Section 1 Basic Techniques Procedures

Part I: Laboratory Etiquette and Rules

A laboratory can be a very productive and enjoyable place to work depending on how smoothly everything works. Since many people have to accomplish tasks using a common area, equipement and reagents a few simple common sense rules apply. Any question on laboratory policy can be addressed to Dr. Walker.

- **If you do not know how to operate equipment** please ask how before something several times your annual income is broken.

- **Clean up after your self.** Glass ware such as beakers, flasks can be washed in dish washer. Glass ware should be rinsed with distilled water.

- **Put glassware and equipment away** in their proper places when finished with them.

- **Common chemicals used by every one** should be replentished by the person who uses the last amount. { TBS, TBS-T, SDS electrode buffer, gels } It is not fair for one person (especially me) to make up these solutions for everyones use.

- **Work safely**. If you are using a hazardous material or piece of equipment please inform others of the danger.

- **Know the proper handling of infectous and hazardous materials**. A hazardous materials manual is found in the laboratory. If you don't know the proper procedures ask Dr. Walker.

- **Dispose of infectous waste** in orange bags with Bio-Hazards symbols. This waste is taken down to Mr. Ed Budde for ultimate disposal.

- **Dispose of glass waste** in broken glass recepticles. This includes Pasteur pipets and graduated disposable pipets. This is taken downstairs (Floor 1) and disposed of in dumpster out side Chemical Mangment loading dock.

- **Interpersonal problems in the lab**: If you have a problem with another person in the laboratory bring it to my attention and something will be worked out to resolve the problem.

- **Receiving orders** should be documented by initialling and dating the order sheet in the top bin of the organizer at the computer desk. If all the items have been received transfer sheet to reconciled bin (middle bin).

- **Items in freezers and refrigerators** are kept track of by recording their location in a log book that is kept on the Lab Mac them.

Part II: Pipetting and Aseptic Techniques

In this class, you will be exposed to two of the leading micropipettors -- the "Pipetmen" and the "Oxfords". Generally, these instruments are referred to as "pipetman," a name which is based on the Rainin-Gilson instrument. The Pipetman are the navy-blue-handled instruments while the Oxfords have a grayish handle. The two function very similarly, but have a few features unique unto themselves. If at anytime you are unsure as to the operation of one instrument or the other, do not hesitate to ask. They are relatively expensive instruments. If you use them properly, they will not fail you. If you destroy them, then your experiments may also fail. Before proceeding, read the information in Appendix A and sections A and B below. Included in Appendix A is a copy of the instruction manual for the Rainin-Gilson instrument. Please read this in detail. It contains valuable data on the operation of the pipetters which many people do not know! As stated in class, under no circumstances should a student disassemble a pipetman!

You really do not need to take data notes on this section. It may be advisable, however, to write down any relevant notes that you may need concerning the operation of these instruments so that you have this for future reference.

A. Don'ts

- Never rotate volume adjuster beyond the upper or lower range of the pipette, as stated by the manufacturer. Even a few microliters in either direction will destroy the micrometer inside of the instrument.

- Never use micropipettor without tip in place; this could ruin the precision piston that measures the volume of fluid. It may also lead to a subsequent contamination of other solutions or reactions.

- Never lay down pipettor with filled tip; fluid could run back into piston. Same consequences as above.

- Never let plunger snap back after withdrawing or ejecting fluid; this could damage piston. If it doesn't damage the piston, it will surely lead to a very inaccurate measurement. It also causes an excessive generation of aerosol which may contaminate your next solution or reaction.

- Never immerse barrel of pipettor in fluid. Never allow tip to descend more than 3 - 5 mm below the surface of the liquid. Remember Pascal's Principle?

- Never flame micropipetor tip. This will melt the tip and allow inaccuracy to enter the measurement, even if it doesn't appear to melt.

B. Pipetting Directions

1. Choose correct instrument. Rotate volume adjuster to desired setting. Note change in plunger length as volume is changed. Be sure to locate decimal point properly when reading volume setting. The black digits are microliter (µl) quantities; the red digits are not.

2. *Firmly* seat proper sized tip on the end of the micropipettor. The color-coded plunger can aid in this task.

3. When withdrawing or expelling fluid, always hold tube firmly between thumb and forefinger. Hold tube at nearly eye level to observe the change in the fluid level in pipette tip. Do not pipette with tube in test tube rack or have another person hold tube while pipetting. This is especially important when pipetting very small amounts since you will need to see that the liquid does in fact enter the tube.

