Chapter 3

Mastering the micropipette

Welcome to the microworld! In this class, you are working with microorganisms, including yeast and bacteria, millions of which would fit into a period on this page. You will also be working with costly reagents, such as plasmids and enzymes. Therefore, in every experiment, you will be required to accurately measure volumes as small as a few microliters (µL). Micropipettes will allow you to do this accurately and precisely.

Objectives

• Learn to select and adjust micropipettes

• Learn to accurately transfer microliter volumes

• Use the spectrophotometer to measure absorbance

• Understand experimental errors in measurements

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Using micropipettes correctly

Arguably, the most important scientific equipment that you will use this semester are adjustable micropipettes. Micropipettes are precision instruments that are designed to accurately transfer volumes in the microliter range. You may use microliters or milliliters as the units of volume in your lab notebooks and lab reports, but be careful to always state the volume unit that you are using. Recall the relationships between volume units:

1 microliter (abbreviated μL) = 10^{-3} milliliter (mL) or 10^{-6} liter (L)
(A useful tip for Mac users: The keyboard shortcut for the Greek letter μ is Alt-m)

Accuracy and precision

Ideally, micropipettes will deliver liquids with accuracy and precision. Accuracy depends on the micropipette delivering the correct volume. Precise results are reproducible. Let's use a target analogy to demonstrate the difference between accurate and precise results. Imagine that four students try to hit the bulls-eye five times. Students A and B are precise, while students A and C are accurate.

| A	B	C	D
Precise Accurate	Precise Inaccurate	Imprecise Accurate	Imprecise Inaccurate
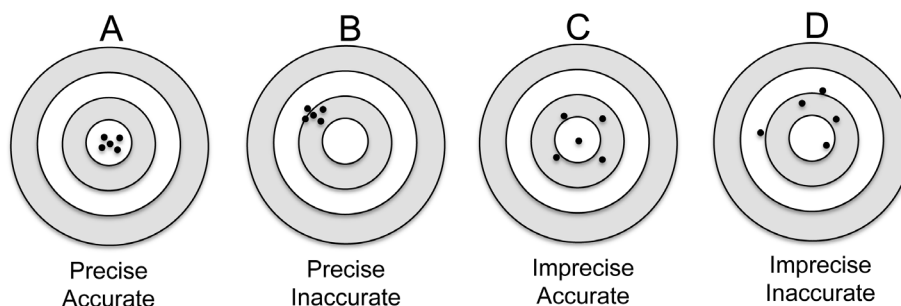
The best way to determine the accuracy and precision of micropipettes is to use them to weigh set volumes of distilled water on an analytical balance. The density of water is 1.0 gram per mL at 25˚C. The process is repeated several times during the calibration process, and the data is used to calculate the accuracy and precision of a micropipette.

Accuracy refers to the performance of the micropipette relative to a standard (the intended) value. Accuracy is computed from the difference between the actual volume dispensed by the micropipette and the selected (intended) volume. Note that this can be a negative or positive value. When micropipettes are calibrated, the accuracy is normally expressed as a percent of the selected value. In general, micropipettes are designed to operate with accuracies within a few percent (generally <3%) of the intended value. The accuracy of a micropipette decreases somewhat, however, when micropipettes are set to deliver volumes close to the lowest values in their range.

Precision provides information about reproducibility, without any reference to a standard. Precision reflects random errors that can never be entirely eliminated from a procedure. Precision is expressed as the standard deviation (σ) of a set of measurements. Assuming random error, ~2/3 of measurements will fall within one standard deviation of the mean, and 95% of measurements will fall within two standard deviations of the mean.
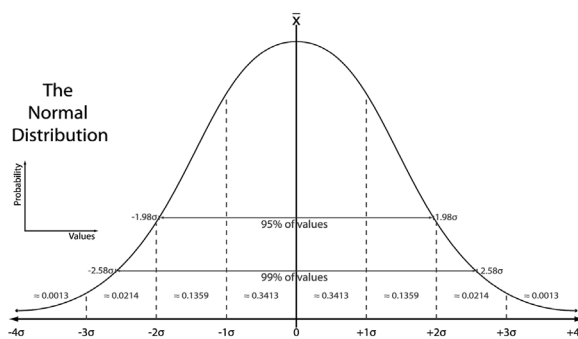
Choosing the micropipette

There are three different sizes of micropipettes in the laboratory, which we will refer to as the P20, P200 and P1000. Our micropipettes have been purchased from several different manufacturers, but the principles of operation are the same. The numbers after the "P" refer to the maximum number of microliters that the micropipette is designed to transfer. Use the chart below to select the correct micropipette for an operation. Note that there is some overlap in the ranges of the different micropipettes. For example, both the P200 and P20 can be used to transfer 15 µl, but the P20 is more accurate within that range. As a rule of thumb, always select the smallest volume pipette that will transfer the volume.

