	SKILLS CENTER STANDARD OPERATING PROCEDURE	A BIOFIZZ PRODUCTON
Protein Assay **Module Hours: 3.0**	**Effective Date: 22/01/2022** **PRQs:** **Buffers and stock solutions**	**Revision 1.2** **Author: Z. Hazlett-Klein** **Checked by: M. Stowell**

Background

Simple, accurate, and reliable methods to measure protein concentration are necessary for protein-related research. As soluble purified protein is not easily observable by the naked eye, modern protein quantification techniques take advantage of the characteristics of light to measure protein concentration. Several methods are common in biology ranging from those that directly measure how much light a protein absorbs to those that use chemical intermediates that turn colors in proportion to the quantity of protein in solution. This SOP will provide instruction on the use of three common protein quantification techniques as well as a comparison of their utility.

<u>**UV Vis Protein Assay**</u>
Protein quantification using ultraviolet-visible spectroscopy, or "UV Vis"**,** is one of the most common techniques for measuring the protein concentration of a solution. Proteins have an intrinsic ability to absorb light at different wavelengths. The aromatic amino acids tyrosine and tryptophan absorb ultra-violet light with a peak at 280 nm. A spectrophotometer can be used to measure a solution's absorbance of light at 280 nm (A280). In order to calculate concentration from an absorbance value, we must look to Beer's Law which states that
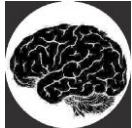
The absorbance, A, is proportional to the path length through the sample, b, and the concentration of the absorbing species, c.[1]

$$A \propto b \cdot c$$

With the addition of the molar absorptivity constant, ε, (also known as the extinction coefficient) which provides information regarding how strongly a particular chemical species attenuates light at a given wavelength, we are left with:

$$A = \varepsilon \cdot b \cdot c$$

As the value of absorption and the extinction coefficient are both dependent on the wavelength of light, λ, used in the measurement, we can write the final equation for Beer's Law as a function of wavelength:

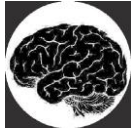$$A(\lambda) = \varepsilon(\lambda) \cdot b \cdot c$$

For our purposes, we can take the absorption reading at 280 nm, find the extinction coefficient of our substance at 280 nm, and with a known path length, solve for our molar concentration.

$$A(280 \text{ nm}) = \varepsilon(280 \text{ nm}) \cdot b \cdot c$$

$$A / (\varepsilon \cdot b) = c$$

While protein quantification using UV Vis is simple, accurate, and reliable, it does have a few significant drawbacks. First, your protein solution must be pure. Any mixture of two or more proteins makes this method an unreliable means of detecting total protein content. Also, if there is contamination of nucleic acids in your sample, which have some absorption at 280 nm, the reading of protein concentration will not be accurate. Next, protein absorption of light at 280 nm is dependent on aromatic amino acids. If your protein of interest has very low or no aromatic amino acids, then there will not be absorption at 280 nm sufficient for accurate measurements. Thankfully many other methods have been established to provide reliable means of protein quantification under these and other types of conditions.

Because this technique is a direct measurement of pure protein in solution, blanked against its buffering conditions, and does not rely on any intermediate steps, this method offers high precision as well as high accuracy if all the proper conditions are met. There are cases where these conditions cannot be met, however, so other techniques have been developed to provide reliable alternative ways to measure protein content.

Bicinchoninic acid (BCA) Protein Assay

The BCA Assay is another easy method to measure protein concentration in solution using a two-step chemical reaction resulting in a measurable color change. In this assay, soluble protein reacts with and reduces Cu^{2+} to Cu^+ in direct proportion to its concentration in solution. The reduced copper reacts with BCA producing a purple-colored product that absorbs light at 562 nm (Figure 1). The assay relies upon first establishing a standard curve of protein concentration versus absorbance at 562 nm for a series of protein standards with known concentration. Then a protein solution of unknown concentration can be measured, and its concentration determined by identifying matching its A562 value with the concentration on the standard curve.