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. Unless absolutely necessary, never grasp the tube near the rim. This almost always leads to contamination. This may be difficult to get used to when using the smallest microfuge tube (0.5 ml tube). Practice makes perfect!!

5. Hold pipettor almost vertical when filling.

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 plunger to *first stop* and hold in this position. Dip tip into solution to be pipetted, and draw fluid into tip by *gradually* releasing plunger. Never enter the fluid by more than 5 mm.

   b. Slide pipette tip out along inside wall of reagent tube to dislodge excess droplets adhering to the outside of tip. Do not let capillary action remove the contents of the tip, but at the same time, don't bring an extra 100 µl with you on the outside of the tip.

   c. Check that there is no air space at the very end of the tip. To avoid future pipetting errors, learn to recognize the approximate level that particular volumes fill the pipette tip.

8. To expel sample into reaction tube:

   a. Touch pipette tip to inside wall of reaction tube into which the sample will be emptied. This creates a capillary effect that helps draw fluid out of tip. When pipetting very small volumes, always try to go as deep as possible into the microfuge tube.

   b. *Slowly* depress plunger to the first stop to expel sample. Depress to second stop to blow out last bit of fluid. Hold plunger in depressed position.

   c. Slide pipette out of reaction tube with plunger depressed to avoid sucking any liquid back into tip. Do not bring the liquid back up the side of the tube.

   d. Eject tip into the glass disposal boxes kept on the lab bench for this purpose. You may move the box to a convenient location for all members of the group. The tip is ejected by depressing a separate tip-ejection button.

9. To prevent cross-contamination of reagents:

a. Always add appropriate amounts of single reagents sequentially to all reaction tubes. You may use one tip to add one reagent to a series of tubes, provided that you don't contaminate that tip by waving it in the air or touching your clothing . . .

b. Use *fresh tip* for each new reagent to be pipetted. In time you will be able to use one tip per reagent. For now, don't worry about the number of tips used. Worry about sterility!

c. If tip becomes contaminated, switch to a new one. If you are not sure, switch to a new one.

10. Eject used tips into the glass disposal box kept on the lab bench for this purpose.

C. Sterile Use of 10-ml Standard Pipette

The following directions include flaming the pipette and tube mouth. It is probably best to learn to flame and then omit flaming when safety or situation dictates. The process is much easier when working as a team: one person handles the pipette, while the other removes and replaces caps of tubes. You will want to practice first as a team, then individually. During the practical you will work alone.

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

Loosen caps so that they are ready for easy removal. Remember, the longer the cap is off the tube, the greater the chance of microbe contamination. Do not place sterile caps on a nonsterile bench. A cap that is unscrewed, but still in place does not compromise the sterility of the solution. As you grow more proficient with these techniques, you will not need two people.

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

1. Light Bunsen burner. There is no need to flame sterile plastic disposable! This will most assuredly lead to melting of the pipette or tube.

2. Void the bulb of the pipette aid or choose a slider.

3. Select sterile 10 or 5ml pipette and attach pipette aid or bulb. *Remember to handle only large end of pipette to avoid touching lower two thirds.* You should practice with the canned, glass pipettes and the plastic disposables.

4. Remove the remaining wrapper if using a disposable pipette, and quickly pass lower two thirds of pipette cylinder through Bunsen flame several times. *Be sure to flame any portion of pipette that will enter sterile container.* Pipette should become warm, but not hot enough to cause glass pipette to crack when immersed in solution to be pipetted.

5. Hold 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. Withdraw >5 or >10 ml of Solution V from conical tube. Replace cap. Into a waste container, expel the extra liquid until the top of the meniscus rests at the 10 or 5ml marking on the pipette.

7. Remove top of sterile 15-ml culture tube or glass test tube with little finger of hand holding pipette. Quickly flame mouth of tube.

