

Introductory Lab: pH Meters, Cary 50 UV-Visible Spectrophotometer, and Variable Volume Pipettors

Reading: Chapters 1 and 2 in Ninfa and Ballou (N&B).

Goal: In this course you will be introduced to the proper use of three very important bio-analytical devices: a pH meter, a scanning UV-visible spectrophotometer, and variable volume pipettors. Please read the appropriate material in Ch. 1 and 2 in the textbook about these instruments and the techniques related to them. The material below should be used as a reference for the use of the pH meters, the Cary-50 spectrophotometers, and the variable volume pipettors that are specific to this lab. We will expand upon this information in the classroom.

Part A. Calibrating the pH Meter and Electrode

Introductory Experiment 1: Determination of pH of Two Buffers.

In this experiment, we will learn how to do a two-pH standard calibration of the VWR SympHony pH meter/electrode using instructions that will be handed out in class. We will then measure the pH of two buffers that will be provided for you.

1. Following the instructions handed out in class, calibrate the pH meter/electrode using pH 4 and pH 7 standards, then determine the pH of the two buffer unknowns you have been given:

Buffer A pH: _____.

Buffer B pH: _____.

2. Repeat the above calibration procedure using the pH 7 and pH 10 standards, then measure the pH of the buffer unknowns again.

3. Did the pH values change? If the answer is YES, consider the following. For most pH meters calibration of the meter and electrode using two pH standards results in a linear function of voltage from the electrode (which is relayed to the analog display or digital screen) vs. pH (or $[H^+]$), which is responsible for the electrochemical potential giving rise to an electric current. Based upon this knowledge, offer a simple explanation for the variation in pH values with the two calibration processes.

4. Suppose you are doing a pH dependent study of a molecule containing a titratable group. If you want to cover a range of pH from 6 to 9, what would be the best standard buffers to use in order to calibrate the meter/electrode, a combination of pH 4 and 7 standards or pH 7 and 10?

BIOC 463a
Intro Lab
2006 - 2007

5. **Thoroughly** rinse the electrode before putting back into storage vial.

Storage: For storage of electrodes the following solutions can be used (in order of preference:

- pH 4 buffer with 1.5 M KCL (the best)
- pH 4 buffer (better)
- pH 7 buffer (not so good)
- H₂O (Very bad. Never store electrode in deionized water!!!)

A few notes about electrodes:

pH electrodes, when used with biological materials (proteins, DNA, cell extracts) eventually become “clogged” or coated with precipitated material. The electrode begins to respond very sluggishly (i.e. the time required for the electrochemical potential to be established increases) and it takes a long time for the electrode to stabilize. Eventually, the electrode will start bouncing around erratically and become unusable. There are chemical ways of cleaning the electrode (for instance, soaking in HF acid for a limited amount of time), that will revive the electrode for a while, but eventually, the electrode will have to be replaced.

Standard pH electrodes are Ag/AgCl (or calomel) electrodes. These electrodes are very practical for virtually everything except for TRIS buffers. TRIS tends to degrade calomel electrodes because Tris is believed to chelate the Ag⁺ ions in the electrode, thereby shifting the equilibrium between Ag metal and Ag⁺. When you measure the pH of a TRIS solution using a calomel electrode, do so as quickly as possible (what some refer to as the “on the fly” method) in order to minimize the amount of time the electrode is exposed to TRIS and thoroughly rinse the electrode when finished. If you are working with TRIS buffers a lot, it is better to use a ROSS electrode that is designed specifically to be used with TRIS. However, these electrodes are considerably more expensive than the calomel variety.

The majority of the pH meters used in our lab are analog devices. Unfortunately, most pH meters sold today are digital varieties, which are instruments of the Devil! Each meter has a computer routine that dictates calibration, temperature factors, etc. Therefore, it is necessary to wade through a lengthy procedure, punching buttons on the meter in their proper sequence, in order to do a two pH buffer standard calibration. Often instructions for these devices leave out “little” points, that make it necessary to call the manufacturer when you run into a problem (that is why they have Customer Help lines!).

Part B: Variable Volume Pipettors (read pps. 17 – 21 in N&B).

