Protein Studies

Part C

Protein Assay

An Outreach Project of the
Fralin Biotechnology Center
Virginia Tech

Toby M Horn, PhD
Coordinator for Biotechnology Outreach

Consider part A to be Gel Electrophoresis of Fish Proteins and part B to be Column Chromatography

Proteins are made by every one of our cells. They do practically all the **work** of life, making and breaking bonds, providing structure, turning food into energy and building blocks. Proteins are essential nutrients in the foods we eat.

Many diseases are due to mutated proteins. Genetic mutations often produce proteins with altered properties. Most drugs interfere with normal - or abnormal - protein action.

How do we know this? Through many different kinds of studies performed by researchers all over the world.

We purify individual proteins, figure out their composition and structure, what they work on or with, when and how they are made, and test how they work under a variety of conditions. We also compare the same type of protein from different kinds of organisms and between "wild type" and mutant variants. Each facet involves long term investigation even though some of the processes may take only a few minutes.

Numerous researchers have earned the Nobel Prize for work on proteins, including Gunther Blobel recently (1999) for his studies on how proteins figure out where to go in (or out of) the cell after a protein is made on the ribosome.

Along with all the other things we want to know, we keep track of how much total or specific protein is in our study sample. Protein quantitation is important to researchers and equally important in the food industry. Manufacturers must list the protein content of the foods they process.

A protein assay is a test for the total amount of protein in a sample. A protein assay does not tell us what kind of protein is present; we need enzyme or antibody assays or gel electrophoresis for that. A good protein assay helps us detect protein even when many other components may be present. For instance, our cells have DNA, RNA, fats, minerals, carbohydrates AND protein. Milk has sugars, calcium, fat, etc.

A hallmark of a good assay is its specificity, sensitivity, and speed. The Bradford protein assay is the 3rd generation after Biuret and Lowry. Using a dye that stains silk and wool blue, the red reagent combines with protein in acid solution to generate tiny blue precipitates. The Bradford reagent, Coomassie Blue, forms complexes with many, many proteins. After more than 20 years of use, scientists have found that there are

some specific proteins that Coomassie Blue does not bind to (including the important enzyme, trypsin). Such proteins are therefore invisible in this test.

Note that we are not talking about a perfect assay -- none exist. We may choose one test over another because of ease, but sometimes our choice is dictated by specific scientific limitations.

Industry needs inexpensive, rapid, sensitive and simple tests. In manufacturing plants, thousands of samples are generated daily. Sound like school?

The Bradford protein assay is well-suited to a classroom situation. Though students must be attentive, their effort will pay off with nearly immediate gratification --- after they have argued about colors and performed a lot of mathematical manipulations --- yes, in biology class.

The Bradford Protein Assay provides an excellent opportunity for students to see the connections between chemistry and biology and math and science. It also helps students to be better informed consumers.

Despite a few limitations, the Bradford protein assay is widely used for research and industry.

1. Only 3 liquids are needed: the sample or standard, water to equalize volumes, and the reagent.

2. Within 5 minutes the color reaction is stable.

3. The shades of blue generated are easy to distinguish, enabling students to obtain accurate data with their own eyes, without using instruments.

4. The test is extremely sensitive, yielding a distinct color reaction in the range of 5-20 _g of protein, much less than can be weighed out on even a research grade balance.

Students need some skills to get satisfactory results. Often students gain a better understanding of these skills and concepts as they perform the assay and analyze their results.

Technique:
- Using a 1 ml pipette and pipettor
- Following instructions
- Recording observations

Math:
- Working with scientific notation (powers of ten)
- Understanding the concept and performing the math for dilutions
- Keeping units straight while making conversions
- Understanding the concept of sampling

Chemical:
- Understanding the distinction between concentration and amount
- Distinguishing types of chemicals
- Understanding that biological substances are chemicals

Investigative:
- Concepts of experimental design
- Standard for comparison
- Blank for control
- Replicates for reproducibility
- Range for assay sensitivity

Analytical
- Observing
- Estimating
- Judging
- Calculating
- Averaging

To begin, use the protein standard, a highly purified, readily available (to scientists) serum protein from cows, Bovine Serum Albumin (BSA). Because it is pure and available in large quantities (powder sample provided for demonstration purposes--please do not open), the powder can be weighed out on a regular balance.

The powder can dissolve in water at relatively high concentrations (for instance 40 mg/ml). For the Bradford protein assay, we need very small increments of 5 µg or 0.0005 mg. Thus, the initial protein solution is highly concentrated for our purposes. However, concentrated solutions

of protein tend to be more stable, so we store small portions (aliquots) of the standard concentrate.

On the days that your students are ready to perform the assays, take the designated small volume and dilute with distilled water as specified (see extra handout for proportions to use depending on the concentration of the stock BSA).

Teaching the Bio-Rad Protein Assay

This rapid and sensitive test is used by research and industrial scientists to detect protein in foods, cells, and purification processes. Its development began in 1963 as a collaboration, in Australia, between microbiologists and textile chemists.

This is a hands-on lab that can be reasonably conducted in 3 class periods. It involves reading, pipetting, observation, and math.

This quantitative method estimates, specifically, the protein amount in a complex mixture that has many different proteins and other substances. Milk, serum, cell homogenates, food samples, fermentation secretions are all complex mixtures of different substances. As a particular protein gets purified, it may show exceptional behavior. For instance, the enzyme trypsin does not bind much to the Bradford protein assay reagent. However, most proteins can be detected by the Bradford dye, Coomassie Brilliant Blue R250, when the dye is in a solution containing phosphoric acid and methanol.

- Ask students about nutritional concerns. (Balance of protein, carbohydrates, fats, vitamins and minerals, water.)
- Hand out empty (and rinsed out) SKIM milk containers. Have teams look them over and tell how much is in the container.
- Students will probably answer 1/2 pint or quart. Remind them that in the lab we use the metric system! Hopefully, they'll shout out 236 ml (or how many ml in a quart).

Students will get plenty of exercise with math, pipetting, teamwork, judgment. They will also see how sensitive certain dyes are for detecting other molecules.

Presentation	**History, vocabulary**	**Materials**
Activity	Pass out milk containers, read the label What information is on the container? — RDA, amounts, volume What is the protein amount? — 8 grams What is the protein concentration? — 8 grams per 236 ml,0.034 g/ml	Milk containers Calculators Assay handout
Assay handout	These are manufacturer's instructions. The Protein Assay detects protein in the range of 1µg to 25 µg in a 1 ml reaction. How would you express the protein concentration of the milk container in µg/ml? — 0.034 (0.0339) g/ml — 33.9 mg/ml — 33,900 µg/ml This is way a lot! Could you weigh this amount of protein? YES, but since it is in a liquid that has many other components, you need a specific test to see the protein in the mixture. So, we have to dilute the milk so that at some point, there will be a certain small amount of protein in the range that the protein assay can detect.	

