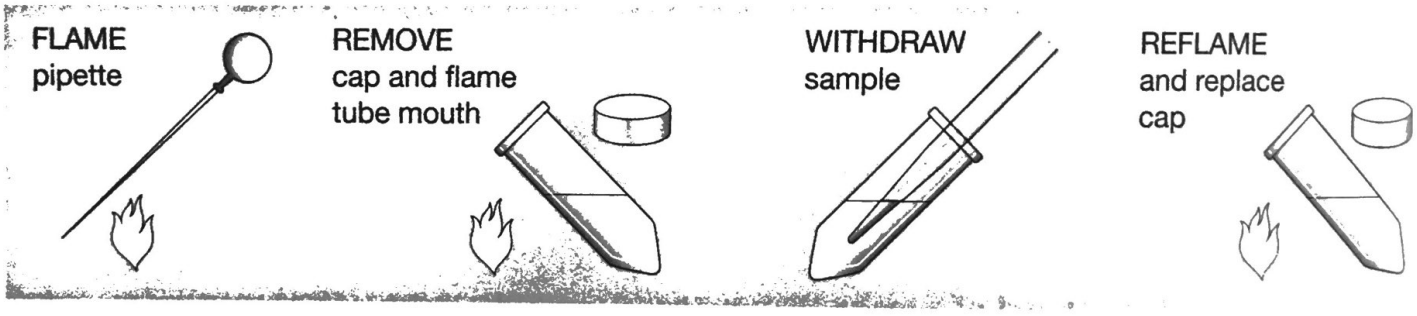
I. Small-volume Micropipettor Exercise

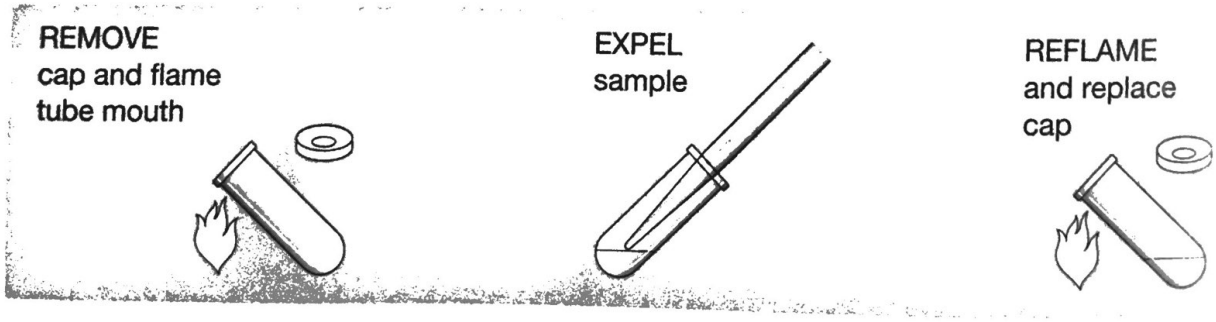


II. Large-volume Micropipettor Exercise



III. Sterile Use of 10-ml Standard Pipette





PRELAB NOTES

Digital Micropipettors

The volume range of digital micropipettors varies from manufacturer to manufacturer. Select both a small-volume micropipettor with a range of 0.5–10 µl or 1–20 µl and a large-volume micropipettor with a range of 100–1000 µl.

Microcapillary Pipettes

Microcapillary pipettes are an inexpensive alternative to adjustable micropipettors. These disposable glass capillary tubes come in sizes that cover the range of volumes used in this course. Several types of inexpensive micropipette aids are available. A thumbscrew micrometer may be easier to use than a pipettor bulb. A no-cost and easily controllable pipette bulb can be made by tying a knot in a length of latex tubing, which is usually provided with the capillary pipettes.

Under conditions of high static electricity, capillary pipetting can be very difficult and, at times, impossible. The reagent droplet adheres stubbornly to the side of the pipette and cannot be transferred to the side of a polypropylene reaction tube. Even under the best of circumstances, microcapillary pipettes are more difficult to master. Allow students sufficient time to become competent with them before attempting any experiments.

Transfer Pipettes

Small polypropylene transfer pipettes are handy because they have an integrated bulb. The smallest size, which holds a *total* volume of approximately 1 ml, has a thin tip that can be used to measure microliter amounts. Before use, calibrate the transfer pipette using a digital micropipettor or microcapillary pipette. Pressing on the pipette barrel, rather than the bulb, creates less air displacement and makes measuring small volumes easier.