Each lab group will be provided with a set of four pipettors that span a range of delivery volumes of 0.5 uL to 1 mL. The following table lists the pipettor and the tips that should be used:

**BIOC 463a
Intro Lab
2006 - 2007**

<i>Pipettor</i>	<i>Tip</i>
0.5 uL to 10 uL	Clear or Yellow
5 uL to 50 uL	Yellow
20 uL to 200 uL	Yellow
100 uL to 1 mL	Blue

These instruments are high precision devices that can be damaged or destroyed if not handled properly. The following guidelines will be followed in this laboratory :

1. Choose the correct pipettor to use. It is best to choose a pipettor whose limits bracket the desired volume. ***For volumes > 1 mL do not use a pipettor, rather choose either a 5 mL or 10 mL disposable seriological pipette.***
2. Choose the correct tip to use. For the 0.5 – 10 uL pipettor we have special micro tips that must be used with caution, especially if you are dispensing 10 uL. If care is not taken you can draw liquid into the end of the pipettor itself, which is not desirable (see below).
3. Slowly adjust the volume to be dispensed by turning the top of the pipettor. These pipettors have a ratchet-type device that can be damaged by rough treatment.
4. Draw liquids into the pipettor slowly. Drawing the liquid rapidly often results in drawing in an air bubble, especially with the 100 uL – 1 mL pipettor.
5. Hold the pipettor at an angle when drawing up the liquid.
6. **Never** lay the pipettor on your lab bench when there is liquid within the tip. This can result in contaminating the pipettor itself. If the pipettor becomes contaminated it must be cleaned thoroughly.
7. **Cleaning the pipettor:** if the device becomes contaminated please let one of the instructional staff know immediately. Some of the reagents used in this course are quite toxic and the pipettor will need to be cleaned right away.
8. **A Pipettor IS NOT: A SWORD, A POINTER, A PRY BAR, A BACK SCRATCHER, or A NOSE PICKER.**

**BIOC 463a
Intro Lab
2006 - 2007**

Part C: Using the Cary-50 UV-Vis spectrophotometers

The Cary-50 spectrophotometers in our lab are high precision single-beam instruments. The light source is a powerful Xenon arc lamp that emits both UV and visible light. There are several distinct advantages that this instrument has over more commonly found spectrophotometers that use a deuterium lamp for UV light and a tungsten light source for visible radiation that we will discuss in class. The resolution of any spectrophotometer (see pages 57-62 in N&B) is limited by the slit width, which is fixed at 1 nm in the Cary-50. Spec-20 instruments, which are commonly found in undergraduate teaching labs, have a bandpass of ~20 nm, thereby its spectral resolution is much less (cf. Fig. 2-6A in N&B).

Like most modern spectrophotometers, the Cary-50 is driven by proprietary software. We will discuss the software and its utilization in much greater detail in class, however the instructions below are sufficient to get you started.

Introductory Experiment 2: Obtaining the Spectrum of Riboflavin (Rf)

In this experiment, we want to obtain the spectrum of oxidized and reduced riboflavin, a biological electron transfer agent. You will be supplied with a stock solution of 0.144 mM Rf, from which you will prepare two 5 mL solutions at concentrations of 0.02 mM and 0.035 mM. In order to calculate the volumes of Rf and the solvent (H₂O) you will employ one of the most useful equations that you will become infinitely familiar with by the end of the semester:

$$(C_1)(V_1)=(C_2)(V_2)$$

Where C_1 is the concentration of the Rf stock solution, V_1 is the volume of Rf stock solution to be transferred, C_2 is the concentration in the final solution, and V_2 is the total volume of the final solution.

Example calculation: Prepare 20 mL of a 20 μ M solution of Rf from a 10 mM stock solution:

$$(10 \text{ mM})(V_1) = (0.02 \text{ mM})(20 \text{ mL})$$
$$V_1 = (0.4 \text{ mL})/10 = 0.04 \text{ mL}$$

Therefore: Add 0.04 mL (= 40 μ L) of 1 mM stock to 19.96 mL of H₂O. (For all practical purposes, 19.96 mL ~ 20 mL; it is highly unlikely that one could accurately differentiate between the two volumes).

BIOC 463a
Intro Lab
2006 - 2007

1. Initial Set-up Procedure for Spectra Acquisition: (Note: <DC> = “double click”)

On the main Windows screen **double click** (<DC>) on the **Cary WinUV** icon. This will open the suite of programs that can be used to collect data on the Cary-50.

<DC> **SCAN** icon. This opens the scanning module.

<DC> **SETUP** button.

- Under the **CARY** tab window set:
- Wavelength interval range: **300 – 500 nm**.
- Scan control (i.e. speed) = **Medium**.

<DC> **BASELINE** tab (at top).

- Set for **Baseline Correction**.

<DC> **REPORTS** tab:

- Set **Display All Traces** on.

<DC> **AUTO STORE** tab:

- Set **Storage off**.

<DC> **OK** at bottom.

<DC> **BASELINE** button: **BASELINE CORRECTION ON**.

<DC> **DONE**.

You are now ready to run a spectrum using the plastic 1 cm cuvettes. In virtually every case, you will run a **baseline spectrum** first (this is stored in computer memory and subsequently subtracted from the sample spectrum). The baseline solution contains everything except the sample molecule. In this case, the baseline will be the spectrum of H₂O.

2. Obtain Baseline and Sample Spectra.

- Fill a semi-micro disposable cuvette with water and place in the cell holder. Return to the **SCAN** window.

<DC> **BASELINE**.