Protein quantification using BCA is slower than direct measurements of intrinsic protein absorbance at 280 nm but provides additional benefits that are not available in the UV Vis assay. These benefits include the ability to measure concentration of a protein without any knowledge of what the proteins is, i.e. one does not need an extinction coefficient for the protein being measured. With the BCA assay one could even measure the total protein concentration of a solution containing more than one purified protein. Lastly, this assay is more resistant to contamination with other non-protein molecules such as nucleic acids as the copper reduction step is specific to protein chemistry. However, the presence of thiols, phospholipids, and/or ammonium sulfate interferes with the measurement.[3] If these are known components of your solution, consider alternative methods for protein quantification.
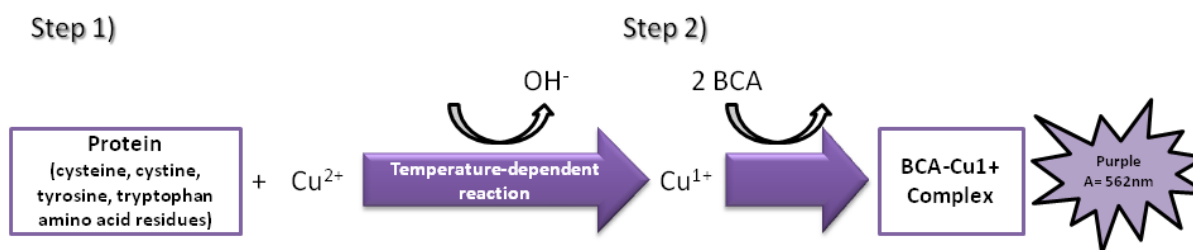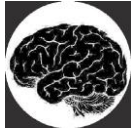

Figure 1. Two Step BCA reaction resulting in a purple color change that absorbs light at 562 nm.[2]

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Where the BCA assay provides a more versatile means of measuring protein concentration, this comes at the cost of reduced precision and accuracy. To ensure the highest degree of precision and accuracy, one must prepare all standards and reagents carefully and consistently. Slight errors in pipetting can lead to less than accurate results. One must consider these variables in terms of their desired experimental outcomes and make their judgements accordingly.
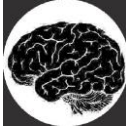
Pierce 660 nm Protein Assay

The Pierce 660 nm protein assay is another dye-based protein assay compatible with detergents and reducing reagents.[5] The Pierce 660 nm protein assay uses a ready-made reagent containing a proprietary dye-metal complex that binds to protein in acidic conditions causing a shift in the dye's absorption maximum at 660 nm. The principles of determining protein concentration using this assay are similar to that of a BCA assay, except the reagent comes ready-to-use and the wavelength at which the absorption is measured is 660 nm. If you plan to use this assay to measure total protein concentration of a cell lysate containing detergents, there are some specific details that must be followed such as mixing the reagent with an Ionic Detergent Compatibility Reagent (IDCR). For our purposes here, our buffers do not contain detergents, so steps such as these are not required.

With the greater versatility for buffering conditions that the pierce 660 nm protein assay provides, there are still drawbacks. According to ThermoFisher Scientific, this protein assay has a higher protein-to-protein variability between measurements (around 37%) compared to other assays[5], making the accuracy and precision of readings even less than that of BCA and UV Vis. However, given the right context, this assay can be very useful for routine measurements of protein concentrations in which the protein solutions contain components incompatible with other assays.

1. Purpose

The purpose of this SOP is to instruct students on the concepts and techniques of two common protein quantification assays.

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2. Scope

This procedure applies to qualified skills center users.

3. Responsibility

3.1. It is the responsibility of the user to understand and perform the procedure described in this document.

3.2. It is the responsibility of the user performing the procedure to fully document any deviations from the written procedure.

3.3. It is the responsibility of the user to become trained on the procedure.

4. Definitions

4.1 <u>Spectroscopy</u> – The categories of science concerned with how matter interacts with or emits light.

