

College of the Canyons: “Introduction to Biotechnology” Custom Lab Exercises



Protein Standard Curve

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- Standard curves are used in many assays in biotechnology.
 - The idea involves making a series of standard solutions that are then assayed for their value. These values can be used to generate a graph, which can then be used to determine the concentration of unknown protein samples.
 - The most typical assay is called a colorimetric assay. This is the type of assay that was used in the gel filtration lab.
 - In this experiment you will create multiple protein standard curves using albumin protein with a known concentration (2mg/mL, undiluted) as your standard.
 - Replicates (repeated experiments) will be compared with data from single experiments to help demonstrate the significance of replicates in determining confidence in experimental results.
 - Graphs with a “small range” and a “undefined / large range” will be prepared and compared to unknown samples that can be assessed using either the small or undefined /small range graphs.
 - Data will be generated using a colorimetric assay with Cu^{++} ions reacting with amine groups in the protein. A 96 well micro plate reader will be used to allow for rapid replicate work and reading of samples @ 562 nm light wavelength.
- For more information on College of the Canyons’ Introduction Biotechnology course, contact Jim Wolf, Professor of Biology/Biotechnology at (661) 362-3092 or email: jim.wolf@canyons.edu. Online versions available @ www.canyons.edu/users/wolfj

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I. Objectives:

1. To understand the importance of standard curves and assays in biotechnology.
2. To become proficient at making standard solutions for a both a broad range of samples (unknown /large range, 0.0156– 2 ug/ml 3 orders of magnitude) and with in a narrow, defined range (small range, 0.2-2 mg/ml, one order of magnitude).
3. Become proficient at operating a microplate spectrophotometer including setting absorbance values, defining “reading cells” and “blanks” and generating a raw data report which is then loaded in Excel.
4. To create a series of protein standard curves after obtaining the above data. Using Excel, compare the effect of replicates on “R values” and to understand the role of “R values” (confidence values) in generating statistical rigor.
5. To be able to use these standard curves to determine the protein concentration of provided unknown samples and subsequent samples extracted from you soon to be cultivated cell cultures.

II. Background:

Standard curves are used in many assays in biotechnology. In addition to protein concentration assays, other types include: DNA/RNA assays that use UV light to visualize the nucleic acids directly in the spectrophotometer and isotopic assays that count the amount of radioactive product in particular sample (as in “pulse / chase” assays used to identify Krebs cycle compounds).

The protein assay lab will consist of three distinct phases:

- 1. Standard preparation:** Your team will be given an ampule of protein. This protein is albumin, and is at the concentration of 2mg/ml. This assay technique will work for protein concentrations from 20-2000 μg . If the protein concentration is higher or lower, the data becomes nonlinear and the standard curve needs to be adjusted. To adjust for concentrated samples, a dilution is done, and for very dilute samples, either a more sensitive spectrophotometer is needed, or the samples are concentrated (via osmotic dialysis or other techniques).
- 2. Develop the samples:** A 50 to 1 solution of protein color development solution will be prepared. The solution is unstable and must be mixed within a few hours of its use. It is a redox reaction that involves the reduction of Cu^{+2} to Cu^{+1} . The amount of this reduction is dictated by the amount of protein (in an alkaline media) and the Cu^{+1} then interacts with bicinchoninic acid to create a color change. This is essentially a modified Biuret reaction similar to that employed in the gel filtration lab. The name of the particular test kit we use is a BCA Protein Test Kit.

3. **Collect the data:** Once you have prepared your samples you will use a spectrophotometer to collect the ABS 562 data for the samples and two unknowns. You will then graph your known samples and, using the standard curve you have just created, determine the unknown sample protein concentration by interpolating the ABS to the protein concentration. You will also assess your unknown using other group's graphs in order to get a feel for the accuracy of this technique.

NOTE: The plate reader and computer need to be turned on and set up. The first students in lab (or reaching this point in the lab protocol) should turn on the device(s) and set-up the 96 plate well to allow for the protocol to be executed in the manner described in this protocol. The protocol for this is under section 3. Plate Reader.

REMEMBER TO USE A MICROPIPETTE or GRADUATED CYLINDER FOR ALL STEPS.

On the last page of this protocol are some blank plates and accompanying excel spreadsheet of all 96 wells. This may be useful in helping you keep track of what samples went where, etc. Remember, a standard curve is only as accurate as the technician is meticulous.