Micropipette	Recommended range (µL)	Smallest increment (µL)
P20	1 - 20	0.02
P200	20 - 200	0.2
P1000	100 - 1000	2.0

Micropipettes use disposable plastic tips. The P1000 tips are larger than those used with P200s and P20s. P1000 tips may be either natural/clear or blue, depending on the vendor, while P20 and P200 tips may be either yellow or natural/clear in color.

Specifying the transfer volume

There are three numbers on the volume indicator. With each of the micropipettes, you will specify a volume to three digits by turning the volume adjustment knob. You will also be able to extrapolate between the lowest numbers with the vernier marks on the lower dial. Most of the measurements you will make with the micropipettes will be accurate to four significant figures!

NEVER turn the indicator dial beyond the upper or lower volume limits of the micropipette! This could damage the piston.

P 200

Set to deliver
153 µL

Vernier
scale
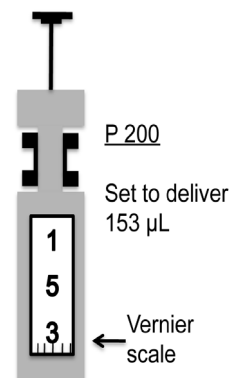
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Transferring volumes accurately

Micropipettes work by air displacement. The operator depresses a plunger that moves an internal piston to one of two different positions. The first stop is used to fill the micropipette tip, and the second stop is used to dispense the contents of the tip. As the operator depresses the plunger to the first stop, an internal piston displaces a volume of air equal to the volume shown on the volume indicator dial. The second stop is used only to dispense the contents of the tip.


|		
Start	First stop	Second stop

Filling the micropipette

- Remove the lid from the box containing the correct micropipette tips.
- Attach the tip by inserting the shaft of the micropipette into the tip and pressing down firmly (figure on right). This should produce an airtight seal between the tip and the shaft of the micropipette.
- Replace the lid of the tip box to keep the remaining tips sterile. ***Avoid touching the tip (especially the thinner end), because the tips are sterile.***
- Depress the plunger of the micropipette to the FIRST stop.
- Immerse the tip a few millimeters below the surface of the solution being drawn up into the pipette. Pipetting is most accurate when the pipette is held vertically. Keep the angle less than 20˚ from vertical for best results.
- Release the plunger *S L O W L Y*, allowing the tip to fill smoothly. Pause briefly to ensure that the full volume of sample has entered the tip. Do NOT let the plunger snap up. This is particularly important when transferring larger volumes, because a splash could contaminate the shaft of the micropipette. If you inadvertently contaminate the shaft, clean it immediately with a damp Kimwipe.
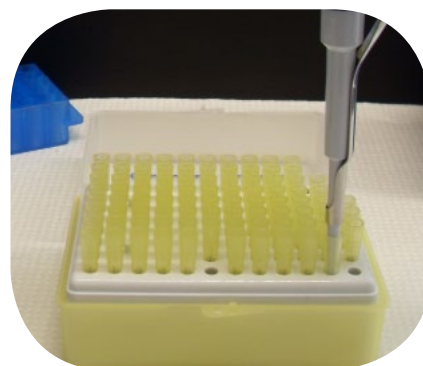
NEVER rest a micropipette with fluid in its tip on the bench!
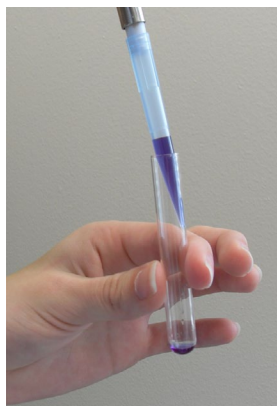
Dispensing the contents of the micropipette
- Place the micropipette tip against the side of the receiving test tube. Surface tension will help to dispense the contents of the micropipette. Do NOT attempt to eject the contents of the micropipette into "thin air."
- Smoothly depress the plunger to the first stop. Pause, then depress the plunger to the second stop. The contents of the pipette should have been largely released at the first stop. The second stop ensures that you've released the "last drop."
- Use the tip ejector to discard the tip.