8. Expel fluid into culture tube. Re-flame mouth of tube and replace top.

9. Once you have practiced with solution V, it is time for the real test. Can you transfer 5 ml of LB Broth to a sterile test tube and incubate it overnight without any bacterial growth? LB Broth is a media used for growing up bacterial cells. To try, each member should follow the above procedure, but instead of 5 ml of solution V, substitute 5 ml of LB Broth. Sterilely transfer 5 ml of LB Broth to a sterile, glass, green-capped tube. Replace the green cap. (Avoid flaming the glass too long or it will melt the plastic green cap.) Label the tube with your name and the date. (When labeling materials for use in a situation where they may be confused with another group's materials, one should use tape to label. Marker sticks better to tape then it does to glass or plastic.) Incubate them overnight at 37^{o}C with shaking at 350 rpm to affect bacterial growth. The incubator is in the centrifuge room on the left side of the back wall. If the broth remains clear, then your technique is excellent! You should check the cultures the next day. I will refrigerate any cultures that have not been removed after 24 hours of shaking.

Inaccurate pipetting and improper sterile techniques are the chief contributors to poor laboratory results. If you are still uncomfortable with micropipettors and/or sterile technique, take time now for additional practice. These techniques will soon become second nature to you. Each member of the group should perform these exercises. When you feel confident at these techniques go to your instructor and ask to be graded on Lab Practical I.

Part III: pH and Buffers

The pH meter is a device used to measure the electrical potential developed in a Galvanic cell which employs the glass electrode as one of its half-cells. The glass electrode is responsive to the hydronium ion activity of the test solution according to the standard Nernst relation. We will use a pH meter in this experiment to study the effect of several parameters on the practical pH of aqueous solutions. pH meters require a warm-up period of 20-30 minutes and therefore are usually left on and kept in the "standby" position (ring symbol). The glass electrode is fragile, even though protected by a plastic shield. Handle it with care. Before making a pH measurement, rinse the electrode probe thoroughly with distilled water and dry them gently with a Kim-wipe tissue. After the measurement, again rinse it. Then resuspend it in its storage buffer at pH 7. Dr. Walker will demonstrate any procedures that you are not familiar with. The model of pH meter we use in this laboratory is Corning 215.

A. Glass Electrode Care.

The type of pH electrode used in this laboratory requires saturated KCl in the electrode chamber. If this chamber is not filled, add some saturated Kcl through the 'fill hole' near top of electrode. Make readings with the 'fill hole' unstoppered, but stopper it for storage. Make sure the tip guard (orange plastic ring) is in place to protect electrode tip. When using the electrode with a stirring bar, be careful to have the electrode elevated well above the stir bar. Likewise, be cautious about bumping the electrode bulb on the bottom of the vessel containing a test solution.

B. pH Meter Calibration and Use.

1. Select pH mode.
2. Place electrode in pH 7.0 buffer standard (yellow) and adjust ˚C control to temperature of buffer. Set Cal 2 to 100%
3. When reading stabilizes set meter to 7.0 with **Cal 1** adjust.
4. Remove electrode, rinse with dH2O place in either pH 4 or pH 10 buffer standard and wait for reading to stabilize.
5. Set pH to second pH by adjusting the **Cal 2**.
6. you are now ready to use meter, (the above calibration procedure should be done if it has been more than two days since last calibration or if you suspect protper readings.
7. Remove electrode from buffer, rinse and gently dry with kim wipe. Place in solution to be analyzed.
8. Make sure the mode is set at pH setting. Read meter. If titration is required follow procedure below.

C. Buffer Titration.

1. If buffer is more acidic than desired, add a concentrated base solution (concentrated NaOH) drop wise to the buffer while stirring.
2. If buffer is more basic than desired, add a concentrated HCl (concentratedHCl is 12.1 M) drop wise to the buffer while stirring.
3. Titrate very slowly if it is the first time titrating a new buffer.

E. Buffer Preparation and the Henderson-Hasselbach Equation.

The H-H equation is given as: $pH = pK_a + \log([\text{conjugate base}]/[\text{acid}])$

1. Find the pK_a value for phosphate in the appendix to this section. Calculate the amounts of 0.1M base and 0.1M acid for phosphate buffer you would need to mix together to prepare a phosphate buffer at pH 7.3.

