EXERCISE 8B - Lab Procedures

Turn on the Spec-20 and set the wavelength to 595 nm. Allow the Spec-20 to warm up for at least 15 min before taking any readings. Review the procedure for operating the Spec-20 in Appendix D, if necessary.

Place 20 Spec-20 cuvettes in a rack and use tape to label them "1" through "15" and "A" through "E". Consult you instructor if insufficient Spec-20 cuvettes are available.

I. Prepare the samples that you will analyze by gel electrophoresis next week

1. Use a permanent marker to label 8 clean Eppendorf tubes as follows: "Milk", "Pellet", "Whey", and "C" followed by the fraction number of each of the 5 column fraction that you saved (e.g. column fraction 9 would be labeled "C9."). Also label each tube with your group name. Write directly on the Eppendorf tubes; do not use label tape

2. Add 50 µL of the appropriate milk fraction to each Eppendorf tube. Save the remainder of each milk fraction for use later during this lab.

3. Locate the Eppendorf tube labeled "2X Sample Buffer" at your workstation and add 50µL of "2X Sample Treatment Buffer" to each of the 8 Eppendorf tubes.

4. Close the lids on the Eppendorf tubes tightly and mix the contents by repeatedly tapping the side of each tube with your finger.

5. Insert all 8 Eppendorf tubes into a holder and place them in a boiling water bath. Heat for 5 minutes, then carefully remove the tubes and bring them back to your work area.

6. With their caps securely closed, place all 8 Eppendorf tubes into a small container for storage. Have your instructor check the contents of your container to make sure that you have everything you will need for next week's lab.

7. Cover the container with a piece of plastic wrap and secure the plastic wrap with a piece of label tape that goes all the way around the container. On the label tape, write the following:

   - **Your instructor's name (very important!)**
   - **Your lab day and time**
   - **Your group name**
   - **Today's date**

8. Give the container containing your milk fractions to your instructor, who will store them at -20° C until the next lab period.

II. Prepare BSA dilutions for your standard curve

1. You have been provided with a stock solution containing 2 mg/mL of BSA.

2. Dilute the BSA stock solution as shown in the table below. Accuracy is **CRITICAL**, so make sure you use an appropriate measuring device and you adjust it correctly.

Preparation of 0.1 mL of each BSA dilution from a 2 mg/mL stock solution of BSA			
Cuvette #	**Final BSA concentration (mg/mL)**	**Amount of stock BSA needed**	**Amount of dH_2O needed**
1	**0**		
2	**0.1**		
3	**0.2**		
4	**0.4**		
5	**0.6**		
6	**0.8**		
7	**1.0**		
8	**1.2**		
9	**1.4**		

III. Prepare dilutions of the 8 milk fractions you collected in Lab 8A

1. Dilute samples of your milk fractions as shown in the table below. Accuracy is **CRITICAL**, so make sure you use an appropriate measuring device and you adjust it correctly. Also, be sure not to cross-contaminate your samples. Use a clean measuring device each time you switch to a different type of sample. This includes using a separate, clean, measuring device for each of your column fractions.

Preparation of 0.1 mL of each diluted milk fraction			
Cuvette #	**Milk fraction (dilution)**	**Amount of milk fraction needed**	**Amount of dH_2O needed**
10	Nonfat milk (2.0 %)		
11	Nonfat milk (3.0 %)		
12	Pellet (3.0 %)		
13	Pellet (5.0 %)		
14	Whey (50.0 %)		
15	Whey (100.0 %)		
A	Column fraction # ____ (100 %)		
B	Column fraction # ____ (100 %)		
C	Column fraction # ____ (100 %)		
D	Column fraction # ____ (100 %)		
E	Column fraction # ____ (100 %)		

IV. Perform the Bradford assay on your BSA dilutions and your diluted milk fractions

Important note: After the Bradford reagent is added to your protein samples, the mixture will develop sufficient color for an accurate reading after about five minutes. The color change will give accurate measurements for up to an hour. However, for the measurements to be consistent, each of the protein samples must have reacted with the dye for approximately the same period of time. That is, if the A_{595} of **all** of your samples is measured 10 minutes after the dye is added, then the assay is accurate. If the A_{595} of **all** of your samples is measured 20 minutes after the dye is added, then the assay is accurate. However, if some samples are measured 10 minutes after the dye is added and others are measured 20 minutes after the dye was added, then the results will not be reliable.