III. SOP/Lab Activities: Standard Curve Within a Known Range.

Important: Always note any addenda that are posted by the instructor. These should be noted in the instructions part of your notebook and reflect recent changes to the procedure that may not have made it into the written protocol. A standard curve with in a known range is sort of a "unknown with in a known". This may seem odd, but is actually very common. Most tests on unknowns have an expected range (i.e. glucose level in blood, hormone level in water sample, etc.)

The **BSA** protein reagent is commercially available in a wide range of concentrations and the type we are using in lab is 2 mg/ ml. The upper limit is dictated by how soluble the albumin is, how dark the BSA/BCA solution becomes, the cuvette volume (and therefore the amount of sample the light must pass through). The lower limit is determined by the resolution of the test. The **BSA** test can give you readings from 2 mg/ml all the way down to 0.002 ug/ml. Despite this large range many tests never need this wide range of resolution. Most blood tests, many food tests, routine testing etc. fall within a expected range commonly, (so, are an unknown within a known). To help find this small needle is a smaller haystack complete the first phase below.

1. Standard Preparation: (Unknown with in a known range).

- 1.1 Aliquot the following solutions in 10 labeled microfuge tubes. (Be sure and use the 1.5 ml, the pointed bottom tubes). ****Note:** this particular set of standards is not prepared by executing a serial dilution (compared to the next set of samples).

Tube #	Protein (µL)	0.9 Saline (µL)	Concentration (mg/mL)**
1	100	0	2 mg/ml
2	90	10	mg/ml
3	80	20	mg/ml

4	70	30	mg/ml
5	60	40	mg/ml
6	50	50	mg/ml
7	40	60	mg/ml
8	30	70	mg/ml
9	20	80	mg/ml
10	10	90	mg/ml

- 1.2 Now make ***two extra tubes*** (ensuring the total of each tube is 100ul) of any two of the above 10 concentrations. These will be unknowns that you will give to your lab colleagues so that they may deduce the concentrations using the standard curve. Remember to clearly label these tubes and to mark down the concentrations in your lab book. It goes without saying that these values should be kept confidential until later in the lab. Save your remaining albumin solution in case you make a mistake in later steps. As an extra security measure, write your unknowns here:

Unknown _____, CONC. _____ Unknown, _____ CONC: _____

1.3 Vortex for 30 seconds to mix.

- 1.4 ** Do not perform the concentration calculations yet as you will have time to do this later in the lab, during incubation.
- 1.5 Place the samples on ice until you develop all of the samples in a later step. Remember to trade unknowns with another group and record your unknowns somewhere for easy reference.

2 SOP/Lab Activities: Standard Curve Within a Unknown Range.

Often a unknown becomes a “super-unknown”. As a cell culture grows, the amount of protein produced can vary wildly. In many Haz-Mat responses, the amount of contaminating reagent is not known. Unlike lots of medical tests, which routinely fall within an expected range (see above), the test is not able to rely on the fact that there is an expected range for the sample to be in. Sometimes, tests completed as noted above fall out of range. In these rare cases, a broader range of test can be conducted to help find this “outlier”. To complete these standards, you will do a serial dilution (see metric lab for clarification). Depending on what type of range we are looking at, the test reagents being used, the device being used, this will decide the type of serial dilution you will be doing. Sometimes a simple 1 to 1 dilutions is done. Other times, 1 to 3, 1 to 10, etc. For the purposed of this lab, we will do one to one and dilution. Starting with the most concentrated sample (2 mg/ml stock BSA) and dilute it one to one. As you go down this series, you will eventually get to the lower limit of the test, device, etc. Also, the unknowns assess with these types of curves tend to be more likely to be off by a significant amount (as the concentration axis spans orders of magnitude, not just say 2 – 0.2 mg/ml, as seen in the previous experiment). For the sake of expediency, we will not be doing unknowns for this graph, but we will be doing replicates and actually will be using this graph later when we revisit this lab/ graph when we look at the protein concentration of your cell cultures.

2. Standard Preparation: (Unknown with in a unknown range).

- 2.1 Aliquot the following solutions in 6 labeled microfuge tubes. (Be sure and use the 1.5 ml, pointed bottom tubes). NOTE: start with tube 1X, you will using this tube to prepare the following sample. Tube 1X will contain 600ul. So, for sample 2, take 300 ul from tube 1X and add to tube 2X. Mix with 300 ul of saline by vortexing, etc.

