UNKNOWN PROTEIN CONCENTRATION: USING A STANDARD CURVE

STANDARDS
- 3.1.10B, 3.1.12B – Mathematical models applied to biological science.
- 3.2.10B, 3.2.12B – Testing controlled experiments, recognizing variables, and evaluating experimental data correctly within experimental limits.
- 3.3.10B, 3.3.12B – Analyze the organic molecules found in living organisms.

INTRODUCTION
Cellular proteins are essential to the functioning of a healthy cell. They are responsible for processes as diverse as the replication of DNA or the detection of an invading virus. One way scientists study the function of an individual protein is to extract it from the cell and isolate that single protein from all the other cellular proteins. This process, known as protein purification, involves many steps. At each step, there is the chance of technical or human error, both of which can affect the overall yield (amount) of protein recovery. With this type of variability, it is essential to determine the final concentration of each purification procedure. Determination of the concentration is usually done by comparing the protein solution to a standard curve. Once the concentration is determined, this information can be correlated with an enzyme assay to give an overall picture of the activity and purity of a particular protein purification procedure.

Proteins, or polypeptides, are made of amino acids joined by peptide bonds. The protein concentration assay used in this lab is based upon an acidic dye that will bind to basic and aromatic amino acid residues. The amino acids that would be bound by the dye are arginine, lysine, histidine, tyrosine, tryptophan and phenylalanine (Fig. 1). The dye has a differential color change in response to various concentrations of protein, turning from a rust to a blue color. The more protein there is in a solution, the darker the blue color of the bound dye. The color change is detectable using a visible light spectrophotometer set at an absorbance of 595 nm.

A standard curve is created by testing a protein of known concentration with the dye-binding assay to be used. A linear range is determined and these data points are used to create a linear plot. A graphing calculator or spreadsheet application (like Microsoft Excel) can determine the best-fit straight line using the least-squares method (see equation, Fig. 2). The straight line equation produced is based on the formula:

\[ y = mx + b \]

where \( y \) is the absorbance; \( x \) is the protein concentration in \( \mu g/mL \); \( m \) is the straight line slope; and \( b \) is the straight line intercept (the value of \( y \) when \( x = 0 \)). Using this equation, it is possible to determine the concentration of an unknown protein solution based on the absorbance reading (solve equation for \( x \)).
Figure 1. Amino Acids Bound by Dye

BASIC AMINO ACIDS

Lysine (Lys or K)
Arginine (Arg or R)
Histidine (His or H)

AROMATIC AMINO ACIDS

Phenylalanine (Phe or F)
Tyrosine (Tyr or Y)
Tryptophan (Trp or W)

Figure 2. Protein Concentration Standard Curve

\[ y = 0.0413x - 0.0061 \]
It is possible obtain a linear best-fit without a calculator or spreadsheet. To create a best-fit line, make a scatter plot with the data points obtained from the standard curve. Instead of connecting the points, estimate the best place to draw a line between all the data points. The intersection of the absorbance reading and the hand-drawn line will indicate the approximate concentration of the unknown protein solution (see Fig. 3). Your instructor may have you plot your data on the graphing paper provided at the end of this lab.

**Figure 3.** Hand-Drawn Best-Fit Line

![Hand-Drawn Best-Fit Line Diagram]

**GUIDING QUESTIONS**
- How can dyes be used to estimate protein concentration?
- What is a standard curve?
- How is a standard curve used to determine how much protein is in an unknown solution?

**MATERIALS**
- Spectronic 20 Genesys
- disposable plastic cuvettes
- Kimwipes
- 200-1000 µL pipettor
- BSA standards (0,2,4,8,12, 16, 20 µg/mL)
- 1 unknown protein solution (letter)
- wash bottle with deionized water
- cuvette holder
- BioRad Protein Assay Dye Reagent
- 200-1000 µL pipet tips
- microcentrifuge tube rack
- 20 mg/mL BSA protein solution

**SAFETY**
The dye contains phosphoric acid and methanol, both of which will irritate the skin and eyes, and also stain clothing. Caution should be used when handling this reagent.
PROCEDURE

Preparing the Spectronic 20 Genesys
1. Turn on the spectrophotometer. The spectrophotometer should warm up for at least 10 minutes. Use the same instrument for the entire experiment.