An efficient way to make sure all of your samples have reacted with the dye for about the same amount of time is the following:

1. Before adding Bradford reagent to any of the cuvettes, make sure your Spec-20 is warmed up, set to A_{595}, and ready to go. Also, all of your cuvettes (1-15 and A-E) should contain the proper dilutions of BSA and milk fractions.

2. Cut 20 small squares of Parafilm–just large enough to cover the tops of your cuvettes when stretched.

3. Make a table for recording the absorbance values for your 20 cuvettes.

4. Add 3.0mL of Bradford reagent to the first cuvette and cover with a small square of Parafilm. Note the time. Mix the contents of the tube by holding your thumb over the Parafilm-covered top of the cuvette and ***gently and quickly*** inverting it 2-3 times. Replace the tube in the rack.

5. Add 3.0mL of Bradford reagent to your second cuvette, cover and mix as you did the first cuvette. Replace it in the rack.

6. Repeat with the remaining cuvettes, adding 3.0mL of Bradford reagent to each cuvette, one after another, in numerical order. Try to keep a steady pace as you move from one cuvette to the next.

7. After you have mixed the Bradford reagent with the contents of all 20 cuvettes, check the time. When at least five minutes have passed since you added Bradford reagent to cuvette #1, proceed to the next step.

8. Remove the Parafilm from the tops of the cuvettes. Wipe the sides of cuvette #1 (your blank) with a Kimwipe and use it to calibrate the Spec-20. **Have your instructor check your calibration before you proceed.**

9. Wipe the sides of Cuvette #2 with a Kimwipe, insert it into the Spec-20, and read and record the A_{595}.

10. Keeping a steady pace (try to keep about the same pace as when you were adding and mixing the Bradford reagent to your cuvettes), read and record the A_{595} values of your remaining cuvettes in numerical order. **Have your instructor check your data before continuing.**

11. After your instructor has checked your data, pour the contents of the cuvettes into the sink. Place the cuvettes in the fume hood and fill them with ethanol. Allow them to soak in ethanol for at least 10 minutes to remove any adhering blue dye. After the tubes have soaked for at least 10 minutes, pour the ethanol into the designated waste container. Rinse the cuvettes 3 times with tap water and then 3 times with dH_2O. Finally, place the cuvettes upside down in a test tube rack to drain.

    Remember! Never use brushes, scouring pads, or abrasive cleansers on Spec-20 cuvettes! Rinse with water only!

12. Leave the Spec-20 on and keep all of your samples, the Bradford reagent, cuvettes, etc. handy – you may have to repeat the assay for one or more samples after examining your standard curve.

V. Plot the standard curve for your Bradford assay

1. Using a sheet of graph paper or a computer with spreadsheet program, make a scatter diagram of the data from your BSA dilutions **(tubes 1 through 9 only)** by plotting the A595 values of these dilutions on the y-axis and the protein concentration of these dilutions on the x-axis. **Make sure your graph has a title and you adequately label both axes of your graph.**

2. Examine the 9 points on your scatter diagram. They should form a straight line. Using a hand-held calculator or a computer with spreadsheet program, carry out linear regression to determine the equation of the "best fit" straight line for your data points. Remember, **protein concentration** is the x value at each point and the corresponding **A595 value** is the y value.

3. After you complete linear regression, examine the **linear correlation coefficient** for your data. As mentioned in previous labs, most scientists will conclude that 2 variables are linearly related if the absolute value of the linear correlation coefficient is greater than 0.95. If the absolute value of your linear correlation coefficient is less than 0.95, this may indicate that the linear relationship is "breaking down" at the highest protein concentrations. (Why might this happen?) If this is the case, the points on your scatter diagram at highest protein concentrations will seem to "flatten out". If they do, you may need to repeat the linear regression without these points. However, you should not arbitrarily omit points in the middle of your curve or at lower protein concentrations. Ask your instructor for help if you omit the points at the highest protein concentrations and the absolute value of the linear correlation coefficient remains below 0.95.