WARNING: The most common - and serious - operator error is depressing the plunger to the second stop before filling the micropipette tip.

DO NOT DO THIS!!!

Using the spectrophotometer to evaluate your pipetting skills

Since you will be using micropipettes for all of your experiments, the quality of your results will depend on proper operation of the micropipette. Today's laboratory will lead you through some exercises that will show you how to use micropipettes correctly and point out some common pitfalls associated with their use. Your results will also provide information about whether the pipettes are functioning properly.

In these exercises, you will be using the spectrophotometer to determine if your pipetting is accurate and precise. You will be using micropipettes to combine various volumes of water and solutions of a blue dye, bromophenol blue (BPB). You will measure the absorbance of the resulting solutions at 590 nm (A_{590}), which is close to the absorbance maximum of bromophenol blue at neutral pH. Measuring errors will be reflected in the spectrophotometer readings.
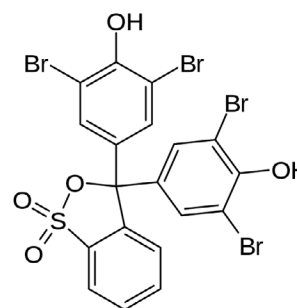
The spectrophotometer readings provide an indirect measurement of pipette performance. The proper way to calibrate the micropipettes would be to weigh out volumes of water, which has a specific gravity of 1.0 g/mL. Unfortunately, we do not have enough balances with sufficient accuracy for the class to perform the measurements. If you suspect inaccuracies in the micropipettes that you are using, refer them to the teaching staff, who will test them properly.

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Light spectroscopy

Spectrophotometers measure the amount of light absorbed by a sample at a particular wavelength. The absorbance of the sample depends on the electronic structures of the molecules present in the sample. Measurements are usually made at a wavelength that is close to the absorbance maximum for the molecule of interest in the sample.