10-ml Pipettes

Presterilized, disposable 10-ml plastic pipettes are most convenient and are supplied in bulk pack or individually wrapped. Bulk-packed pipettes should be opened immediately before use. To dispense, cut one corner of the plastic wrapper at the end opposite the pipette tips. Avoid touching and contaminating the wrapper opening; tap bag to push the pipette end through the cut opening. Reclose with tape to keep sterile for future use. To use individually wrapped pipettes properly, peel back only enough of the wrapper to expose the wide end of the pipette and affix the end into the pipette aid or bulb. Completely peel back the wrapper immediately before use.

To Flame or Not to Flame?

There is general disagreement about whether it is necessary to flame pipettes and mouths of tubes as part of the sterile technique. Flaming warms the air at

the mouth of the container, creating an outward convection current that prevents microorganisms from falling in. Even so, the effect of flaming may be primarily psychological when fresh sterile supplies are used and manipulations are done quickly. Especially when using individually wrapped supplies, flaming can be omitted without compromise to sterility. When flaming plasticware, do so briefly to avoid melting the plastic.

Microfuge

Although not essential, a microfuge is very useful for pooling and mixing droplets of pipetted solutions in the bottom of a 1.5-ml reaction tube.

PRELAB PREPARATION

1. To simplify initial practice with a micropipettor, use colored solutions that are easily visible. Prepare five colored solutions using food coloring or other dyes mixed with water.
2. Prepare for each experiment:
   - Four 1.5-ml tubes, each containing 1 ml of a different colored solution, marked I, II, III, and IV.
   - One 50-ml conical tube containing 25 ml of colored solution, marked V.

MATERIALS

REAGENTS

Solution I (1 ml), colored
Solution II (1 ml), colored
Solution III (1 ml), colored
Solution IV (1 ml), colored
Solution V (25 ml), colored

SUPPLIES AND EQUIPMENT

Beaker for waste/used tips
Bunsen burner (optional)
Conical tube (50-ml)
Culture tube (15-ml)
Microfuge (optional)
Micropipettor (0.5–) + tips
Micropipettor (100–) + tips
Permanent marker
Pipette (10-ml)
Pipette aid or bulb
Test tube rack
Tubes (1.5-ml)

METHODS

Metric Conversions

Become familiar with metric units of measurement and their conversions. We concentrate on liquid measurements based on the liter, but the same prefixes also apply to dry measurements based on the gram. The two most useful units of liquid measurement in molecular biology are the milliliter (ml) and microliter (µl).

1 ml	=	0.001 liter	1,000 ml	=	1 liter
1 µl	=	0.000001 liter	1,000,000 µl	=	1 liter

Complete the following conversions:

1 µl	=	_____ ml	_____ µl	=	1 ml
10 µl	=	_____ ml			
100 µl	=	_____ ml			

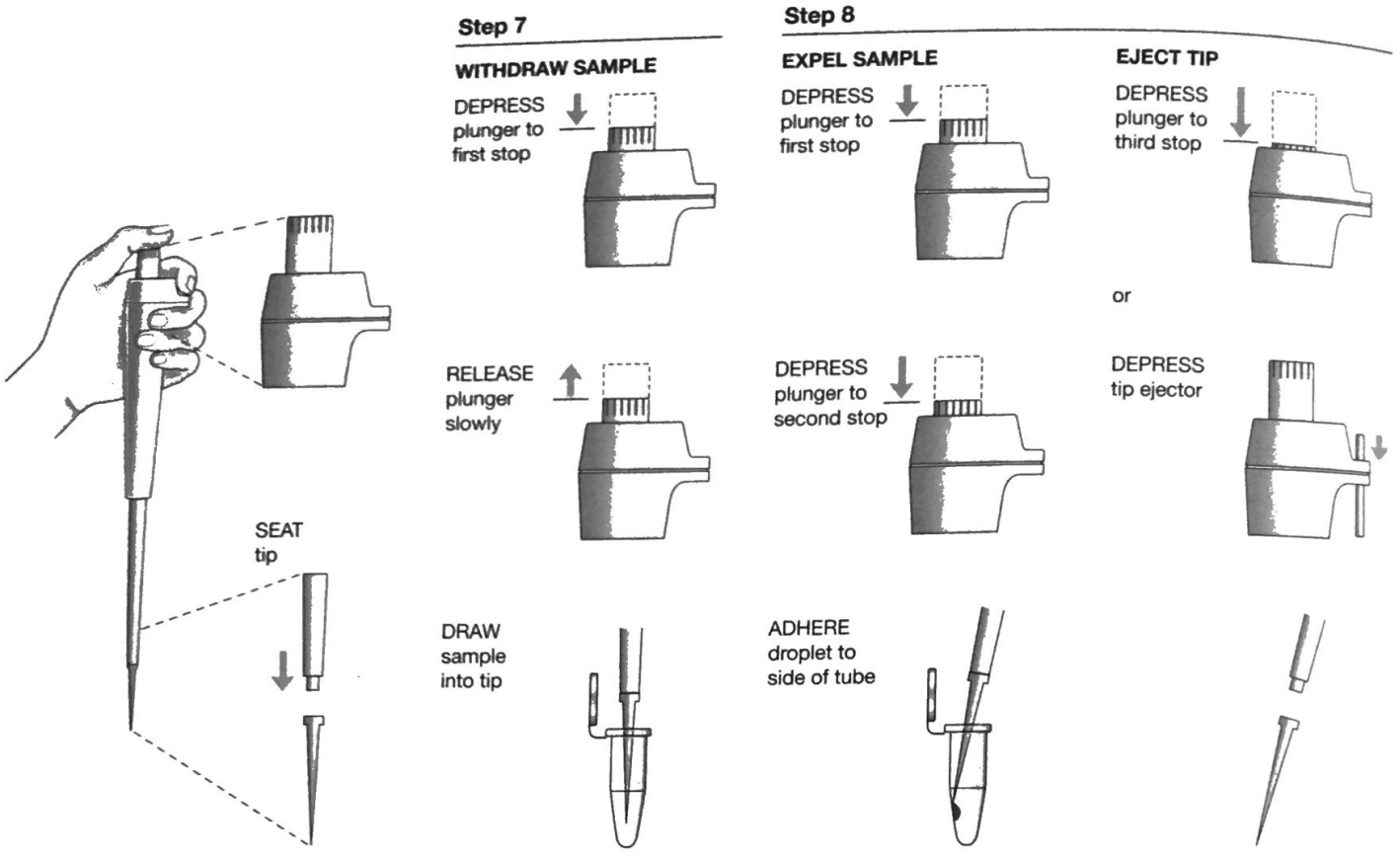
Use of Digital Micropipettors (10 minutes)

