

Using the Spectrophotometer

Any colored solution is colored because it absorbs some wavelengths of light and transmits others. If you put blue food coloring in water, the solution will **absorb** most of the light passing through it but will **transmit** light that has wavelengths in the blue range. Blue light passes through the solution, other colors do not. Even apparently colorless solutions may absorb specific wavelengths of light (perhaps in the ultraviolet range, where we can't see it).

The **spectrophotometer** is designed to measure the amount of light absorbed by a solution. This sensitive instrument is a very important tool for a biologist. It can be used to determine a molecule's **absorption spectrum**: the wavelengths of light that the particular molecule absorbs (for example, DNA is best at absorbing UV light with a wavelength of 260 nm). Furthermore, a more concentrated solution will absorb more light than a less concentrated one (so the more blue food coloring you put in water, the darker it looks). Using the spectrophotometer, we can quantitatively measure absorbance, and this information can be used to determine the concentration of the absorbing molecule.

With a little clever experimental design, we can use the spectrophotometer as a way to quantitate what's going on in all kinds of biological processes. For example, if an enzyme reaction produces or destroys a colored molecule, the spectrophotometer can be used to measure how much of that molecule is present and therefore can quantitate the activity of the enzyme. Here are just a few ways that a spectrophotometer might be used in biology:

- Calculate the concentration of DNA in a solution
- Quantitate photosynthesis
- Measure amounts of chlorophyll in a leaf
- Determine the rate of an enzyme-catalyzed reaction
- Measure protein concentration
- Follow the growth of bacterial cells

A spectrophotometer (see Figure 41) works by passing light from a lamp through a "monochromator" (a prism or diffraction grating) that breaks it up into individual wavelengths. An adjustable slit is then used to allow only light of a single wavelength to reach the sample, which is placed in the light path in a transparent **cuvette**. On the other side of the cuvette is a photoelectric tube which measures the amount of light that got through the sample.

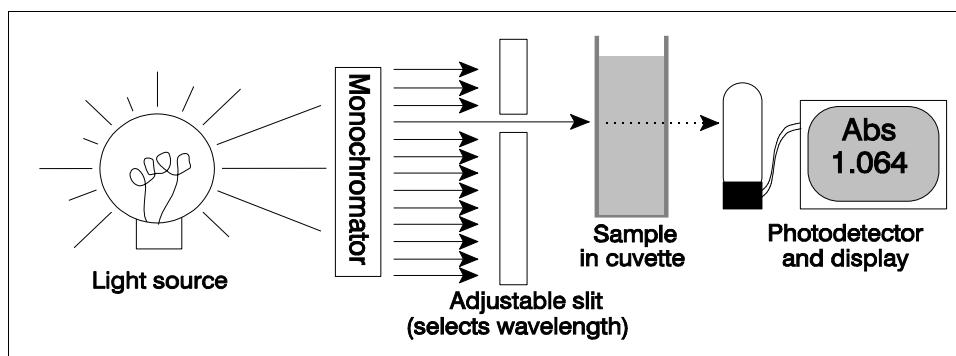


Figure 1. How a spectrophotometer works.

Technically, then, what the spectrophotometer is measuring is not how much light is *absorbed*, but rather how much is *transmitted*. However, the percentage transmitted can be easily converted to absorbance by using this formula:

$$\text{absorbance} = \log\left(\frac{1}{\% \text{ transmittance}}\right)$$

Most spectrophotometers do this conversion for you automatically. There are no units for absorbance, but you should report the wavelength used whenever you give absorbance data (e.g., a graph axis might be labeled "absorbance at 260 nm").

You will find several types of spectrophotometer in the Biology and Chemistry departments. One is a fairly simple "Spec-20" machine which measures only the transmittance or absorbance of visible light (wavelengths above 340 nm) and can be used with square

cuvettes, round cuvettes that look like test tubes or even culture tubes used to grow bacteria. We also have computerized Shimadzu spectrophotometers and SmartSpec spectrophotometers from BioRad. Both of these are UV-VIS spectrophotometers, meaning that they can measure absorbance of either visible or ultraviolet light.