The baseline spectrum will be obtained. Note: The only indication that a scan is occurring will be seen in the Wavelength indicator box in the upper right hand corner of the screen. When the Wavelength indicator returns to the upper wavelength, you may obtain your sample spectrum.

- Remove the cuvette from the cell holder, shake out the water, and fill the cuvette with your lowest concentration of Rf using a Pasteur pipette. Place the sample back in the cell holder and close the lid.

<DC> **START** button at top of **SCAN** Window.

- Repeat this process for the highest concentration.

BIOC 463a
Intro Lab
2006 - 2007

What is the highest wavelength peak, λ_{\max} : _____?

Absorbance of 1st sample at λ_{\max} : _____.

Absorbance of 2nd sample at λ_{\max} : _____.

Using the Beer-Lambert relationship (see N&B p. 55), determine the milli-Molar extinction coefficient at this wavelength for both spectra: _____.

Are the extinction coefficient values determined from the two spectra similar?
What is the percent error between the two values?

3. Examine the effect of varying scan speed on spectral resolution.

Due to a variety of instrumental reasons, the speed at which you obtain a spectrum may greatly affect the spectral resolution of a trace as seen in Figure 2-6A of your text for the affect of scan rate on the observed spectrum of reduced cytochrome c. In this case, spectral resolution is related to three parameters: the position of λ_{\max} in nm, the absorbance at λ_{\max} (A_{λ}), and the half band width (hbw). In this experiment you will see if these parameters for the spectrum of Rf change with an increase in the scan rate on the Cary 50.

Re-open the **SETUP** window and find the **SPEED CONTROL** box (**SETUP>CARY>SPEED CONTROL**).

- Press the **MEDIUM** button. Click on the **ADVANCED** button.
- A new window will appear listing **Ave. Time, Data Interval, Scan Rate**. Make a note of the numbers for each one of these parameters for the scan that you just obtained (see above).
- Click on OK or Cancel.
- Increase the scan rate to **FAST, FASTEST, and SURVEY**. For each scan rate, obtain the information for **Ave. Time, Data Interval, Scan Rate** as described above.

Does λ_{\max} , A_{λ} , and hbw change with an increase in the scan speed?

Comparing your spectra with those shown in Fig. 2-6A in N&B, do you see a dramatic effect on the Rf spectrum with an increase in scan rate as is seen with cytochrome c? If so, at what **SCAN RATE** do you begin to notice a significant decrease in spectral resolution. How is this related to **Ave. Time, Data Interval, and Scan Rate**? If no, why is there a difference in the observed effect for the two spectra? (Hint: compare the hbw for the two spectra and the first derivatives of the two spectra).

4. Obtain a Fixed Wavelength Kinetic Trace:

In several experiments this semester we are going to monitor the change of absorbance at a single (fixed) wavelength as a function of time. Experiments performed in this manner are referred to as kinetic experiments. One of the nice

BIOC 463a
Intro Lab
2006 - 2007

attributes of the Cary UV-Win software is that you can easily go from **SCAN** to **KINETICS** without losing any data. Assuming you are in the **SCAN** module, minimize the **SCAN** window so you can see the CARY UV-Win window.

<DC> **KINETICS** icon: this opens the Kinetics module, but notice the **CONNECT** at the top of the window. This tells you that you have another module (i.e. **SCAN**) “connected” to the spectrophotometer. In order to connect the **KINETICS** module:

<DC> **CONNECT**: The **CONNECT** will be replaced by **START** when this module is connected (and **SCAN** is disconnected).

<DC> **SETUP** button:

- Set **Wavelength**: chose the wavelength maximum determined above.
- Under **Collecting Time**, set for **Simple Collection**, then set the # of minutes for the **STOP time = 1 minute**.
- **AUTO STORE**: off.
- <DC> **OK**.

Once you have the program set up, put a cuvette containing water in the cell holder and:

<DC> **ZERO** button (left side). This will “zero” the instrument at the wavelength you have chosen to monitor. Zeroing means setting the absorbance = 0 at the wavelength you have chosen for the cuvette and the baseline solution.

To begin your time-dependent trace:

<DC> **START**: answer the first box, then you will see the **Sync Start** box appear. The actual data collection will start when $t = 0$ on the timer or you click “**OK**”.

Place the sample containing the highest concentration of Rf in the spectrometer.

<DC> **OK** on timer window: the window will then show you the absorbance at the chosen wavelength as a function of time. We will demonstrate how to adjust the y-axis to view the trace.

Do you see any change in absorbance as a function of time? Why or why not?

Finally, reactivate the **SCAN** window and re-**Connect** to this software. Run a spectrum of the RF at a **FAST** scan speed. Then add a pinch of Sodium Dithionite (a very powerful reducing agent) to the solution in the cuvette and gently mix using a Parafilm square. The solution should change color. Run a spectrum of this solution. Did the Rf spectrum change?