"Nevers"

- Never rotate volume adjustor beyond the upper or lower range of the micropipettor, as stated by the manufacturer.
- Never use a micropipettor without the tip in place; this could ruin the precision piston that measures the volume of fluid.
- Never lay down a micropipettor with a filled tip; fluid could run back into the piston.
- Never let the plunger snap back after withdrawing or ejecting fluid; this could damage the piston.
- Never immerse the barrel of the micropipettor in fluid.
- Never flame the micropipettor tip.

Micropipetting Directions

1. Rotate the volume adjustor to the desired setting. Note the change in the plunger length as the volume is changed. Be sure to locate the decimal point properly when reading volume setting.
2. *Firmly* seat proper-sized tip on the end of the micropipettor.
3. When withdrawing or expelling fluid, always hold the tube firmly between thumb and forefinger. Hold the tube at nearly eye level to observe the change in the fluid level in the pipette tip. Do not pipette with the tube in the test tube rack or have another person hold the tube while pipetting.
4. Each tube must be held in the hand during each manipulation. Grasping the tube body, rather than the lid, provides more control and avoids contamination from the hands.
5. Hold micropipettor almost vertical when filling.



Use of Digital Micropipettor (Steps 7 and 8)

6. Most digital micropipettors have a two-position plunger with friction "stops." Depressing to the first stop measures the desired volume. Depressing to the second stop introduces an additional volume of air to blow out any solution remaining in the tip. Pay attention to these friction stops, which can be felt with the thumb.
7. To withdraw sample from reagent tube:
   a. Depress the plunger *to the first stop* and hold in this position. Dip the tip into the solution to be pipetted, and draw the fluid into the tip by *gradually* releasing the plunger.
   b. Slide the tip out along the inside wall of the reagent tube to dislodge excess droplets adhering to the outside of the tip.
   c. Check that there is no air space at the very end of the tip. To avoi ure pipetting errors, learn to recognize the approximate level that p lar volumes reach in the tip.
8. To expel sample into reaction tube:
   a. Touch the tip to the inside wall of the reaction tube into which ple will be emptied. This creates a capillary effect that helps draw of the tip.
   b. *Slowly* depress the plunger to the first stop to expel sample. Depr second stop to blow out the last bit of fluid. Hold plunger in d position.

c. Slide the pipette out of the reagent tube with the plunger depressed to avoid sucking any liquid back into the tip.

d. Manually remove or eject the tip into a beaker kept on the lab bench for this purpose. The tip is ejected by depressing the measurement plunger beyond the second stop or by depressing a separate tip-ejection button, depending on the particular micropipettor being used.

9. To prevent cross-contamination of reagents:

   a. Always add appropriate amounts of a single reagent sequentially to all reaction tubes.

   b. Release each reagent drop onto a new location on the inside wall, near the bottom of the reaction tube. In this way, the same tip can be used to pipette the reagent into each reaction tube.

   c. Use a *fresh tip* for each new reagent to be pipetted.

   d. If the tip touches one of the other reagents in the tube, switch to a new tip.