2. Prepare this buffer according to your HH-derived recipe, and measure the pH values to check accuracy of preparation .

Appendices

Appendix A
Selected References

Publications

Matsudaira, P.T. and Burgess, D.R. (1978) *Anal. Biochem.* 87:386-396 {mini SDS-PAGE gels}

Laemmli, U.K., (1970) *Nature* ˆ227:680 { SDS-PAGE gels}

Alberts, Bray,Lewis, Raff, Roberts, Watson, *Molecular Biology of the cell* , 3rd ed. (1994), Garland Publishing, Inc. {general reference}

Berger and Kimmel, Editors, *Guide to Molecular Cloning Techniques,* Methods in enzymology Vol. 152 (1987). {molecular biology}

Lowry *et al*, *J. Biol. Chem.* 177:751 (1951). {protein assay}

Ferguson, K.A. Meatbolism 13: 21 (1964) { molecular weight determination}

Folin and Ciocalteau, *J. Biol. Chem.* 73:627 (1927). {protein assay}

Harlow and Lane, *Antibodies: A Laboratory Manual* . Cold Spring Harbor Laboratories (1988) {Immunochemistry and Immunology Techniques}

WEB Sites

http://expasy.hcuge.ch/www/tools.html (protein analysis and molecular biology resources)
http://www.dl.ac.uk/SEQNET/databases.html (sequence data base)
http://www.tiac.net/users/pmgannon/ (Cell and Molecular Biology Online)
http://www.faseb.org/ascb/ (American Society for Cell Biology)

Appendix B
Use of the Pipetman/Oxfords

The accurate delivery of minute amounts of liquid is an indispensable part of modern biochemical lab technique. A wide variety of routine, analytical procedures involve the manipulation of volumes on the order of a microliter (abbreviated µl or λ and representing one thousandth of a milliliter). For example, the recipe for a restriction digestion will typically involve combining four or five separate components in a total volume of ten microliters.

Thus, the availability of a device that delivers microliter volumes quickly, accurately and reproducibly is critical to the practice of biochemistry. That device is called a Pipetman. ("Pipetman" is a registered trademark of Gilson Medical Electronics; a number of equivalent although less commonly used devices are available from other manufacturers.) The name "Pipetman" refers to a family of continuously adjustable digital air-displacement micropipets whose individual members are called the P-2, P-10, P-20, P-200, P-1000, and P-5000; the number in each name indicates the largest volume (in microliters) which can be handled by that device. All of these instruments use an air displacement mechanism to effect transfer of liquid. A digital micrometer is used to set the displacement distance or "stroke" for a stainless steel piston. After extension of the piston inside a sealed chamber within the Teflon shaft of the Pipetman, a disposable plastic "pipette tip" mounted on the end of the shaft is lowered into a solution. Retraction of the piston causes liquid to be drawn up into the tip as the pressure of the

gas inside the air-tight compartment consisting of the pipette tip and the shaft is equalized with the atmospheric pressure acting on the surface of the solution. Obviously, the displacement distance of the piston determines the volume of solution which will be drawn up.

Successful biochemical experimentation requires effective use of the Pipetman. Thus, the fate of your experiments in the lab course this semester will depend in part on your mastery of this device. There are two points to keep in mind: First, you must observe several simple guidelines in order to insure maximum accuracy in volume delivery (specific details will be described below); you must develop, in effect, a "feel" for the proper use of the instrument. Second, you need to develop a sensitivity to the fact that the Pipetman, like any measuring device, is subject to failure in a variety of ways. Failure can range from something obvious, such as fluid dribbling out of the pipette tip each time it is drawn up, to something much more subtle, such as a 20% breakdown in calibration.

Before proceeding, it is important to establish the difference in the meaning of the terms "accuracy" and "precision." Although these terms are often used interchangeably, they connote very different concepts in scientific or statistical usage. The term "accuracy" refers to the extent to which values from a particular measurement device or process correspond to an external, fixed standard. Thus, in this case, the accuracy of your Pipetman in delivering one microliter of water depends on how closely the volume actually delivered matches one cubic millimeter, one acceptable definition for the microliter. The term "precision," on the other hand, refers to the extent to which repeated measurements with a given device vary from one another. Thus, it would be possible for your Pipetman to be very inaccurate and yet very precise simultaneously if it delivered 2 µl of solution when the dial on the micrometer was set for 1 µl but delivered exactly 2.00±0.02 µl for each of many repeated deliveries. In conclusion, accuracy is a question of calibration, whereas precision is a question of reproducibility.