The diagram below shows the elements present in a typical spectrophotometer. The light sources used in most spectrophotometers emit either ultraviolet or visible light. Light (I_0)passes from a source to a monochromator, which can be adjusted to allows only light of a defined wavelength to pass through. The monochromatic (I) light then passes through a cuvette containing the sample to a detector.
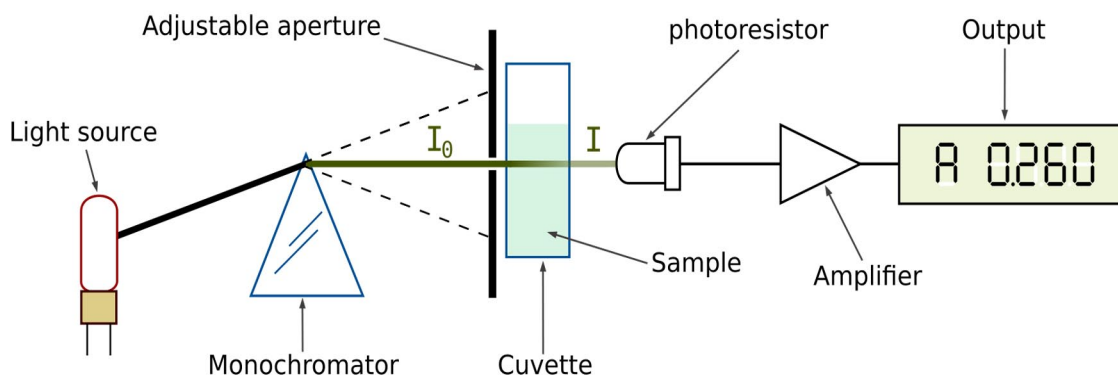
The spectrophotometer compares the fraction of light passing through the monochromator (I_0) to the light reaching the detector (I) and computes the transmittance (T) as I/I_0. Absorbance (A) is a logarithmic function of the transmittance and is calculated as:
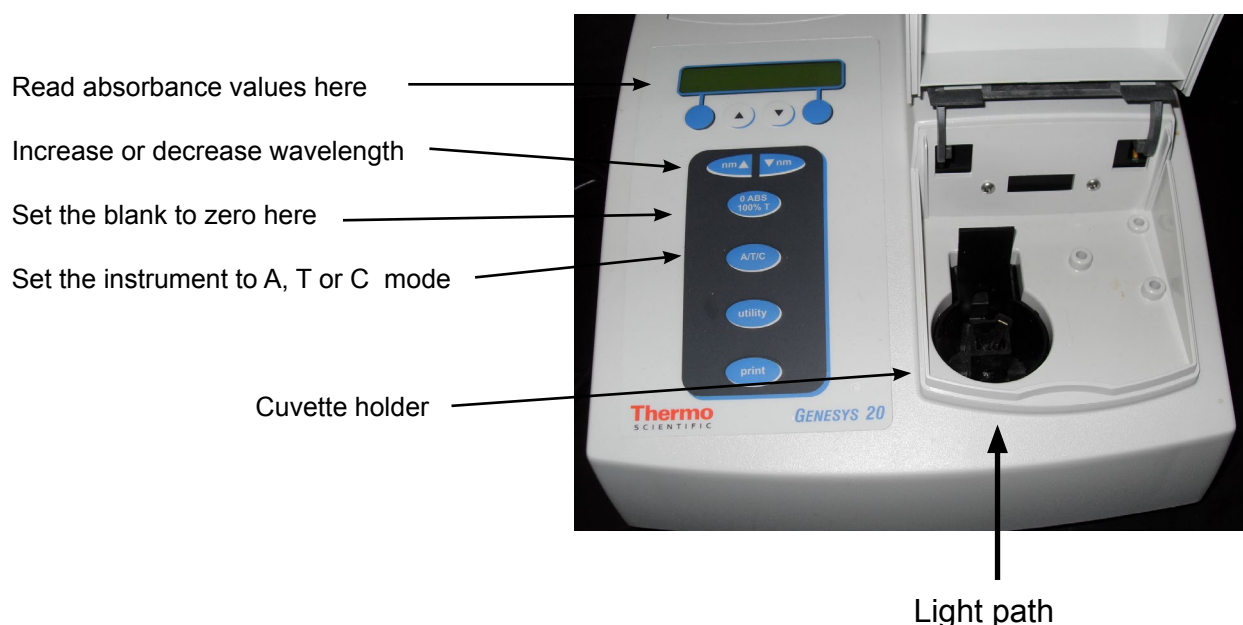
$$A = \log_{10}(1/T) = \log_{10}(I_0/I)$$

Spectrophotometers can express data as either % transmittance or absorbance. Most investigators prefer to collect absorbance values, because the absorbance of a compound is directly proportional to its concentration. Recall the Lambert-Beer Law, traditionally expressed as:

$$A = \varepsilon \, b \, C$$

where ε is the molar extinction coefficient of a compound, b is the length of the light path through the sample, and C is the molar concentration of the compound. Cuvettes are formulated to have a 1 cm light path, and the molar extinction coefficient is expressed as L/moles-cm. Consequently, absorbance is a unitless value.

Using the GeneSys 20 spectrophotometer

Our labs are equipped with GeneSys 20 spectrophotometers. Programming is very simple and is accomplished with a few buttons, as shown in the figure below. In our labs, we will be using the instruments in absorbance (A) mode, rather than the transmittance (T) or concentration (C) modes.

Read absorbance values here

Increase or decrease wavelength

Set the blank to zero here

Set the instrument to A, T or C mode

Cuvette holder

Light path

1. Set the mode to absorbance.

2. Adjust the wavelength to the proper value (590 nm) using the arrow keys.

3. Prepare a cuvette containing deionized water to serve as a blank. Insert the cuvette into the cuvette holder. Be sure the cassette is oriented correctly in the light path. ***The light path in the GeneSys 20 is from front to the back of the instrument.*** Close the lid.

4. Press the 0 Abs/100% T key. This will zero the instrument. Remove this cuvette. Save this blank for others to use.

5. Place a cuvette containing your sample into the cuvette holder. Read the absorbance values and record them in your notebook.