Tube #	Protein (µL)	0.9 Saline (µL)	Concentration (mg/mL)**
1X	200	0	2 mg/ml
2X	100 µl of 1X	100	1.0 mg/ml
3X	100 µl of 2X	100	0.5 mg/ml
4X	100 µl of 3X	100	0.25 mg/ml
5X	100 µl of 4X	100	0.125 mg/ml
6X	100 µl of 5X	100	0.0625 mg/ml
7X	100 µl of 6X	100	0.0312 mg/ml
8X	100 µl of 7X	100	0.0156 mg/ml

- 2.2 Place the samples on ice until you develop all of the samples in a later step.
- 2.3 Collect the samples from both curves and be sure each is clearly labeled. You should have 10 samples labeled 1-10, 2 unknowns and 8 samples label 1X-8X. Make sure they have been vortexed and now, centrifuge them to gather all of the samples to the bottom of the microfuge tube.
- 2.4 Get about 300 ul of 0.9% saline for blanks, and label tube accordingly.

STOP POINT: The next few steps will take AT LEAST one hour. Depending on your comfort with computers, pipetting, etc. you may need to freeze your samples and read them next time In the interim, read ahead and complete other exercises as noted in the lab syllabus.

The following steps may not all need to be completed by you. If you are NOT the first group to use the plate reader today, you can “jump in” at step 6 and get your data. Do take a moment and review the overall idea (steps 1-10), but *for the purposes of getting data, only steps 7-10 need to be addressed.*

3. PLATE READER:

This needs to be turned on (and the computer too) to allow for it to warm up. This procedure will only get the device started. One student should set up the “field”. It basically involves telling the computer what to read, what is a blank and what is can ignore. Here is the general SOP for this and ask the instructor if not sure.

1. Turn on the micro plate Bio-Rad Model 680 Plate Reader by reaching around to the back of the device and flipping the switch.
2. On the Reader, enter the password (0000), and then press ENTER.
3. Turn on the PC, after allowing it to warm up, select the program “Micro plate Manager 5.2.1.” Also select to open Excel, and as the program comes up, minimize the window for later use.
4. Select **File**, then scroll over **New Template**, and click on **12x8**
5. Fill the micro plate template (you will fill the plate itself later) according to the table provided (on next page). Once you have filled your microplate data field accordingly, return to these instructions to obtain your results. It is now time to review the field and set each well to read accordingly:

- a. Select the circle containing an “S” in it and click on the wells which are to be read as “Standards”
 - b. Select the square containing an “X” in it and click the wells which are to be read as “Unknowns”
 - c. Select the diamond containing a “B” in it and click the wells which are to be read as “Blanks”
 - d. Select the option with an “A” on it, then high-light all the wells contained which are made up of your first dilutions (samples number 1-10). These will be marked as a different “Assay” than those, which are not highlighted (1X-8X). This will make looking at the spreadsheet of data a little easier, but will not affect the data in any way.
 - e. Once this has been set up once, these steps can be ignored. However, it is a good idea to check if the correct wells have been set appropriately.
 - f. Before you move on, take some time to mark down what you have put in each well. You can do this on the sheet that is provided to you on the last page of this protocol. It is important you do not skip this steps for several reasons. The most important is that you may not be able to finish the lab today, and you will learn that it is difficult to remember the contents of each well when they all look the same.
6. Next, click **File**, and select “**New Endpoint Protocol**” and Select **550 nm** (closest pre-set filter to 562 nm) for the wavelength.

NOTE: This set up of the microplate reader is explained in step 3(previous), and the samples are prepared in step 4 (below). Be sure you understand this idea, as simply placing a plate into the reader without the proper sample prep will result in ZERO results!...So...read ahead and know what you are doing for these crucial steps..

7. Place your plate into Biorad Machine. Be very gentle and note the beveled (cut edge) that ensures you put the plate in with the correct orientation. Close the lid to the plate reader.
8. Hit **Run** on the endpoint protocol screen. The device will take a minute or so to complete its analysis.
9. Another box will appear on your screen. This contains the data from your micro-plate. Highlight and copy the raw data report (from the spreadsheet on the screen), and paste it into an Excel spreadsheet. If the computer will not allow for transfer of the data, print up a data report from the plate reader itself. Use this report to review when entering the data into Excel.
10. Save the file, Print up a hard copy, email your self the file as well. Remember to do the same for your lab partner and when in doubt...make extra copies...Label everything and DO NOT ASSUME ANYTHING!