The Shimadzu and BioRad spectrophotometers require 1 cm square cuvettes. Plastic or glass cuvettes can be used for measuring absorbances in the visible range, but UV light is stopped by plastic and glass, so UV absorbance (below 340 nm) must be measured in expensive, fragile quartz cuvettes. Be sure you are using an appropriate cuvette for the volume of solution you want to measure and for the wavelength you need to use!

Using the BioRad spectrophotometers for single-wavelength measurements

1. Turn on the unit using the rocker switch located on the back.
2. Press the λ key for single-wavelength measurement.
3. Press 1, to read the absorbance at one wavelength, followed by ENTER.
4. Enter the wavelength that you wish to read.
5. Tell the instrument not to subtract a background measurement.
6. Prepare your blank cuvette and place it into the spectrophotometer.

⚠ Notice that the light path runs from front-to-back, so you want your cuvette oriented correctly. The cuvette needs to be at least half full, so that your solution is actually in the light path. Additionally, try not to touch the sides of the cuvettes where the light will pass through—you don't want to measure the absorbance of your fingerprints!

7. Press the READ BLANK key and wait a moment while the zero is set. Then press the right arrow key to move on.

⚠ The job of the blank is to tell the spectrophotometer what “zero” is. If you're planning to measure a colored molecule dissolved in water, then the absorbance of plain water would represent “zero,” and you should use water as your blank. However, you might be dissolving your molecule in a solution which is colored to start with. In that case, your blank should be the solvent with nothing added to it, so that the spectrophotometer will subtract out the color of the solvent itself.

8. Remove your blank cuvette and insert the cuvette with your sample. Press the READ SAMPLE key. Note the absorbance that is displayed.
9. Continue reading as many samples as you have.

Determining an absorption spectrum with the BioRad spectrophotometers

In some cases, you won't know in advance the wavelength(s) absorbed by the solution you're interested in. In that case, the first step would be to measure its **absorption spectrum** and find out experimentally what wavelengths are absorbed best. The spectrophotometer has a program which will automatically try every wavelength and give you the absorbance at each one. Here's the procedure:

1. After the spectrophotometer warms up, press the SCAN key.
2. Enter the wavelengths that you wish to scan.
3. Press ENTER to choose to not subtract a background setting.
4. Press ENTER to select the 'slow' scan.
5. Put your blank cuvette in the spectrophotometer and press READ BLANK.
6. Insert your sample cuvette and press the READ SAMPLE key. It will take a few moments to scan.

7. Press the PRINT key to get a small printout of your results. You can press PRINT again for your partner's notebook. Notice that the wavelength which your sample had the maximal absorbance (max) is listed near the top.

Following absorbance over time with the BioRad spectrophotometers

In some cases, you will want to measure how the absorbance of your solution changes over time. This is particularly useful for enzyme assays where your product (that absorbs light) is being made. Rather than watching the clock and reading out the absorbance as time passes, we can program these spectrophotometers to do that for us. Here's the procedure:

1. After the spectrophotometer warms up, press the KINETICS key.
2. Enter the wavelength that you wish to read.
3. Choose the total duration of the reaction. For example, if you want to collect the absorbance values every five seconds for one minute, enter 60 seconds as the duration.
4. Choose the interval between successive readings. In the example above, this would be five seconds.
5. Press ENTER to choose to not subtract a background setting.
6. Put your blank cuvette in the spectrophotometer and press READ BLANK.
7. Insert your sample cuvette, start the reaction and press the READ SAMPLE key. Wait patiently while the spectrophotometer records your data.
8. Press the PRINT key to get a small printout of your results. You can press PRINT again for your partner's notebook.

Using the Shimadzu spectrophotometers

1. Open the sample compartment on the left side and be sure no one has left a cuvette in the holder (which would mess up the self-check).
2. Switch on the instrument and wait for its warm-up and self-check procedures to be completed. The Main Menu will appear.
3. Decide whether you will be measuring absorbance in the UV or visible range, and select the appropriate lamp.

◇ *The transition from UV to visible occurs at about 340 nm. Below this wavelength, use the deuterium (D₂) lamp for UV light; above it, use the tungsten (W) lamp for visible light.*

- From the Main Menu, press 3 (Utilities).
- If the correct light source is selected, simply press RETURN.
- If not, press 6 (Light Source) to change the selection, then Return to the Main Menu.