6. Repeat step 5 with all of your samples, recording the absorbance readings in your notebook.

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Exercise 1 – Getting the feel of micropipettes

Concept: Micropipettes work by an air displacement mechanism

1. Set the P200 to deliver 200 µL. Be careful not to overshoot, which could damage the pipette piston.

2. Grip the pipette by wrapping your fingers around the barrel. Use your thumb to depress the plunger to its first stop.

3. Next press the plunger to the second stop. *Compare the distance that the plunger moved during the first and second strokes.*

4. Set the P200 to deliver 20 µL and depress the plunger to its first stop. *Compare the distance that the plunger moved when the P200 was set to 200 or 20 µL.*

5. Depress the plunger to the second stop. *How does the distance between the first and second stops compare for 200 and 20 µL?*

6. Set the P20 to deliver 20 µL. Depress the plunger to the first stop. *Compare the distance to the first stop when a P20 and P200 are set to deliver 20 µL.*

Concept: The filling and dispensing strokes are different.

1. Place a tip on the shaft of the P200.

2. Set the P200 to deliver 50 µL.

3. Draw up 50 µL of 0.05% BPB solution into the pipet.

4. Dispense the BPB into a microcentrifuge tube down to the first stop, holding the tip against the wall of the tube. Note whether all of the dye has been expelled. Push the plunger down to the second stop to release any remaining BPB.

Lab exercise 2 – How NOT to pipette!

1. Use the P1000 to add 990 µL of water to two microcentrifuge tubes. Label the tubes A and B. Dispose of used tips in the containers provided.

2. Use a P20 to correctly transfer 10 µL of 0.05% BPB to tube A. Make a mental note of what fraction of the pipet tip is filled with the dye. Use the vortex mixer to disperse the BPB in the water.

3. Use a P20 to **INCORRECTLY** transfer 10 µL of 0.05% BPB to tube B. Do this by depressing the plunger to the second stop before you take up the BPB solution. Make a mental note of how well the dye fills the tip this time.

4. Set the wavelength of the spectrophotometer to 590 (A_{590}). Pipette 1 mL of water into a plastic cuvette and blank the spectrophotometer at this wavelength.

5. Read the A_{590} of the solutions in tubes A and B, in the spectrophotometer. *How do the two readings compare? What kind of error results from drawing solution into the pipette incorrectly?*

Exercise 3 – How precise and accurate are your transfers?

Work in groups of three. One person in the group should work with the P-20, another with the P-200 and the third with the P-1000. Each person should prepare three identical samples and then determine the A_{590} of the three samples. From the data, you will be able to determine if the micropipette is measuring volumes correctly.

1. Each person in your group of three will work with a different micropipette and perform the same transfers in triplicate, as detailed below. The final volume (water + BPB) in each tube will be 1.0 mL. Calculate the volume of water that will need to be combined with each of the following to give 1.0 mL, and record your calculations in your lab notebook:

   Group member A: Use the P-20 to transfer 10 µL of 0.05% BPB to _____ µL of water.
   Group member B: Use the P-200 to transfer 100 µL of 0.005% BPB to _____ µL of water.
   Group member C: Use the P-1000 to transfer 300 µL of 0.005% BPB to _____ µL of water.

2. To minimize our plastic waste, strategize how to minimize the number of tips that you use without contaminating the stock solutions. A tip can be used multiple times, but a tip that has been used for BPB cannot be used to subsequently transfer deionized water. Combine the BPB solution and water to give a final volume of 1.0 mL.

3. Measure the A_{590} of the three solutions with the spectrophotometer and record the data in your notebook.

4. Compute the mean and standard deviations for your three measurements, using either a calculator or Excel. The standard deviation reflects the precision of your measurements.

5. Enter these values on the chart that your TA has prepared on the whiteboard. Compare the values that your group obtained each of the three pipettes with the aggregated class measurements. If the averages that your group obtained are significantly different than those that other groups obtained with the same micropipette, your micropipette may not be transferring volumes accurately.

Notify your TA if any of the micropipettes are not performing properly. Your TA will follow up on your observations and test the micropipettes with the gravimetric test described earlier in the chapter.

Test yourself

As part of an interview for a research position, three applicants are asked to transfer 150 μL of distilled water with a P-200 micropipette to a weighing paper and to determine the weight of each drop with an analytical balance.   The three measurements made by each of the applicants are listed below. Use the space below to calculate the mean and standard deviation of the measements made by each student.

Applicant A: 0.161 g, 0.147 g, 0.142 g
Applicant B: 0.158 g, 0.156 g, 0.157 g
Applicant C: 0.143 g, 0.153 g, 0.150 g

Which applicant makes the most precise measurements?

Which applicant makes the most accurate measurements?