10. Eject used tips into a beaker kept on the lab bench for this purpose.

I. Small-volume Micropipettor Exercise (15 minutes)

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 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.

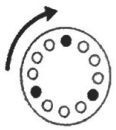
Tube	Sol. I	Sol. II	Sol. III	Sol. IV
A	4 µl	5 µl	1 µl	–
B	4 µl	5 µl	–	1 µl
C	4 µl	4 µl	1 µl	1 µl

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 on 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 tops. 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.



An empty 1.5-ml tube can be used to balance a sample with a volume of 20 µl or less.

8. A total of 10 µl of reagents was added to each reaction tube. To check that the measurements were accurate, set the pipette to 10 µl and very carefully withdraw solution from each tube.
   a. Is the tip just filled?

      or

   b. Is a small volume of fluid left in tube?

      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.)
9. If several measurements were inaccurate, repeat the exercise to obtain a near-perfect result.

II. Large-volume Micropipettor Exercise (10 minutes)

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 reaction tubes D and E.

2. Use the matrix below as a checklist while adding solutions to each reaction tube.

Tube	Sol. I	Sol. II	Sol. III	Sol. IV
D	100 µl	200 µl	150 µl	550 µl
E	150 µl	250 µl	350 µl	250 µl

3. Set the micropipettor to add appropriate volumes of Solutions I–IV to reaction tubes D and E. Follow the same procedure as for the small-volume micropipettor.

4. A total of 1000 µl of reactants was added to each tube. To check that the measurements were accurate, set the micropipettor to 1000 µl and carefully withdraw solution from each tube.
   a. Is the tip just filled?

      or

   b. Is a small volume of fluid left in tube?

      or

   c. After extracting all fluid, is an air space left in the tip end? (The a
      displaced and actual volume determined simply by rotating volu
      ment to push fluid to very end of tip. Then, read the volume dir
5. If the measurements were inaccurate, repeat the exercise to obtai
   perfect result.

III. Sterile Use of 10-ml Standard Pipette (10 minutes)

The following directions include flaming the pipette and tube mouth. It is probably best to learn to flame, then omit flaming when safety or situation dictates. The directions also assume one-person pipetting, which is rather difficult. The process is much easier when working as a team: One person handles the pipette, while the other removes and replaces the caps of the tubes.

The key to successful sterile technique is to work quickly and efficiently. Before beginning, clear off the lab bench and arrange tubes, pipettes, and culture medium within easy reach. Locate Bunsen burner in a central position on the lab bench to avoid reaching over the flame.

Loosen caps so that they are ready for easy removal. Remember, the longer the top is off the tube, the greater the chance of microbe contamination. Do not place a sterile cap on a nonsterile lab bench.

> CAUTION
> Always use a pipette aid or bulb to draw solutions up the pipette. Never pipette solutions using mouth suction: This method is not sterile and can be dangerous.

Nonsterile pipettes may be used for this practice exercise.

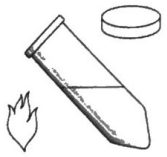
1. Light Bunsen burner.
2. Set pipette aid to 5 ml or depress pipette bulb if using a pipette.

This expels contaminated air and prepares vacuum to withdraw fluid.

3. Select a sterile 10-ml pipette and insert into pipette aid or bulb. *Remember to handle only the large end of the pipette; avoid touching the lower two thirds.*

When using an individually wrapped pipette, be careful to open wrapper end opposite the pipette tip. Unwrap only enough of the pipette to attach end into pipette aid or bulb.

4. Quickly pass the lower two thirds of the pipette cylinder through the Bunsen flame several times. *Be sure to flame any portion of the pipette that will enter the sterile container*. Pipette should become warm, but not hot enough to melt the plastic pipette or to cause the glass pipette to crack when immersed in solution to be pipetted.



5. Hold a 50-ml conical tube containing Solution V in free hand and remove cap using little finger of hand holding pipette aid or bulb. *Do not place cap on lab bench.*
6. Quickly pass mouth of conical tube through the Bunsen flame several times, being careful not to melt the plastic.
7. Withdraw 5 ml of Solution V from the conical tube.
8. Reflame the mouth of the tube and replace top.

9. Remove the top of a sterile 15-ml culture tube with little finger of hand holding the pipette. Quickly flame the mouth of the tube.
10. Expel fluid into the culture tube. Reflame the mouth of the tube and replace top.

RESULTS AND DISCUSSION

Inaccurate pipetting and improper sterile technique are the chief contributors to poor laboratory results. If the handling of micropipettors and/or the use of sterile technique are still uncomfortable or difficult, take time now for additional practice. These techiques will soon become second nature.