◇ *For these spectrophotometers, it is always necessary to manually select the correct lamp. You may notice that it also has an "Auto" function, which you might think would switch lamps as needed. Unfortunately, this function just leaves both lamps on constantly, which will unnecessarily shorten their lives.*

4. Be sure the spectrophotometer is completely warmed up before attempting to measure your samples. Warm-up time for the tungsten lamp is about 15 minutes; for the deuterium (UV) lamp, it's 20-30 minutes.
5. Prepare a blank.

◇ *The job of the blank is to tell the spectrophotometer what “zero” is. If you’re planning to measure a colored molecule dissolved in water, then the absorbance of plain water would represent “zero” and you should use water as your blank. However, you might be dissolving your molecule in a solution which is colored to start with—in that case, your blank should be the solvent with nothing added to it, so that the spectrophotometer will subtract out the color of the solvent itself.*

6. Place the blank and the samples you intend to measure in cuvettes. The cuvettes need to be at least about three-quarters full for an accurate measurement. Handle the cuvettes only by the sides (keep your fingerprints away from the light path) and be sure they are clean and dry.
7. From the Main Menu, press 1 to select normal photometric measurement.
8. Press the GOTO λ key and type in the wavelength you want to measure, then press ENTER.
9. Place the cuvette containing your blank solution in the slot closest to you and close the lid. The light moves from right-to-left in these spectrophotometers. Be sure that the clear sides of the cuvette face left and right.
10. Press AUTO ZERO to set the absorbance of the blank to zero. Wait a few seconds until the display shows zero.
11. Remove the blank and place the sample to be measured in the machine, close the lid, and press START to measure and display its absorbance.
12. Repeat step 11 until all samples are measured.

Determining an absorption spectrum with the Shimadzu spectrophotometer

In some cases, you won’t know in advance the wavelength(s) absorbed by the solution you’re interested in. In that case, the first step would be to measure its absorption spectrum and find out experimentally what wavelengths are absorbed best. The spectrophotometer has a program which will automatically try every wavelength and give you the absorbance at each one. Here’s the procedure:

1. When the Main Menu appears, insert the Spectrum Program Pack into the card slot and select “Option Prog Pack” from the menu.
2. You should now see a screen on which spectrum parameters can be set. Set the Scanning Range to the range of wavelengths you want to measure. The Recording Range sets the limits for the absorbance measurement, so if you expect your absorbance values to be, say, less than 2, you would set this to go from 0 to 2. The Scan Speed can usually be left at “Medium.”
3. Place the blank in the machine, close the lid and press BASECORR to scan the blank.
4. Replace the blank with the sample to be scanned and press START.
5. When the scan is complete, you will see a graph showing the absorbance of your sample at each wavelength. You can press the ZOOM key to change the scales of the axes, for example to get a “close-up” of a particular region. If you press the PEAK key, you will get a listing of the wavelengths at which the most absorbance was measured (peaks on the chart) and the absorbance at those points.

Using the Spec-20 spectrophotometer

1. Turn on the spectrophotometer by turning the left knob clockwise until it clicks. Use this knob to adjust the pointer to zero on the transmittance scale (left end of the scale), or to set the digital display to zero in transmittance mode.
2. Let the spectrophotometer warm up for 15 minutes.
3. Use the large knob on top of the spectrophotometer to set the desired wavelength.
4. Again adjust the zero point with the left knob.
5. Place a blank in the sample holder and use the right knob to adjust the reading to 100% transmittance (or zero on the absorbance scale). Remove the blank.
6. If you are using the digital spectrophotometer, use the Mode switch to choose absorbance or transmittance mode as needed.
7. Place your sample in the sample holder and read absorbance or transmittance.

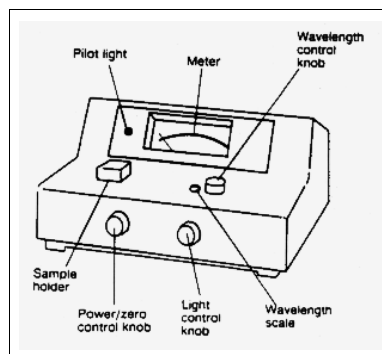


Figure 2. Diagram of a Spec-20 spectrophotometer.