4.2 <u>Beer's Law</u> – A law that connects the magnitude of light absorbance to the quantity of absorbing material, a constant regarding how the material interacts with light (extinction coefficient), and the length of the path through the material. Gives us the useful equation "$A = \varepsilon \cdot b \cdot c$". A, absorbance. ε, extinction coefficient. b, pathlength of light. c, concentration.
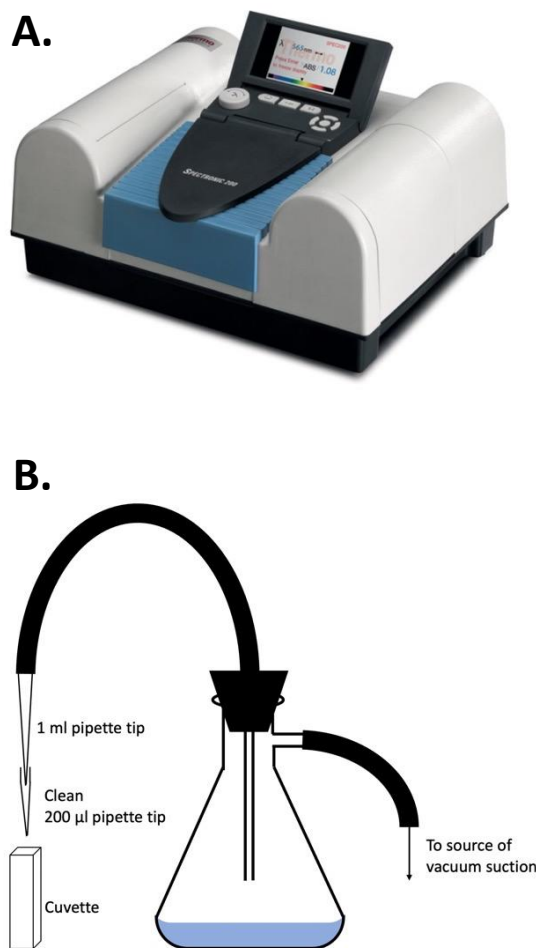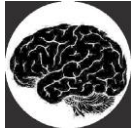
A.

B.

Figure 2. A) Spectronic 200. Source: Thermo Fisher Scientic Cat # 840-281700. B) Vacuum aspiration apparatus used here to clean cuvettes.

4.3 <u>Extinction Coefficient (ε)</u> – A constant of a material determined based on its ability to attenuate light.

4.4 <u>Standard Curve</u> – Also known as a calibration curve. A graphical representation that establishes the outcomes of a set of known standards and is used to determine the unknown variable of a test sample using the same outcome variable. For example, one can measure the absorption of light at 280 nm for a set of BSA protein standards of known concentrations. Comparing the A280 of a sample with unknown protein concentration to the standard curve can help in the determination of that sample's concentration.

5. Materials/Equipment

5.1 <u>UV Vis + BCA</u>
    5.1.1 Spectrophotometer (Spectronic 200; Figure 2A)
    5.1.2 Pipettes and tips
    5.1.3 BSA sample to be measured
    5.1.4 Clear plastic cuvettes (Total volume 50-200ul)
    5.1.5 Double distilled water (ddH20)
    5.1.6 Cuvette cleaner
        5.1.6.1 We use a ddH20 squirt bottle and vacuum aspirator (Figure 2B)

5.2 <u>BCA or Pierce 660 nm assays</u>
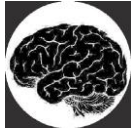   5.2.1 0.5 ml Test tubes
    5.2.2 96 well plate
    5.2.3 Plastic (Saran) wrap
    5.2.4 BCA reagents or Pierce 660 nm reagents
    5.2.5 BSA standards
    5.2.6 Microsoft Excel

6. Procedures

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UV Vis Protein Assay

6.1 Turn on spectrophotometer or plate reader

6.2 Pipette 100 µl ddH20 into cuvette well or plate well. Be careful not to introduce bubbles or they will disrupt the path of the light and the absorbance reading. Keep in mind that some cuvettes take larger volumes.

6.3 Open spectrophotometer hood, insert water filled cuvette, close the hood, set wavelength to 280 nm with left dial, and blank the system by pressing the "0.00" button. Remove blanking cuvette.

Figure 3. Path of light through a cuvette. Image source: Cuvette photo/shutterstock.com

    6.3.1 Consider the direction of light through the spectrometer (left-to-right). Place your cuvette such that the light path enters and exits through the clear sides of the cuvette (pathlength 10 mm). See Figure 3.
    If using the plate reader you can fill multiple wells but ensure you are using a UV transparent plate.
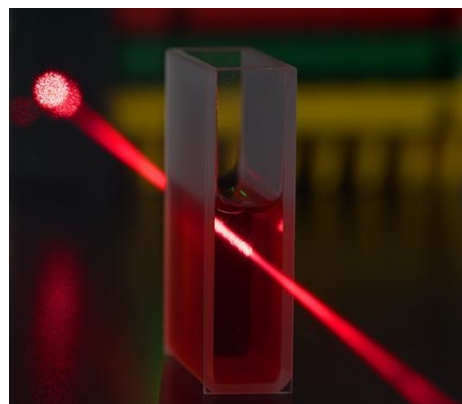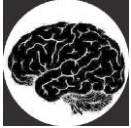
6.4 Aspirate water from cuvette with desktop vacuum aspirator. Fill new clean cuvette with 100 µl protein solution of unknown concentration.