4. Developing the Samples:

On the last page of this handout are a few blank plates templates. Use them to record what samples went where. Record which wells had samples that were standards, blanks, unknowns, and those left unfilled.

1. Get a micro plate well array (96) and take a moment to note the letters and numbers. There are 8 letter lines, and 12 lines numbered one to twelve. Starting

the first set of unknowns (tubes 1-10). You will first need to add your protein samples into all the indicated wells. Use the below grid to guide this process. Like wells can use the same pipet tip (i.e. A1, B1, C1). Rows A,B and C will have your 10 samples from the 2-0.2 mg/ml BSA standards. Rows D,E,F will have the 8 samples (labeled with number and X) with BSA samples ranging from 2 mg/ml to 0.0156 mg/ml. The unknowns will go into the lane G (3 samples of each unknown) and blanks will go at the ends of lanes D-E and F. 6 blanks are sufficient.

	1	2	3	4	5	6	7	8	9	10
A	25 μ l tube 1	25 μ l tube 2	25 μ l tube 3	25 μ l tube 4	25 μ l tube 5	25 μ l tube 6	25 μ l tube 7	25 μ l tube 8	25 μ l tube 9	25 μ l tube 10
B	25 μ l tube 1	25 μ l tube 2	25 μ l tube 3	25 μ l tube 4	25 μ l tube 5	25 μ l tube 6	25 μ l tube 7	25 μ l tube 8	25 μ l tube 9	25 μ l tube 10
C	25 μ l tube 1	25 μ l tube 2	25 μ l tube 3	25 μ l tube 4	25 μ l tube 5	25 μ l tube 6	25 μ l tube 7	25 μ l tube 8	25 μ l tube 9	25 μ l tube 10
D	<u>25</u> μ l tube 1X	<u>25</u> μ l tube 2X	<u>25</u> μ l tube 3X	<u>25</u> μ l tube 4X	<u>25</u> μ l tube 5X	<u>25</u> μ l tube 6X	<u>25</u> μ l tube 7X	<u>25</u> μ l tube 8X	BLA NK	BLANK
E	<u>25</u> μ l tube 1X	<u>25</u> μ l tube 2X	<u>25</u> μ l tube 3X	<u>25</u> μ l tube 4X	<u>25</u> μ l tube 5X	<u>25</u> μ l tube 6X	<u>25</u> μ l tube 7X	<u>25</u> μ l tube 8X	BLA NK	BLANK
F	<u>25</u> μ l tube 1X	<u>25</u> μ l tube 2X	<u>25</u> μ l tube 3X	<u>25</u> μ l tube 4X	<u>25</u> μ l tube 5X	<u>25</u> μ l tube 6X	<u>25</u> μ l tube 7X	<u>25</u> μ l tube 8X	BLA NK	BLANK
G	Un- known 1	Un- known 1	Un- known 1	Un- known 2	Un- known 2	Un- known 2				
H										