BioRad Protein Assay Reminders

Very important reminder: you will receive a 100 point grade for this work (equivalent to two test grades). The only thing that I will evaluate will be your data record. Make it complete!!! I will look for everything you did to be included and dated. Include trips to the grocery store and planning and calculating times. Include detailed procedures, observations and results for work done in the lab. Don't forget to record and discuss possible sources of error in your data.

Preparing a BSA standard curve:

1. Test protein standards in increments of 5 (0-25 ug/ml BSA) to prepare a standard curve in the region where color v. protein is linear. For BSA this is 1.2- 10 or 20 ug protein/ml.

2. The microassay requires a total volume of 1.0 ml, which must contain
   0.2 ml of Bradford reagent
   0.8 ml of sample (standard)

3. 0.1 ml increments are used for the standard concentrations, so prepare a BSA solution with a concentration of 5 ug/.1 ml=50ug/ml from the BSA standard stock. Aliquots of the BSA standard stock are in the freezer. The concentration of the standard stock is 5mg/ml protein.
   - Determine how much standard you will need for one day only; calculate how you will dilute a portion of the standard to make this amount. (Actually, the standard will last several days if refrigerated, but no longer.)
   - Take one frozen micro test tube of standard out of the freezer. Thaw it in your hands.
   - Dilute (as calculated) the standard in a sterile plastic centrifuge tube, using distilled water. (Why should the tube be sterile?) Keep refrigerated until needed.

Points to consider for the protein assay:

- larger quantities are needed for spectrophotometric analysis (at least 3ml)

- stock standard BSA is frozen 1 ml aliquots in microtubes at a concentration of 5 -50 mg/ml

- BioRad reagent stock needs to be stored in a refrigerator. Determine how much you will need for an assay; take about 1 ml more than this amount with a sterile pipette to use and put into a labeled plastic test tube (this tube does not need to be sterile, just clean) before dispensing from THAT tube to student dishes.

- protein standard solutions have a storage life of about 1 week in the refrigerator if they are kept sterile.

- if the protein samples you are testing are liquid, then using the milk example, prepare serial 1:10 (10X or 10-fold) dilutions of your selected protein or protein mixture for testing.

- **remember that a standard curve must always be done at the same time that you test your protein samples. Why?**

If your protein samples are solid:

- proteins can be extracted in aqueous solution for Biorad testing since most proteins you will be testing are water soluble.
- proteins can be released from cells into water when the cell membranes are disrupted. This can be done chemically and/or physically. For the purposes of practicing the Bradford assay, simply disrupt the cells by grinding or blending with water.
- these protein solutions must be tested immediately because they will break down rapidly for the following reasons: they are mixtures of many proteins (including proteolytic enzymes); they are not buffered so protein degradation will change the pH; they are not sterile so microbes will multiple, feeding on the proteins and producing waste products which also change pH.

(grams, milligrams, micrograms, nanograms, picograms, etc.)

Getting from the protein content in the milk container to the range of protein the Bio-Rad protein assay can detect

The milk container says it has 8 grams of protein. The container holds 236 ml.

8 g /236 ml = 0.0339 g of protein per ml.

The protein assay detects ug of protein per ml. Therefore **convert g to ug**.

1 g is 1 million ug (micrograms, greek letter mu).

The milk protein concentration is **33,900 ug/ml (33.9 milligrams/ml).**

To get from here to a concentration detectable by the assay, you need to divide by 10,000, or, **dilute 10,000-fold**. However, we only assay a portion of any sample, so we can assay 0.1 ml (or 0.2 ml for good luck!).

So, we can make a 1:1000 dilution. We can add 1 ml of milk to 999 ml of water, but this is really wasteful since we may only test 0.1 ml! So we can get to the same place by making

3 serial 1:10 dilutions, resulting in a solution with 33.9 ug/ml.
(Serially/Cereally diluting the milk --- Ha Ha!)

Using 0.1 ml of the 1:1000 dilution will provide 3.4 ug of protein.
Using 0.2 ml of the 1:1000 dilution will provide 6.8 (7) ug of protein.
Using 0.3 ml of the 1:1000 dilution will provide 10.4 (10) ug of protein.

Getting from the protein assay values to milk

Students assayed duplicate 0.2 ml samples of the A, B, and C dilutions of milk. A and B were both very blue but C tubes were gray blue. By comparison with the BSA standard, the color looked between the colors of the 5 and 10 ug samples, so the students chose 8, because the colors looked more like the 10 ug standards than the 5 ug standards.

The students estimated that their **0.2 ml sample of milk dilution C had 8 ug of protein in it.**

To calculate the protein content per ml of dilution C, multiply by 5, since
(1 ml/0.2 ml) = 5. Thus, **dilution C has 40 ug of protein per ml.**

Dilution C is a 1:1000 dilution of milk
(0.1/10 = A, X 0.1/10 = B, X 0.1/10 = C).
Thus, multiply 40 ug/ml by 1,000. Therefore, milk has 40,000 ug of protein per ml or 40 mg of protein per ml.

TEACHER CHARTS

Chart I. STANDARD (BSA Protein Concentration is 50µg/ml)

Tube Number	Water Volume = (ml)	Sample = BSA Volume = (ml)	**DYE Reagent** Volume = 0.2 ml	Protein Amount added= (**µ**g)	Color
1,2	**0.8 ml**	**none**	0.2	0 **µ**g	brick red/brown
3,4	**0.7**	**0.1 ml**	0.2	5 **µ**g	gray blue
5,6	0.6	0.2	0.2	10 **µ**g	more blue
7,8	0.5	0.3	0.2	15 **µ**g	more blue
9,10	0.4	0.4	0.2	20 **µ**g	bright blue

Chart II. Serial Dilutions for Unknown = MILK

Sample Dilutions	**Water Volume (ml)**	**Sample =Milk Volume (ml)**	**Calculated** Protein Concentration (**µ**g per ml)	**Calculated** Protein Amount (**µg per 0.2 ml**)	In the RANGE? (Y or N)
MILK	**none**		**33900**	6780	N
A	**0.9 ml**	**0.1 ml Milk**	**3390**	678	N
B	0.9	**0.1 ml A**	**339**	67.8	N
C	0.9	0.1 ml B	**33.9**	6.8	Y

Chart III. Milk Protein Assay

Tube Number	Water Volume = (ml)	Sample = Milk Dilution Volume= 0.2ml	**DYE Reagent** Volume = 0.2 ml	**Color**	Protein Amount (**µ**g) cf. standard
11,12	**0.6 ml**	**0.2 ml C**	**0.2 ml**	slate blue	?betw 5 and 10, perhaps 8
13,14	0.6 ml	**0.2 ml B**	0.2 ml	bright blue	too high
15,16	0.6 ml	**0.2 ml A**	0.2 ml	bright blue	too high

Pre-Lab Activity: Reading the Label / Calculating Amounts, Concentrations and Dilutions.