    6.4.1 If ever needed, you can dilute a stock concentration (2-fold, 10-fold, or 100-fold) with ddH20 and take your measurements with that sample. Make sure to correct for the dilution at the end when solving for concentration.

6.5 Insert cuvette, close hood. Measure sample absorbance at 280 nm. **Collect (pipette) sample back into test tube for measurement using below colorimetric assay.

6.6 Calculate concentration of sample using Beer's Law.

6.7 Clean cuvettes by aspirating remaining contents, rinsing with ddH20, aspirating the water, then leaving out to dry. Subsequent measurements can be made with a single

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cuvette if you are confident it was cleaned thoroughly and all the water has been aspirated/dried out.

BCA Protein Assay
(Adapted from ThermoScientific - Pierce[TM] BCA Protein Assay Kit User Guide[4])
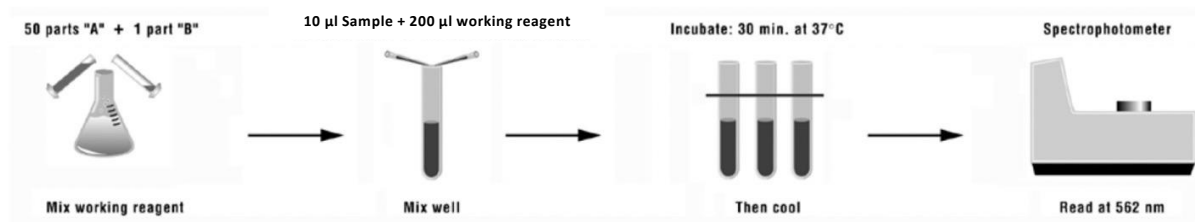

Figure 4. Procedure summary for the BCA Protein Assay[4]

6.8 Fill an insulated ice bucket with ice. Gather all reagents, thaw any frozen stocks, and keep protein solutions on ice until needed.
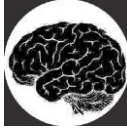
6.9 You are given a frozen stock of 100 ul of 100 mg/ml BSA. Use this to make a series of 6, 1:2 serial diluted BSA stock solutions diluted with ddH20 starting at 1 mg/ml. Reference serial dilutions in the "Buffers, stocks and solutions" SOP if needed. Set up your plan including the math and concentrations of each serial diluted stock prior to making these stock solutions.
    6.9.1 Hint: You will first need to dilute the stock 1:100 to make a solution of 1 mg/ml. I recommend doing this stepwise (1:10 dilution twice) to improve accuracy. Use this 1 mg/ml solution to then serial dilute 5 times to make 6 total BSA standards. Add a 7th sample of just water to have a baseline (no protein) measurement.

6.10 Preparation of the BCA working reagent (WR)
    6.10.1 Use the following formula to determine the total volume of WR required:

(# standards + # unknowns) ✕ (# replicates) ✕ (volume of WR per sample*) = total volume WR required.

Example: for the standard test-tube procedure with 3 unknowns and 2 replicates of each sample:
(9 standards + 3 unknowns) ✕ (2 replicates) ✕ (0.2 mL) = 4.8 mL WR required

*Note: 2.0 mL of the WR is required for each sample in the test-tube/cuvette procedure, while only 200 µl of WR reagent is required for each sample in the microplate procedure.

6.11 Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B).
For the above example, combine 5 mL of Reagent A with 0.1 mL of Reagent B.

Note: When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).

6.12 For each reaction, add 0.2 ml WR to 10 µl sample in a well in your 96 well plate. Mix well by pipetting up and down slowly.
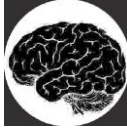
6.13 Incubate at 37ºC for 30 minutes. Then cool.

6.14 Measure absorbance at 562 nm (the absorbance of the BCA-copper complex) of each sample in the provided cuvettes, first for your standards, then for your unknown(s). Record the values for each.
Remember to use a clean cuvette for each sample or clean the cuvette between samples.