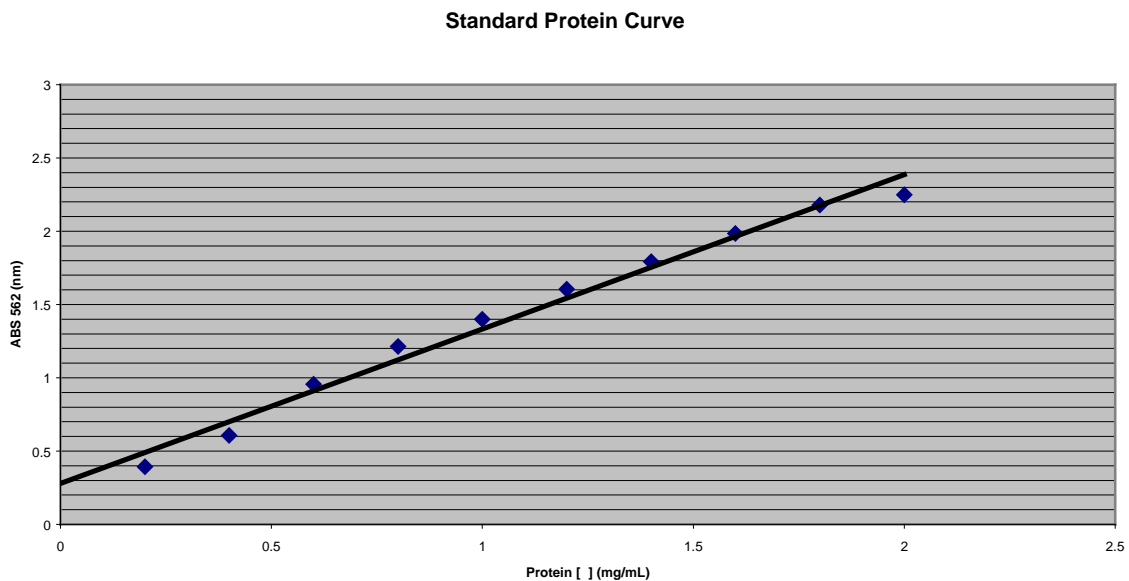
- Now take a moment to prepare the BCA reagent. It is a 50 to 1 mixture that needs to be prepared fresh. A quick tally of the wells shows that we have 30 for three sets of standard curves 1, 24 for standard curve 2, 6 blanks and 6 unknowns. This should give us 66 wells. Each well will require 175 ul of the BCA solution. So...lets prepare for 70 wells (allows for some overage). For the samples we are working on, a 1;8 ratio is recommended...so 25ul of protein and 175 ul of BCA is good.)
- Label to 15 ml falcon tube, "BCA working reagent". 175 ul times 70 wells equals 12.25 mls. Round to 13 mls (again for ease of measuring and some overage). The BCA is a two-part reagent with a 50:1 preparation ratio. BCA reagent, part A. 50/51 of 13 mls is 12.74 mls. I would recommend a 100-1000 ul pipet and for the 740 ul ul and a graduated cylinder for the 12 mls. Part B is 1/51 of 13 mls or 255 ul. Use a fresh tip and 100-1000 ul pipet. There is likely a set of this equipment next to the BCA, FYI... Put cap on solution and vortex to mix. The solution turns a bright green and is stable for a few days if kept refrigerated.
- Working quickly, add 175 ul of the BCA reagent to each of the wells (using a 20-200 ul pipet). Do not forget the blank wells at the end of the "X-series samples, and also add to the unknowns. Save any unused samples for colleagues who may get caught short. Avoid letting the tip or the pipet touching the well or its contents. This way, you will not need to change tips between samples. Be sure the tip is far enough into the well so as to avoid spillage, but not so far in as to contaminate the tip with the protein.

5. With the plate sitting on the counter top, slowly swirl the plate to allow mixing. Do this for about a minute.
6. Place a plastic tape cover on top of the plate, and using a “brayer” (a roller) to smooth the tape cover onto the plate. Using a small piece of masking tape, label your plate (but sure to place it on the tray in such a way that it can be easily removed as needed).
7. Place plate in 60 °C heat bath for 15 minutes. The incubator is set-up to accommodate your plates. During this time, make sure the plate reader is good to go and if there is time review the following:
8. During this time determine the protein concentration of the samples you prepared by using $C_1V_1=C_2V_2$. For example, tube #2 contains 90 μ L of the 2mg/mL stock protein solution and 10 μ L of the saline solution. Therefore:

$$\left(2 \frac{\text{mg}}{\text{mL}}\right)(270 \mu\text{L}) = (C_2)(300 \mu\text{L}) \quad ? \quad C_2 = 1.8 \frac{\text{mg}}{\text{mL}}$$

3. Graphing the data:

1. Collect the ABS data for the various samples and your unknowns, etc. using the above noted SOP for the plate reader. Please note: if the computer is acting up...a raw data report can be printed as needed.
2. Using Excel, graph the net protein concentration verse absorbance. This is an example of a completed standard curve. Here is an example graph:
3. Make sure that your graph has a bet fit line and ensure that both axis are as high a resolution as possible (they should have lots of divisions in the units of the axis).
4. While is Excel, note the following page, as you will be making 4 distinct graphs with R values. The example graph below is lacking the R-value and extra “ticks” on the X-axis. This exercise is designed to help you better understand the idea and need for replicates.