2. Set the spectrophotometer to 595 nm using the ▲ nm and ▼ nm buttons.

Creating a Standard Curve
3. Your instructor will provide a set of labeled microcentrifuge tubes labeled: 0, 2, 4, 8, 12, 16, and 20. These are the standard curve tubes, with the number on top indicating the amount of BSA standard (in µg/mL) per tube. You will also receive a tube with an unknown concentration of protein; this tube will have an uppercase letter on it. Record this letter in the place provided on your data sheet.

4. Add 200 µL of the protein dye reagent to all (standards and unknown) the tubes, recap the tubes and invert to mix the contents. Let the samples incubate for at least 5 minutes for proper color development.

5. After 5 minutes, a color change should have occurred, resulting in a range of blue coloration depending on the protein concentration. If no color difference is apparent in the standard curve samples, see the instructor.

6. The “0 µg” sample is considered the blank for this experiment. Using a disposable pipet bulb, transfer the entire sample into the cuvette labeled “0”. Place the blank cuvette into the sample compartment of the spectrophotometer with the triangle on the cuvette facing the front of the instrument. Note: Before inserting a cuvette into the spectrophotometer, wipe it clean and dry with a kimwipe, and make sure that the solution is free of bubbles. Do not touch the clear sides of the cuvette.

7. Press the “0 ABS/100%T” button on the spectrophotometer. By doing this, any background absorbance from the dye and buffer in your samples is subtracted away. (This is equivalent to buying strawberries by the pound. When you go to the field to pick the berries, you weigh the containers first. Then you subtract that weight from the filled container, so that you pay for only the berries.)

8. Record a 0 in the data table in the row for 0 BSA standard under the Absorbance A column. Remove the “0” cuvette from the instrument.

9. Transfer the “2” sample to the cuvette labeled 2. Place the cuvette containing the “2” sample into the spectrophotometer. Make sure that the triangle on the cuvette is facing the front of the instrument. Do not press 0 ABS 100%T. Record the absorbance reading in the data table row for BSA standard 2 in the Absorbance column.
10. Continue to take readings for samples 4 – 20, recording the absorbance in the Absorbance column.

11. Once you have recorded the data for the standard curve, repeat Step 9 for the protein sample of unknown concentration. Record the absorbance reading in the table provided on the Data Sheet.

12. Plot the results of the standard curve using the graph paper provided. The $x$-axis is BSA concentration (µg/mL) and the $y$-axis is the absorbance reading at 595 nm. All these samples are within the linear range of this particular assay, so if the precision for the standard curve is good, then connecting the data points should approximate a straight line. (The standard curve may also be graphed on a graphing calculator or a spreadsheet program, if these are available.)

13. Your instructor will show you how use the standard curve to determine the concentration of your unknown sample.

14. **Optional:** Determine the linear equation using a graphing calculator or a spreadsheet application.

**REFERENCES**


**CREDITS**
This lab was designed based on the Bradford Protein Quantitation Assay. Revisions and adaptations were made by Dr. Stephanie Corrette-Bennett.
DATA SHEET

Name: _______________________
Group: _______________________
Date: _______________________

DATA ANALYSIS

TABLE 1 Standard Curve Absorbance Data

<table>
<thead>
<tr>
<th>BSA standard:</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 2 Unknown Protein Absorbance Data

<table>
<thead>
<tr>
<th>Unknown Protein: (letter)</th>
<th>Absorbance</th>
</tr>
</thead>
</table>

Linear Equation \( y = mx + b \): _________________ (not used if hand plotting the data)

Use the standard curve graph (or linear equation) to determine the protein concentration of the unknown sample.

Unknown Protein (letter) ___: Concentration: ________µg/mL

QUESTIONS

1. How precise was your BSA standard curve? Were your data points mostly in a straight line?
2. What are possible sources of error (technical or human) that affect the precision of the standard curve and how could they be corrected?

3. Why is it necessary to determine the concentration of each new protein purification?

4. Why is it necessary to have a “0” sample, with no protein, as part of the standard curve?