Milk is a fine source of dietary protein. Even school milk containers have the protein content listed.

A few days before class, ask students to begin collecting a few milk containers after lunch. Ask them to PLEASE rinse out the containers before bringing them to class.

Instruct students to read the label and determine how much protein s reported to be in the container (AMOUNT).

Ask students next to figure out the protein CONCENTRATION in the container. Concentration will be the ratio of protein amount to volume (grams per milliliter, g/ml)

Remind students that we use metric values, so for the volume they should come up with 236 ml, not 1/2 pint.

8 grams per 236 ml calculates out to 0.033 g/ml. Most milk containers list 8 grams per portion.

Now, the Bradford protein assay helps us to detect MICROGRAM amounts of protein.

The relationship of micrograms (µg) to milligrams is 1:1000, that is 1,000 µg = 1 mg.

So, the concentration of protein in µg will calculate to be 33,900 µgg/ml. This is WAY more concentrated than the protein assay can detect. This also means we can use only a tiny amount of milk sample. This is a feature that makes the protein assay a good assay.

Very little material needs to be sacrificed to simply determine the protein amount in this protein assay. Thus, almost all the milk can be used as food. When we are purifying rare proteins, which most enzymes are, a sensitive assay is very helpful even though we may have to sacrifice a larger proportion of the sample.

At this juncture, students should realize that we can convert terms so the units are comparable and the distinction between amount (just one unit) and concentration (amount per unit volume).

By CONVENTION, protein concentration is given in amount per MILLILITER, especially in a research and development laboratory.

So the students have worked out the math, but now need to figure out an operation to obtain a sample of milk that can provide only a few (less than 20 µg) m__of protein.

They have calculated that the milk container has 33,900 µg/ml of protein. The assay uses a total volume of 1 ml and only 0.8 ml can be used for the sample. The other 0.2 ml is reserved for the dye reagent.

Thus, in less than 0.8 ml, students need to figure out how to deliver less than 20 _g of protein.

By inspection, 3.39 µg is certainly less than 20 µg of protein, and 3.39 is related to 33,900 --- how?

3.39 is 1:1000. This would mean that 1 ml of a 1:1000 dilution of milk would supply an appropriate amount of protein to be in the detection range of the assay.

But…there's only room for 0.8 ml of test sample, and that would mean the potential to detect 0.8 ml X 3.39 µg/ml or 2.7 µg. Now this is at the low end of the assay, and the first assay standard point will be at 5 µg.

So, back to the drawing board! Some students may notice that if the milk test sample is more concentrated, a smaller volume can still supply enough protein to be detected in the range of the assay.

For instance, a 1:100 dilution will yield 33.9 µg/ml. Using 0.1 ml of this dilution will give 3.39 µg. A volume of 0.2 ml will provide 7.8 µg. Using 0.2 ml for each test sample leaves room to add 0.6 ml of water, with room for the 0.2 ml of dye reagent.

Yes this math seems tough to go through, but these are everyday and multiple-times-a-day operations in a research laboratory.

So making a 1:100 dilution of the milk can enable us to take a 0.1 ml or 0.2 ml sample to test in the protein assay with ease.

Now how to actually make the dilution? Students will first say "add the milk to a larger volume of water." This will result in a spaghetti pot full of diluted milk, way, way more than would be needed for assays by even your entire grade level!

At this juncture, introduce the concept of serial dilutions (double-entendre possible here, since we are diluting milk!).

Serial dilutions conserve the sample AND water and enable you to get very dilute product in just a few steps. **See figure and overhead.**

To make a serial dilution, first add a small, known volume, of sample to a larger, known volume of diluent (water). For instance, a 1:4 dilution would mean you add 1 volume of milk to 3 volumes of water --- frozen orange juice or lemonade are 1:4 dilutions (1 can juice plus 3 cans water).

Then, add a small known volume of the first dilution to a larger known volume of diluent. A 1:12 dilution can be made in 2 steps by making a 1:4 dilution and then a 1:3 dilution.

What can figure in how dilutions get made will be the tools you have available for liquid transfer. The kit comes supplied with 1 ml pipettes. Pipettes are a scientific/technical limitation for assay design. If micropipettors were available, students could use more concentrated protein samples and add 5-50 microliter volumes to 0.8 ml of water in the assay. Using 1 ml pipettes provides the best accuracy with best conservation of materials.

To make a 1:100 dilution, the easiest way is to make 2 serial 1:10 dilutions. Add 0.1 ml of sample to 0.9 ml of water. Mix to make dilution A. Then take 0.1 ml of dilution A and add to 0.9 ml of water (and mix!) to get dilution B. Dilution B is a 1:100 dilution of the original sample and you have used only 1.8 ml of water and 0.1 ml of the original sample!

(Remember, dilution B is a serial dilution from dilution A and does NOT use any more of the original sample -- a common misconception of students.)

Depending on your students, you may assign the calculations as homework, have students work in groups and report out OR simply TELL students. In class group work works best, since students who understand less can catch up. Doing the math is like learning to ride a bike!

Standard

In all tests, we strive to have a standard for comparison. This tells us the test is working and also provides up-to-date information using the materials and equipment on hand.

In Colorado the water sometimes had high levels of ions that even made it through the distillation process, which affected the color of the standards AND the unknowns. Since the standards were run at the same time as the unknowns, we could make appropriate comparisons --- and THEN figure out what ion was contaminating our distilled water supply and find out how to selectively precipitate it! Stuff was turning purple!

The assay standard will be the BSA solution that is diluted as specified. The specified final concentration will be 50 µg/ml. This will provide 5 µg in every 0.1 ml volume, so we can generate a standard for 5 µg intervals (0, 5, 10, 15, 20, 25 µg). Remember the assay detects protein in the range of 5-20 µg.