All of these instruments must be fitted with either a yellow or a blue plastic disposable tip. Which tip fits which pipetman should be self-evident. **Never dial a pipetman below 0 or above the number on top of the plunger. That is a P-1000 should never be dialed above 100**! The black numbers on the digital micrometer are calibrated in microliters; A P-20 is accurate from 1 µl - 20 µl, a P-200, from 20.1 µl - 200 µl, and a P-1000, from 200.1 µl - 1000 µl. The pipetman should never be used for a volume outside of those ranges. Depress the piston, and make sure you can feel the two "stops" along the piston stroke. Moving the piston between the top of its stroke and the first stop (i.e. the point at which the back-resistance increases abruptly) corresponds to transferring the volume set on the dial. Pushing the piston beyond the first stop purges the pipette tip with extra air and can be used to purge residual solution that gets stuck in the tip.

To adjust an instrument, fit a proper tip to the delivery tube, using a slight twisting motion to seat the tip firmly. Set the micrometer by holding the body of the pipette in one hand while rotating the knurled knob to the desired micrometer reading. Depress the plunger button to the first positive stop. This part of the stroke is calibrated according to your setting on the dial. Immerse the end of the tip into the liquid to be dispensed, not more than 3 mm below the surface, then slowly release the plunger while holding the pipette in a vertical position. This draws liquid into the tip. Never allow the plunger to snap back as this could allow liquid to enter the shaft of the unit. It is well to wait a few seconds to ensure complete filling of the tip, especially if the liquid is viscous; this prevents the introduction of air bubbles.

To dispense the measured sample, depress the plunger button to the second positive stop, withdrawing the tip from the container by drawing the free tip end along the side of the vessel. Discard the tip by pressing the ejector button. Tips should be changed with every measurement. Air bubbles may occasionally be noted within the tip during liquid intake. If this occurs, return the liquid to its container, then try again. If it occurs again, discard the tip.

Appendix C
Solution and Reagent Preparation

Rules for Making Solutions and Working with Chemicals

A. Frozen Chemicals and Solutions

1. When removing any liquid solutions from the freezer, they should be allowed to thaw at room temperature, until no ice crystals remain. Most salts and buffers can be thawed in a 37^{o}C water bath, especially if a precipitate can be seen. Before use, every solution should be vortexed to be certain that the solution is homogeneous and that all of the precipitate is once again dissolved. Never use a solution that might not be homogeneous! Once mixed the solutions can then be placed in an ice bucket or simply left at room temperature. Solutions that do not have miscible layers should be centrifuged to separate the layers, i.e. phenol solutions.

2. When removing solids from the freezer, always allow the vial to warm up to room temperature before opening. If you open a cold vial in a humid room the sample will condense water into itself. The solid will not then be homogeneous. Return to the freezer ASAP.

3. Exceptions. Enzyme solutions that are frozen should be allowed to thaw on ice ONLY! DNA/RNA modifying enzymes are supplied in glycerol and should never be removed from the freezer! They may be removed in a freezer storage box designed to keep tubes cold at the bench! Return to the freezer ASAP. Never vortex a protein/enzyme containing solution unless explicitly told to do so.

4. In this teaching lab, students should only store their solutions in the designated area of the freezer or refrigerator. Always use Styrofoam racks for storage. If you use the good plastic/metal racks then you will soon find that there are none left for your use at the bench. Also -- consolidate your samples! If you have one freezer rack, then you should not need two. When a project is finished, you should clean out all of the areas that you had samples stored. When I need room in either the freezer or the refrigerator, I may throw out all of your samples, not just the outdated ones! This could result in disposal of something of value to you and a lower grade if it affects your end result. Never use another group's reagents without their permission!

5. When you open a reagent vile for the first time, place the date opened on the vial and your group identification. This will be yours for the term! Don't waste! Follow proper storage recommendations at all times!

B. Refrigerated Chemicals and Solutions

1. Always vortex a solution before use to insure homogeneity.

2. Solids should be allowed to warm-up as explained above.

3. As always return the original container to the refrigerator ASAP.

4. Never use another group's reagents without their permission.

C. Room Temperature Chemicals and Solutions

1. All room temperature solutions issued to students should be stored in the appropriate lab drawer. Do not leave solutions out on the bench or on the shelves. Do not remove solutions from the instructor's

bench. Do not place anything on the instructor's bench, even for the lab period! Label all solutions with date opened and group identification. Never use another group's reagents without their permission.

2. Most room temperature chemicals and solutions need no special care or maintenance. Only desiccated chemicals require special attention: storage in a dessicator.