6.15 Generate standard curve

6.15.1 Plot the known concentrations of your BSA standards against their absorbance values at 562 nm.

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6.15.2 Insert trend line for the graph and determine the equation of that trend line. This is your standard curve you can use to find the concentration of your unknown sample(s), based on their absorbance values.

6.16 Determine the concentration(s) of your unknown sample(s) using the standard curve.

6.17 Clean up lab space.

Pierce 660 nm Protein Assay
(Adapted from ThermoScientific - Pierce™ 660 nm Protein Assay User Guide[5])
6.18 Prepare BSA standards for standard curve as explained in the BCA assay (Step 6.9).

6.19 Add 10 µl of each standard and unknown into wells in a 96 well plate. Keep track of/document each samples location in 96 well plate.

6.20 Add 150 µl of Protein Assay Reagent to each well

6.21 Cover plate with plastic wrap or plate cover and mix on a plate shaker (or gently by hand) for 1 minute. Incubate at room temperature for 5 minutes.

6.22 Use the blank sample to zero the plate reader. Measure the absorbance of each sample, both standards and unknowns, at 660 nm.
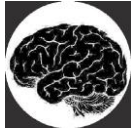
6.23 Generate a standard curve as described in 6.15. Determine concentration of unknown samples using standard curve.

6.24 Clean up lab space.

7. Troubleshooting
7.1 My BSA standards have precipitated a little or a lot.
    7.1.1   As protein precipitates out of solution, the concentration will be less than what is expected, as less protein is dissolved in solution.

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7.1.2 You get to decide here how accurate you desire to be with your measurements.

7.1.3 Sometimes protein standards will show a little precipitate that comes naturally from multiple freeze/thaw cycles. It is possible to centrifuge these samples at 4⁰C to pellet the small bit of precipitate and get results still close to the real concentration.

7.1.4 However, it is also possible that the precipitation of protein in your standards can be great enough to cause inaccurate results.

7.1.5 This type of discretion can be gained over time. For the time being, centrifuge your samples and determine concentration using the soluble fractions.

7.1.6 Observe your resulting standard curve carefully. If the results are too far from expected, consider asking a proctor for new standards.

7.2 My standard curve is not a straight line.

7.2.1 Consider the shape of your standard curve.

7.2.2 If it is straight but tapers of slightly at higher concentrations, this could be due to precipitate of standards at high concentrations.

7.2.2.1 In this case, you are free to delete these 1-3 last points, to establish a straight line of conc. vs absorbance of your standards. The more points you delete, however, the less confidant you will be in your measurement.
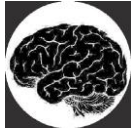
7.2.3 If your standard curve is composed of points scattered all over leading to something that is not close to a straight line, then there could be a problem in your setup or measurements.

7.2.3.1 Walk through each step you've taken up to this point and consider what might have been done wrong.

7.2.3.2 Redo from the beginning if necessary.

8. References

1. D. W. Ball, Field Guide to Spectroscopy, SPIE Press, Bellingham, WA (2006).
2. MATER METHODS 2012;2:115
3. *BCA Assay and Lowry Assays*. Thermo Fisher Scientific. Retrieved from: https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-assays-analysis/protein-assays/bca-protein-assays.html

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4. *Pierce^TM BCA Protein Assay Kit User Guide*. Thermo Fisher Scientific. Retrieved from: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0011430_Pierce_BCA_Protein_Asy_UG.pdf
5. *Pierce™ 660nm Protein Assay Reagent.* Thermo Fisher Scientific. Retrieved from: https://www.thermofisher.com/order/catalog/product/22660

9. MODULE MASTERY TASK

9.1 Choose a sample of unknown protein concentration located in the rack with the BSA standards, either A, B, or C.

9.2 Measure the concentration of your unknown sample using UV Vis and one of the two colorimetric techniques described.

> NOTE: You will need to pipette and collect the sample after the UV Vis to use in your BCA assay.
> Use online resources to determine the extinction coefficient of BSA for the UV Vis assay.

9.3 Document all steps along the way.

9.4 Consider the difference in concentration (if any) determined from each technique. Write a brief statement on why you think there is a difference, which result you have greater confidence in, and why.

9.5 Provide an explanation of the pros and cons of each technique, when it might be beneficial to use each of these techniques, and which situation you might want to use a completely different technique.

9.6 Submit all documentation along with standard curve, results, and discussion to proctor for review.