To provide you with the protein standard concentrate, generally, milligram amounts of protein were weighed out on a balance with the exact amount determined---for instance 580 mg. To make a highly concentrated solution, say 40-50 mg/ml, I calculated that for 580 mg I would need 14.5 ml of water to get 40 mg/ml. So I pipetted 14.5 ml of water into a tube and added the 580 mg of BSA, then let it dissolve slowly without stirring (to minimize formation of bubbles!).

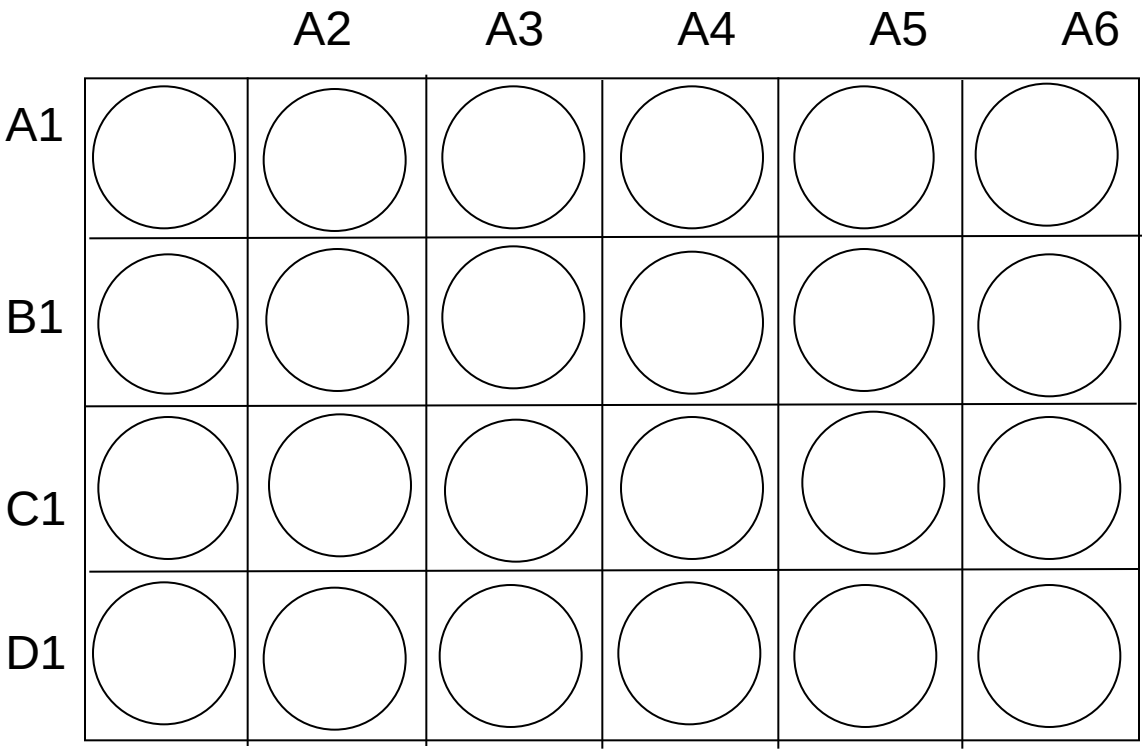
At 40 mg/ml of BSA, 0.1 ml added to 80 ml will give 50 µg/ml, the appropriate concentration, and useful amount for your class.

Each team of 3 or 4 will need to add 2X 0.1, 0.2, 0.3, 0.4, 0.5 ml for a total of 3 ml so you need to figure that teams will use about 4 ml of the standard.

55 ml of standard will provide enough for 18 teams. Prepare the standard for 1 day only and keep it chilled. Protein is excellent microbe food!

Template for Assays

24 wells provide plenty of space for the different solutions you will need to add to prepare the final assay samples. Keep the assay samples along the outside wells so it will be easier to compare the plate of the samples with unknown protein content to the plate with the standard.



BSA STANDARD (ug protein) **PLATE**

	A1	A2	A3	A4	A5	A6
A1	**0**	**0**	**5**	**5**	**10**	**10**
B1	*water*	*water*	*water*			**15**
C1	*BSA*	*BSA*				**15**
D1			**25**	**25**	**20**	**20**

MILK DILUTIONS and ASSAY PLATE

	A1	A2	A3	A4	A5	A6
A1	sample**C**	**C**	**B**	**B**	**A**	**A**
B1	*water*	*water*	*water*			
C1	*milk*	dilA	dilB	dilC		
D1			***DANGER***!		***dye reagent***	***dye reagent***

use the reagent for the 6 samples and the BSA standards

ONLY plain **BOLDFACE** wells get dye reagent added

Preparation and Clean-up

Pipettes

Before using for the first time

Remove the cotton plugs from the 1 ml pipettes so that you can decolorize and rinse them reasonably. Use the paperclips (opened) to tease the plugs out**. (15 minutes)**

We have provided 2 sets of pipettes so that while the second class is working, you can complete the washing and rinsing of the first set in time for the 3rd class.

After the first use

1. Fill a cut 1 plastic liter soda bottle to the top with tap water and add a few drops of liquid detergent.
2. Ask the students to put their used pipettes in tip down!!!
3. At the end of class, pull the pipettes up and down a few times to be sure that each one is wet. Then, holding the pipettes, pour out the soapy water and fill with fresh water keeping the pipettes in the cylinder. The pipettes will fill with water, which helps the rinsing process.
4. Repeat 6-7 times or until no soapy bubbles appear.
5. Shake the stack of pipettes down HARD on some paper towel.
6. Roll on the paper towel to dry the outside.
7. Shake down again.
8. Inspect for residual water and shake only those pipettes down a 3rd time. **(10-20 minutes)**

Protein Standard

Thaw the protein standard

1. Put the centrifuge tube in a beaker with some room temperature water or carry it around in your warm hands. Be certain that the cap is on tight!
2. Do NOT vortex to mix, but invert gently several times to be sure that the thawed solution is uniform. Concentrated protein solutions make lots of bubbles! **(10 minutes)**

Prepare the standard working solution (50 µg/ml 50 micrograms)

1. Fill one of the 50 ml centrifuge tubes nearly full (55 ml). Add 0.1ml of the thawed BSA STOCK solution.
2. Invert gently several times to mix the concentrated stock BSA with the water diluent.
3. Refrigerate if you need to store the working standard solution overnight.