4. Write the **equation** for the "best fit" straight line on your scatter diagram. Next to the equation, write down the **linear correlation coefficient**. Finally, plot the "best fit" straight line in the "linear region" of your scatter diagram. This is your **standard curve** for the Bradford assay.

Do not clear the entries in your calculator yet! You will use them in Part VI.

VI. Use your standard curve to determine the protein concentrations of your diluted milk fractions

1. For your BSA standard curve, enter the range of absorbance readings where the relationship between A595 values and protein concentration remains linear:

   The relationship between A595 values and protein concentration remains linear for A595 values from zero_________ to _____________.

2. Examine the table where you recorded the A595 values for your diluted milk fractions (cuvettes 10-15 and A - E). Circle the A595 values that fall within the linear range of your BSA standard curve. Make sure the A595 value for at least one milk fraction of each type (nonfat milk, pellet, whey, and column fractions) falls within the linear range of the standard curve. If not, repeat the Bradford assay using a greater or lower dilution of that milk fraction.

   NOTE: If you must repeat the Bradford assay for one or more milk fractions, make sure you use a dilution that will give you 0.1 mL of a sample that contains a *sufficiently lower* concentration of protein than your original dilutions. If making this dilution requires measuring less than 2 μL of the milk fraction, you will have to make more than 0.1 mL and then transfer 0.1 mL to your cuvette to be analyzed by the Bradford assay.

3. The linear regression equation for your BSA standard curve should have the following general formula,

$$y = mx + b$$

   where "y" represents A595 values and "x" represents protein concentrations.

Substitute the A_{595} value of each diluted milk fractions (cuvettes 10-15 and A - E) into your linear regression equation for "y" and then calculate the value of "x". The value of "x" equals the protein concentration of the diluted milk fraction. Record your results in a table. **NOTE: When calculating the protein concentrations of the diluted milk fractions, make sure you use only the A_{595} values that fall within the linear region of the BSA standard curve.**

VII. Calculate the protein concentrations of your undiluted milk fractions

1. To calculate the protein concentration of each **undiluted** milk fraction, simply take the protein concentration of the corresponding **diluted** milk fraction and divide it by the dilution percent. For example, if a 1% dilution of nonfat milk had a protein concentration of 380 µg/mL, then to calculate the protein concentration of the **undiluted** nonfat milk simply divide 380 µg/mL by 1% (0.01). This means the undiluted nonfat milk had a protein concentration of 38,000 µg/mL or 38 mg/mL.

   NOTE: When performing the Bradford assay, you used 2 different dilutions of 3 milk fractions: the nonfat milk, the pellet, and the whey. In each case, calculate protein concentrations using only the dilution that produced an A_{595} value that lies within the linear region of your standard curve. If **both** dilutions of a given milk fraction gave an A_{595} value that lies within the linear region of your standard curve, then calculate the protein concentration of both dilutions, and use both results to calculate the protein concentration of the undiluted milk fraction. Obviously, both dilutions should give you the same answer for the concentration of the undiluted milk fraction; if the answers are different, average them.

Clean up

Dispose of your excess solutions as directed by instructor.

Remove label tape and any marks made with a marking pen from all glassware. Wash and rinse all glassware, give it a final rinse with dH_2O, and leave it inverted at your work area in order to drain. Remember, never use brushes, scouring pads, or abrasive cleansers on Spec-20 cuvettes! Rinse with water only!

All disposable glassware goes into the special glass disposal receptacle.

All instruments should be turned off and unplugged.

Wipe off your workspace with a damp paper towel.

Make sure everything that you have used is clean, put away, or discarded. Leave your work area in the same order that you found it in.

Ask your instructor to check your work area before you leave.