You will be asked to make 4 graphs. This exercise is to help familiarize you with Excel as a program. Excel is a **INDUSTRY STANDARD**. Entry- level folks are

expected to have some familiarity with this program. “Mini tab” and “Systat” are used in college classes, but rarely anywhere else. PLEASE NOTE: Every version of Excel is slightly different from the other versions. Sometimes what you need is part of a drop down menu, other versions it is a button. Still other versions have a “chart wizard” to walk you through the process of making a graph. This said, you will struggle some, but I am confident you can find your way through the program and create the 4 graphs requested below. All four graphs are scatter plots and the R-value is also called a “trend-line”.

- **Graph One:** One data set: 0.2-2 mg/ml protein range. Get the R-value for this, and ensure the graph has ticks @ the 0.1 value.

- **Graph Two:** Three data sets: 0.2-2 mg/ml protein range. Get the R-value for this, and ensure the graph has ticks @ the 0.1 value.

- **Graph Three:** One data set: 0.01562 – 2.0 mg/ml protein range. Get the R-value for this, and ensure the graph has ticks @ the 0.1 value (on linear axis only).

- **Graph Four:** Three data sets: 0.01562 – 2.0 mg/ml protein range. Get the R-value for this, and ensure the graph has ticks @ the 0.1 value (on linear axis only).

Remember to back up your information. This can be very frustrating, so it is OK to yell at your computer! It is one of the few things you can yell at with little concern as to repercussion! Ideally, you will print this out in lab, but if you do email or upload your files to another computer, be aware that format / Excel version changes can REALLY alter your graphs.

Note: graphs 3 and 4 have a log axis as compared to graphs 1 and 2, which are linear / linear axis.

Using the standard curve determine the protein concentration of your unknowns by interpolating the ABS to the protein concentration. Be sure to show this interpolation on your graph in your lab notebook.

Compare your unknowns to the other 3 graphs you created. Record the range of values from your interpolations in your lab notebook.

All of the data and the standard curve graph should be placed in to your lab notebook. Remember that you will be using your standard curve to determine the protein concentration of your cell line in a later lab.

IV Post-Lab Questions/Activities: The following post lab questions are for your benefit. The questions will help you to address a range of topics relating to the lab activity. Along with the post lab handouts, these questions will help to ensure that you have both correct information regarding the lab data and crucial lab processes. Complete the post lab questions at the end of the lab and post lab handouts (keys for both of these are available from your instructor) before making any lab-notebook entries.

1. What correlation is apparent between net absorbance and protein concentration?
2. How do replicates affect the confidence one has in their data? Hint: Note the R values in single verse multiple data sets, log/linear, linear / linear.

3. Why is it not appropriate to use the log based graph when assessing samples with a known range between 0.2 and 2 mg/ml? Hint: Mention the axis values and effects of interpolation between the two graph types.

NOTE: Do not answer each question in your lab notebook. Instead, you want not consider them when putting your final ideas on paper.

V: Notebook Entries: Data from the lab should be the focus of this section and if there are any incorrect results, you should discuss this as well as expected results. Section V will contain both your results and discussion. Your data should drive the discussion. An informed discussion is dependent on understanding the post lab questions/activities.

Your intro should: be 6-7 sentences and address the following.

- Define standard curves and use in science.
- Dilution to create standards and visualizing via Bradford's and spectrophotometer.
- Types of unknowns and the standard curves prepared to investigate them.
- Determination of unknowns via use of computer generated standard curves and effects of axis and replicated on accuracy.

Results should be:

- Raw data from spreadsheet. Do not forget to label the data.
- 4 computer generated graphs with best-fit line: 0.1 mg/mL demarcations (on liner axis only), best-fit line, R-values and complete/accurate labels.

Discussion should consider the following:

- Reasons for variation in the curve points (technique, equipment, axis).
- Values for unknown and use of curve to interpolate these values, range of values from different graphs.
- Why were some graphs more accurate than others? Reference replicate # and axis, earlier points (note previous points). Keep discussion to NO MORE the 2 paragraphs!

The previous lab protocol can be reproduced for educational purposes only. It has been developed by Jim Wolf and edits and insights from Brenda Velasco, and/or those individuals or agencies mentioned in the references.

References: BCA Protein Assay Kit; Pierce Inc. 3747 N. Meridian Road, P.O. Box 117, Rockford, IL 61105 www.piercenet.com