(5 minutes)

24-Well Dishes

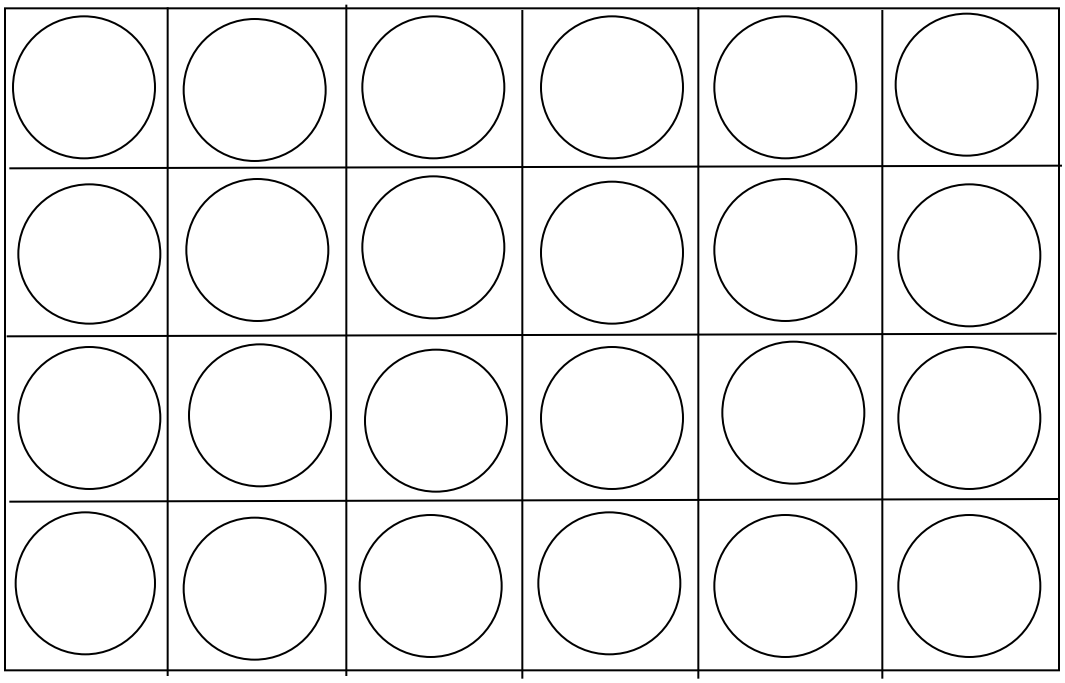
No advanced preparation time is needed the first time.
Shake all the droplets out of the bleached and well-rinsed plates if you are re-using the dishes.

Clean up

1. Immerse in 10% bleach in the sweater box for a few minutes. Bleach solution must cover the bottoms of the wells.
2. Rinse well (6-7 times with running water)
3. Shake excess liquid off to speed up the drying process.
4. Invert over paper towels

(10 minutes)

Cleanup time depends on the number of class sections you teach each day.



Students can make this grid on the computer themselves

1. Make a large rectangle

0 using the vertical and horizontal rulers, make the box 6 inches wide on the horizontal and 4 inches tall on the vertical

2. Make the grid

- ❑ draw on line horizontal and make 3 more using the copy and paste commands. Place along the 1 inch lines on the ruler
- ❑ draw one line vertical, make 4 copies using the cut and paste commands. place along the 1 inch lines

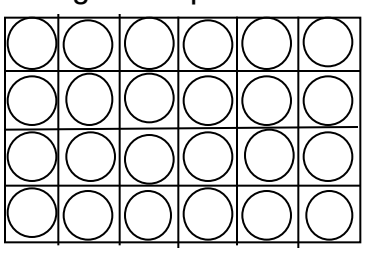
3. Add the wells

- ❑ make a circle that is about the size of the grid block
- ❑ make 23 more using cut and paste
- ❑ place inside the grid

Identifying wells when recording data --- the value of coding

Students can number the wells as they wish, BUT, ask students to look carefully at the plate and note that the wells are already identified by A-D and 1-6. Ask them to indicate where well/box A1 is so that temmates are looking and recording from the same well.

Using a unique number makes it easiest to code the reaction wells.



In their lab books, students can write copious notes on what is happening in each well, while having the easy code for each well: 1-24 or something else simple.

For the protein assay, I strongly suggest that students number their wells as shown here, because it will be much easier to collect and compare data this way.

Protein Assay

The Protein Content of School Milk or...

How'd the Info on the Label Get There, Anyway?

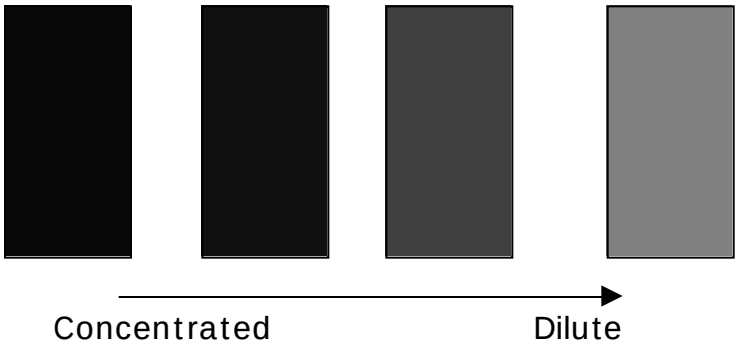
1. Read the milk carton label.
2. Calculate the protein **concentration** in
   milligrams per ml **(mg/ml).**
3. Convert the protein concentration of milk to
   micrograms per ml **µg/ml.**
4. Construct a Dilution Chart for serial **(1:10)** dilutions (10-fold).
5. Conduct serial dilution of the milk.
6. Construct a chart for the Bio-Rad Protein Assay.
7. Conduct the protein assay of the milk dilutions.
8. Calculate the protein content of school milk based on your assay results.
9. Compare with the class results and
   with the information on the label

standard (50 µg/ml) BSA (Bovine Serum Albumin) **0-20 µg** protein amount color of a quantity	**plus** **reagent** **=** **color**	unknown (? µg/ml) sample volume (0.1-0.3 ml) quantity from color

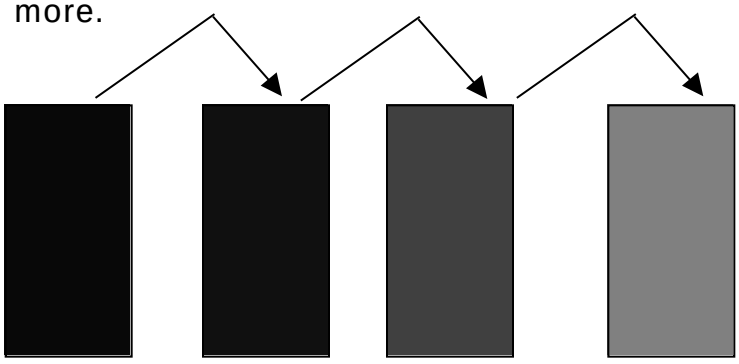
Concentrations, Dilutions

A concentrated solution has a lot of the substance in every unit of volume.