Determining concentration with a standard curve

There are two ways we could measure the concentration of some unknown solution using the spectrophotometer. The first is to generate a **standard curve**: a graph of absorbance vs. concentration for standard solutions whose concentrations are known. We then compare the absorbance of an unknown solution (of the same molecule) to that curve. We don't have to know anything about the molecule in advance. Essentially, we are "calibrating" the spectrophotometer to measure the concentration of the molecule we're interested in.

To make a standard curve, you'll need at least three or four different solutions with known concentrations (called **standards**). You can come up with these easily by taking a single solution of known concentration and making several different dilutions of it (see the section on measurements and solutions on page 67). Then, measure the absorbance of each known solution and draw a graph, plotting absorbance vs. concentration, as shown in Figure 43.

In this graph, concentration is placed on the X-axis, because it's the independent variable—the one we're manipulating. The absorbance is on the Y-axis, because it's the dependent variable: the one whose value depends on the independent variable.

The line on this graph represents the *relationship* between absorbance and concentration. This should not be a "connect-the-dots" line: it needs to be a **best-fit** straight line. Computer programs like Excel can calculate the equation of the best-fit line for you and so can most scientific calculators. See the graphing section of this manual (page 49) for some help with this. Generally, you want to include (0,0) as one of your points.

After generating a standard curve, you can measure the absorbance of the unknown solution and compare it to the absorbances of the known solutions. You could do this by eyeballing where the unknown would fall on the graph, but it's more accurate to use the equation of the best-fit line (in the form $y = mx + b$) to calculate the concentration. Here, y is the absorbance, which you measure, x is the concentration that you want to know, and m and b are the slope and intercept of the best-fit line, which Excel will tell you. By solving for x , you can accurately calculate the concentration of the unknown.

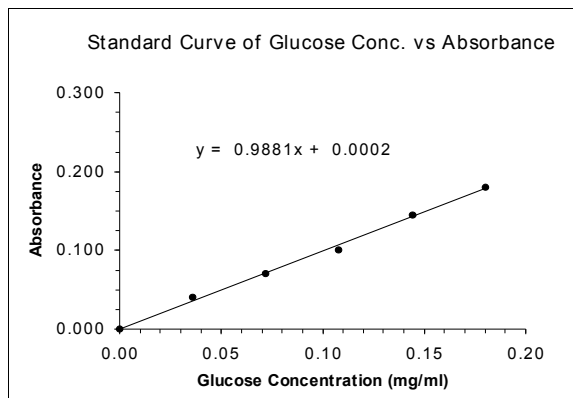


Figure 3. Standard curve used to determine the concentration of an unknown solution

In order for this calculation to be accurate, the absorbance of the unknown has to fall within the range of absorbances of the known solutions—if it doesn't, you may have to dilute the unknown, or make different standards and try again. It's important to note that the relationship between absorbance and concentration is only linear within a certain range, and only when you use the right wavelength of light. The "right" wavelength is λ_{max} , or the wavelength at which the molecule absorbs best. If you don't know which wavelength to use, you can determine λ_{max} experimentally by measuring the absorption spectrum as described above. The peak wavelength is λ_{max} .

Determining concentration by the Beers-Lambert law

The second way to measure concentration using the spectrophotometer is really the same as the first, but with a shortcut. If you (or someone else) has already determined λ_{max} and drawn a standard curve which shows a linear relationship between absorbance and concentration, then you can calculate a number called the extinction coefficient, which you can always use to find concentration for that molecule under those conditions.

The extinction coefficient simply relates concentration to absorbance, using a formula called the Beers-Lambert law. In this equation, the absorbance is equal to the concentration of the molecule of interest (c), times the length of the path the light takes through the cuvette (b ; always 1 cm for our spectrophotometers), times the extinction coefficient (ϵ):

$$\text{Abs} = \epsilon bc$$

The extinction coefficient is the part that is specific for each compound and must be determined experimentally. You can determine it from your standard curve by solving for ϵ , because you know the absorbance and concentration and can measure the width of the cuvette. Once you know ϵ , you can use it for other solutions of the same molecule.