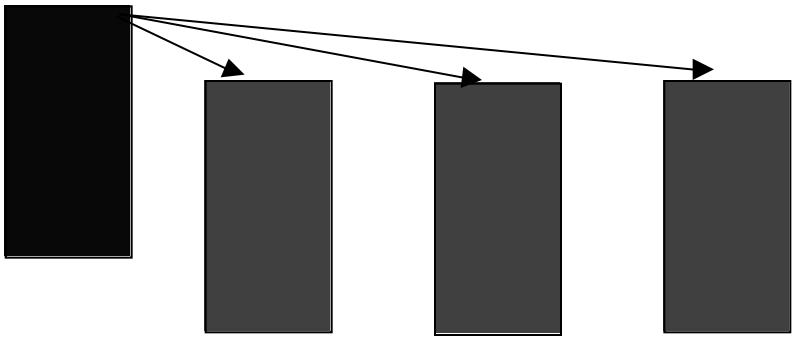
Diluting the substance means there is less per unit of volume. You make a 4-fold or 4X or 1:4 dilution when you add 3 cans of water to one can of frozen orange juice.



You make a serial dilution by taking liquid from the next dilution and diluting it more.



If you keep removing volume from the first sample, you will make a replicate, not serial dilution.



Instructions for Conducting the BioRad Protein Assay

Check Off Here
AS you COMPLETE Each Step

1. Fill in each chart.

2. Label tubes.

Note: You will make duplicate tubes for the BSA Standard and the Milk Protein Assays. Give every tube a different number so you can still distinguish each of the duplicate tubes. You will only prepare one tube each for the milk dilutions, so pipette carefully!

3. Add indicated volumes of water to all the tubes that need water.

4. Prepare serial (1:10) dilutions of the milk using the water pipette.

5. Add the milk dilutions to their appropriate assay tubes.

Mix the tubes by flicking or on the Vortex Genie mixer.

6. Obtain the BSA standard solution (50 µg/ml), and a fresh pipette.

Add the indicated volumes to the standard assay tubes.

7. Obtain DYE REAGENT and a fresh pipette. Add 0.2 ml of the DYE Reagent to all the assay tubes. Mix each tube immediately after you add the reagent.

8. After 5 min., describe the colors of the BSA Standard tubes. Then compare the colors of the milk assay tubes with the colors of the BSA standard. Assign each milk protein assay tube a protein amount. Enter your estimates on chart II.

9. Clean up and resupply the work area with tubes and pipettes.

Record your Numerical ANSWERS Here

10. Calculate

A. the protein amount per assay tube

(Hint: take the average of tubes 11, 12, etc.)

B. the protein concentration per ml of milk dilution

(Hint: what is the portion of a milliliter that you actually assayed?)

C. the protein concentration per ml of undiluted milk and

(Hint: What is the dilution factor?)

D. the protein amount per container.

(Hint: How many ml are in a container?)

DETERMINING THE PROTEIN CONTENT OF SCHOOL MILK

Total Reaction Volume is 1.0 ml

- Prepare serial 1:10 dilutions when the protein amount is unknown.
- Perform steps in the order indicated on the column headings.
- Mix tubes by flicking after each addition. (Shake plate gently to mix contents.)
- Add the DYE REAGENT to all appropriate tubes at the very end. Mix immediately after adding DYE REAGENT to each tube.

Chart I. STANDARD (BSA Protein Concentration is 50µg/ml)

Tube Number	Water Volume = (ml)	Sample = BSA Volume = (ml)	**DYE Reagent** Volume = 0.2 ml	Protein Amount added= (**µ**g)	Color
1,2	**0.8 ml**	**none**	0.2	0 **µ**g	brick red/brown
3,4	**0.7**	**0.1 ml**	0.2	5 **µ**g	gray blue
5,6	0.6	0.2	0.2	10 **µ**g	more blue
7,8	0.5	0.3	0.2	15 **µ**g	more blue
9,10	0.4	0.4	0.2	20 **µ**g	bright blue

Chart II. Serial Dilutions for Unknown = MILK

Sample Dilutions	**Water Volume (ml)**	**Sample =Milk Volume (ml)**	**Calculated** Protein Concentration (**µ**g per ml)	**Calculated** Protein Amount (**µg per 0.2 ml**)	In the RANGE? (Y or N)
MILK	**none**		**33900**	6780	N
A	**0.9 ml**	**0.1 ml Milk**	**3390**	678	N
B	0.9	**0.1 ml A**	**339**	67.8	N
C	0.9	0.1 ml B	**33.9**	6.8	Y

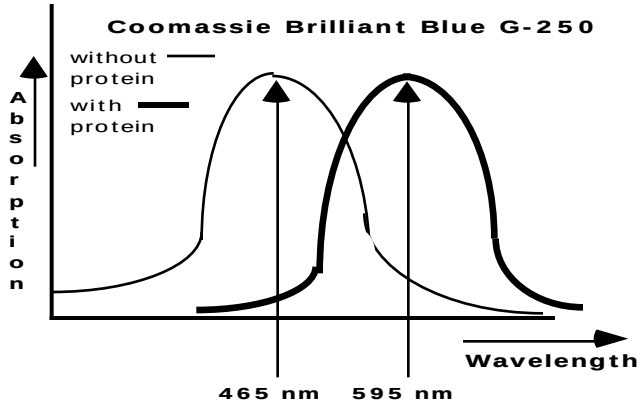
Chart III. Milk Protein Assay

Tube Number	Water Volume = (ml)	Sample = Milk Dilution Volume= 0.2ml	**DYE Reagent** Volume = 0.2 ml	**Color**	Protein Amount (**µ**g) cf. standard
11,12	**0.6 ml**	**0.2 ml C**	**0.2 ml**	slate blue	?betw 5 and 10, perhaps 8
13,14	0.6 ml	**0.2 ml B**	0.2 ml	bright blue	too high
15,16	0.6 ml	**0.2 ml A**	0.2 ml	bright blue	too high

Protein Assay

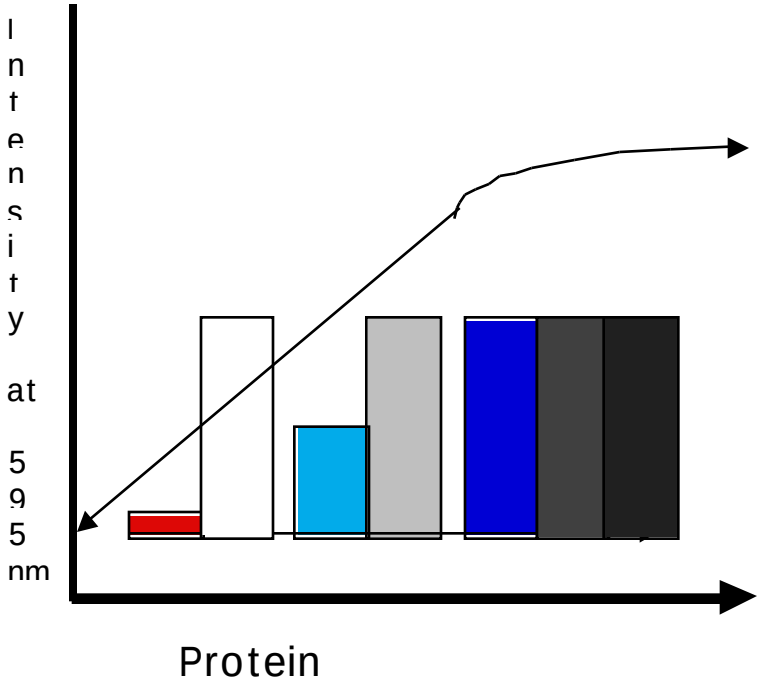
A protein assay is used to test for the presence of protein in a sample. This type of test is based on the ability of a dye to bind specifically to protein, even in a complex mixture like milk or cell homogenate. Some protein assays are qualitative; they indicate the presence or absence of protein. Quantitative protein assays such as the Biuret, Lowry and the Bradford can detect between 1 and 100 ug of protein in a liquid sample. A pharmaceutical scientist may use the information from a protein assay in combination with other tests to obtain the yield or specific activity of a medicine that acts on a protein or of a protein-containing medicine.

The Bradford protein assay is a simple, rapid, and sensitive test that was first reported in 1976. Only three things need to be added, the sample, a volume filler (if needed) and the dye reagent. As robot arms and automatic pipetting devices have become more widely available, the Bradford assay has become automated in many industrial laboratories. Researchers -- and high school students --may still readily conduct the manual assay.



When Coomassie Brilliant Blue is in a dilute acid solution it appears red (maximum absorption at 465 nm) . When mixed with protein, the dye turns blue (maximum light absorption at 595 nm). This sketch shows how the light absorption changes with wavelength.

Using a visible wavelength spectrophotometer, students can determine the absorption spectrum of Coomassie Brilliant Blue G-250 without and with protein. They will find that even in the absence of protein, there is some absorption at 595 nm



At certain protein concentrations, the color shift to 595 nm is directly proportional to the amount of protein. Above this range, the color change is no longer proportional.

In school, it is convenient to perform a 1 ml Bradford protein assay. In a 1 ml assay volume, you can detect from 1 to 20 _g of protein.

Because it is nearly impossible to weigh out between 1 and 20 _ g of solid protein (like powdered egg white) and also impossible to weigh out such a small amount in a complex liquid like skim milk (which contains minerals, sugars, vitamins, etc.), a protein assay that is specific and sensitive is handy and valuable.

An emerging pharmaceutical industry is pharming, which utilizes transgenic female farm animals, like cows, sheep and goats, to produce recombinant proteins in their milk. People might soon drink milk to obtain their medicine!

TRIVIA
Actually "pharming" is a term that dates to the 1930's --- when scientists realized that fungi might be good sources of chemicals. Fermentation processes similar to beer brewing became widely used to produce a number of chemicals and, eventually, antibiotics.

The Bradford BioRad Protein Assay

(developed by Marion Bradford and published in the scientific journal, Analytical Biochemistry, in 1976).

Proteins are one of the major types of cellular macromolecules, besides nucleic acids, lipids, and polysaccharides. There are many different types of proteins and each has one or more roles (enzymatic, structural, signal, receptor, regulator) in the cell. Proteins are polypeptides, chains of amino acids. With this test you can estimate the total amount of protein in a particular sample. Testing companies use robots to perform this assay on thousands of samples a day!	At home, you can find protein information from this test on the label of your milk containers and many other packaged foods.
The Bradford BioRad **DYE REAGENT** contains the dye **Coomassie Brilliant Blue**, which is reddish-brown in acid or when mixed with pure water. This dye turns blue when it comes in contact with (or "sees" or detects) protein. The dye does not detect free amino acids. Almost all proteins make the Bradford reagent turn blue. Almost every other type of molecule (not protein) does not change the color of the reagent to blue.	**DANGER!** The DYE REAGENT contains PHOSPHORIC ACID
This DYE REAGENT is specific for protein molecules, and can quantitate the protein in virtually any sample. When the BioRad DYE REAGENT binds to protein and its color changes to blue, the hue or intensity of the color depends on the amount of protein.	specific for protein
The DYE REAGENT is very sensitive. It can detect as little as 1-20 µg (1-20 millionth of a gram) of protein in a 1 ml assay volume. (Each assay tube contains water, sample and dye reagent.) The test is so rapid that you can read the results after only 5 minutes of color development.	detects from 1 microgram to 20 micrograms of protein
The standard has a known protein concentration, since we can weigh out purified bovine serum albumin (BSA) protein powder and dissolve it in water or buffer to get a solution with 50 µg of protein in each ml.	BSA standard has known amounts of protein (0, 5, 10, 15, 20 µg)
Since we do not know how much protein is in the test samples (unknowns), we prepare serial 1:10 dilutions of them. Then we set up assay tubes for each dilution. After mixing DYE REAGENT with both the BSA standards and the unknowns, wait 5 minutes. Then, compare the colors of the unknown tubes to the colors of the standards.	compare colors of unknowns to the colors of the BSA standard

QUESTIONS:

1. What color is the DYE REAGENT in the absence of protein?
and in the presence of protein?

2. Which of these can the DYE REAGENT detect (Y or N). Explain your answer.:
DNA

RNA

Sugar

Free amino acids

Polypeptides

Most enzymes

3. Why do you need a "standard" for this test?

4. Can you get a protein value for your unknown of 7 µg? Explain.

5. What if your unknown protein develops a color brighter than the highest amount of BSA?

Instructions for Conducting the BioRad Protein Assay

Check Off Here
AS you COMPLETE Each Step

1. Fill in each chart.

2. Label tubes.

Note: You will make duplicate tubes for the BSA Standard and the Milk Protein Assays. Give every tube a different number so you can still distinguish each of the duplicate tubes. You will only prepare one tube each for the milk dilutions, so pipette carefully!

3. Add indicated volumes of water to all the tubes that need water.

4. Prepare serial (1:10) dilutions of the milk using the water pipette.

5. Add the milk dilutions to their appropriate assay tubes.
Mix the tubes by flicking or on the Vortex Genie mixer.

6. Obtain the BSA standard solution (50 µg/ml), and a fresh pipette.
Add the indicated volumes to the standard assay tubes.

7. Obtain DYE REAGENT and a fresh pipette. Add 0.2 ml of the DYE Reagent to all the assay tubes. **Mix** each well immediately after you add the reagent.

8. After 5 min., describe the colors of the BSA Standard tubes.
Then compare the colors of the milk assay tubes with the colors of the BSA standard.
Assign each milk protein assay tube a protein amount. Enter your estimates on chart II.

9. Clean up and resupply the work area with dishes and pipettes.

Record your 10. Calculate
Numerical ANSWERS
Here

A. the protein amount per assay tube

(Hint: take the average of wells 11, 12, etc.)

B. the protein concentration per ml of milk dilution

(Hint: what is the portion of a milliliter that you actually assayed?)

C. the protein concentration per ml of undiluted milk and

(Hint: What is the dilution factor?)

D. the protein amount per container.

(Hint: How many ml are in a container?)

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STUDENT CHART

Chart I. STANDARD (BSA Protein Concentration is 50µg/ml)

Well Number	Water Volume = (ml)	Sample = BSA Volume = (ml)	**DYE Reagent** Volume = 0.2 ml	Protein Amount added= (**µ**g)	**Color** describe colors
1,2	**0.8 ml**	**none**	0.2	0 **µ**g	
3,4	**0.7**	**0.1 ml**	0.2	5 **µ**g	
5,6	0.6	0.2	0.2	10 **µ**g	
7,8	0.5	0.3	0.2	15 **µ**g	
9,10	0.4	0.4	0.2	20 **µ**g	

Chart II. Serial Dilutions for Unknown = MILK

Sample Dilutions	**Water Volume (ml)**	**Sample =Milk Volume (ml)**	**Calculated** Protein Concentration (**µ**g per ml)	**Calculated** Protein Amount (**µg per 0.2 ml**)	In the RANGE? (Y or N)
MILK	**no water**				N
A	**0.9 ml**	**0.1 ml Milk**			N
B	ml	**0.1 ml A**			N
C	ml	ml B			Y

Chart III. Milk Protein Assay

Well Number	Water Volume = (ml)	Sample = Milk Dilution Volume= ??ml	**DYE Reagent** Volume = 0.2 ml	**Color describe**	Protein Amount (**µ**g) cf. standard should be a number
11,12		**C**	**0.2 ml**		
13,14		**B**	**0.2 ml**		
15,16		**A**	**0.2 ml**		

Materials

per team

2 24-well dishes
3 pipettes
pipette pump

index card
labeling pen

to pick up from teacher

protein samples
3 ml of 50 ug/ml **BSA standard**
(1 full well)
0.2 ml of **milk** for dilution and assay of unknown

Bio-Rad reagent DANGER! Acid!
3ml (1 full well)

Use 1 24-well plate for the BSA standard
Use the other plate to make the milk dilutions and to assay the different milk dilutions
Prepare the milk dilutions and milk protein assay in 1 dish and compare to the BSA standard prepared in the other dish.

You may substitute powdered egg white for the bovine serum albumin standard.

SAFETY

The Bio-Rad Bradford Reagent contains about 15% phosphoric acid and 20% methanol. Thus, the reagent is corrosive.

The dye stains protein and so will stain the skin and clothing, especially silk and wool.

Clean up

Soak used plastic pipettes in a plastic cylinder with a soapy solution of Joy detergent. Rinse very well (about 6-10 rinses) with tap water to remove all soap residue (which turns the Bradford assay green!). Shake out to speed up the drying process

Pour spent solutions down the drain and immerse tubes in soapy water. Rinse well.

Pour spent solutions from plates. Rinse in tap water, then soak for a minute in 10% bleach and rinse well in tap water.

Sample calculations

(grams, milligrams, micrograms, nanograms, picograms, etc.)

Getting from the protein content in the milk container to the range of protein the Bio-Rad protein assay can detect

The milk container says it has 8 grams of protein. The container holds 236 ml.

8 g /236 ml = 0.0339 g of protein per ml.

The protein assay detects ug of protein per ml. Therefore **convert g to ug**.

1 g is 1 million ug (micrograms, greek letter mu).

The milk protein concentration is **33,900 ug/ml (33.9 milligrams/ml).**

To get from here to a concentration detectable by the assay, you need to divide by 10,000, or, **dilute 10,000-fold**. However, we only assay a portion of any sample, so we can assay 0.1 ml (or 0.2 ml for good luck!).

So, we can make a 1:1000 dilution. We can add 1 ml of milk to 999 ml of water, but this is really wasteful since we may only test 0.1 ml! So we can get to the same place by making

3 serial 1:10 dilutions, resulting in a solution with 33.9 ug/ml.
(Serially/Cereally diluting the milk --- Ha Ha!)

Using 0.1 ml of the 1:1000 dilution will provide 3.4 ug of protein.
Using 0.2 ml of the 1:1000 dilution will provide 6.8 (7) ug of protein.
Using 0.3 ml of the 1:1000 dilution will provide 10.4 (10) ug of protein.

Getting from the protein assay values to milk

Students assayed duplicate 0.2 ml samples of the A, B, and C dilutions of milk. A and B were both very blue but C tubes were gray blue. By comparison with the BSA standard, the color looked between the colors of the 5 and 10 ug samples, so the students chose 8, because the colors looked more like the 10 ug standards than the 5 ug standards.

The students estimated that their **0.2 ml sample of milk dilution C had 8 ug of protein in it.**

To calculate the protein content per ml of dilution C, multiply by 5, since
(1 ml/0.2 ml) = 5. Thus, **dilution C has 40 ug of protein per ml.**

Dilution C is a 1:1000 dilution of milk
(0.1/10 = A, X 0.1/10 = B, X 0.1/10 = C).
Thus, multiply 40 ug/ml by 1,000. Therefore, milk has 40,000 ug of protein per ml or 40 mg of protein per ml.