The Colorimetric Detection and Quantitation of Total Protein

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ABSTRACT

Protein quantification is an important step for handling protein samples for isolation and characterization; it is a prerequisite step before submitting proteins for chromatographic, electrophoretic, or immunochemical analysis and separation. Colorimetric methods are fast, simple, and not laborious. This unit describes a number of assays able to detect protein concentrations in the low microgram to milligram per milliliter ranges in a variety of formats. *Curr. Protoc. Cell Biol.* 52:A.3H.1-A.3H.28. © 2011 by John Wiley & Sons, Inc.

Protein quantification is an important step for handling protein samples for isolation and characterization, and is a prerequisite step before submitting proteins for chromatographic, electrophoretic, or immunochemical analysis and separation. The methods included in this appendix are colorimetric measurements, whose procedures are fast and simple.

This appendix describes four of the most commonly used total protein assay methods. Three of the four are copper-based assays to quantitate total protein: the Lowry method (see Basic Protocol 1 and Alternate Protocols 1 and 2), the bicinchoninic acid assay (BCA; see Basic Protocol 2 and Alternate Protocols 3 and 4), and the biuret method (see Basic Protocol 3 and Alternate Protocol 5). The fourth is the Coomassie dye binding or Bradford assay (see Basic Protocol 4 and Alternate Prortocols 6 and 7), which is included as a simple and sensitive assay, although it sometimes gives a variable response depending on how well or how poorly the protein binds the dye in acidic pH. A protein assay method should be chosen based on the sensitivity and accuracy of method as well as the condition of the sample to be analyzed.

STRATEGIC PLANNING

Colorimetric Protein Assays

The four colorimetric methods for the detection and quantitation presented in this appendix have withstood the test of time. They are all well-characterized robust assays that consistently work well. The methods were introduced over the past 15 to 50 years. They collectively represent the state of the art for colorimetric detection and quantitation of total proteins in the microgram to milligram range.

When confronted with the need to determine the total protein concentration of a sample, one of the first issues to consider is selection of a protein assay method. The choice among the available protein assays usually is made based upon consideration of the compatibility of the method with the samples to be assayed. The objective is to select a method that requires the least manipulation or pretreatment of the samples due to the presence of substances that may interfere. If the total protein concentration in the samples is high (i.e., in the range of 5 to 160 mg/ml), the biuret total protein reagent is the best choice. If the total protein concentration in the samples is low (i.e., in the range of 1 to 2000 μg/ml), then any one of the other three (i.e., the Lowry, the Coomassie Plus,

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or the BCA method) would be suitable. If the sample contains reducing agents or copper-chelating reagents, the Coomassie Plus Protein Assay Reagent (Pierce) would be the best choice. If the sample contains one or more detergents (at concentrations up to 5%), the BCA Protein Assay Reagent is the best choice.

Sometimes the sample contains substances that make it incompatible with any of the protein assay methods. In those cases, some pretreatment of the sample is necessary.

Each method has its advantages and disadvantages. No one method can be considered to be the ideal or best protein assay method. Because of this, most researchers keep more than one type of protein assay reagent available in their laboratory.

Selection of the Protein Standard

The selection of a protein standard is potentially *the* greatest source of error in any protein assay. Of course, the best choice for a standard is a highly purified version of the predominant protein found in the samples. This is not always possible nor always necessary. In some cases, all that is needed is a rough estimate of the total protein concentration in the sample. For example, in the early stages of purifying a protein, identifying which fractions contain the most protein may be all that is required. If a highly purified version of the protein of interest is not available or it is too expensive to use as the standard, the alternative is to choose a protein that will produce a very similar color response curve with the selected protein assay method.

For general protein assay work, bovine serum albumin (BSA) works well as the choice for a protein standard, because it is widely available in high purity and relatively inexpensive. Although it is a mixture containing several immunoglobulins, bovine gamma globulin (BGG) is also a good choice for a standard when determining the concentration of antibodies, since BGG produces a color response curve that is very similar to that of immunoglobulin G (IgG).

For greatest accuracy of the estimates of the total protein concentration in unknown samples, it is essential to include a standard curve in each run. This is particularly true for the protein assay methods that produce nonlinear standard curves (e.g., Lowry method, Coomassie dye–binding method). The decision about the number of standards used to define the standard curve and the number of replicates to be done on each standard depends upon the degree of nonlinearity in the standard curve and the degree of accuracy required of the results. In general, fewer points are needed to construct a standard curve if the color response curve is linear. For assays done in test tubes, duplicates are sufficient; however, triplicates are recommended for assays performed in microtiter plates due to the increased error associated with microtiter plates and microtiter plate readers.

Preparation of the Samples

Before a sample can be analyzed for total protein content, it must be solubilized, usually in a buffered aqueous solution. The entire process is usually done in the cold, with additional precautions taken to inhibit microbial growth or to avoid casual contamination of the sample by foreign debris such as hair, skin, or body oils. When working with tissues, cells, or solids, the first step of the solubilization process is usually disruption of the sample's cellular structure by grinding and/or sonication, or by the use of specially designed reagents containing surfactants to lyse the cells (i.e., the "POPPERS" line of products, available from Pierce). This is done in a cold aqueous buffer containing one or more surfactants (to aid the solubilization of the membrane-bound proteins), one or more biocides (to prevent microbial growth), and protease inhibitors (to minimize or prevent digestion of the proteins into peptide fragments by endogenous proteases). After filtration or centrifugation (to remove the cellular debris), additional steps such as sterile

filtration, removal of lipids, or further purification of the protein of interest from the other sample components may be necessary.

Calculation of the Results

If calculating the protein concentrations manually, it is best to use point-to-point interpolation. This is especially true if the standard curve is nonlinear. Point-to-point interpolation refers to a method of calculating the results for each sample using the equation for a linear regression line obtained from just two points on the standard curve. The first point is the standard that has an absorbance just below that of the sample and the second point is the standard that has an absorbance just above that of the sample. In this way, the concentration of each sample is calculated from the most appropriate section of the whole standard curve. The average total protein concentration for each sample is determined from the average of its replicates. If multiple dilutions of each sample have been run, the results for the dilutions that fall within the most linear portion of the working range are averaged.

If using a computer program, use a quadratic curve fit for the nonlinear standard curve to calculate the protein concentration of the samples. If the standard curve is linear or if the absorbance readings for the samples fall within the linear portion of the standard curve, the total protein concentrations of the samples can be estimated using the linear regression equation.

Most software programs will allow the experimenter to construct and print a graph of the standard curve as well as calculate the protein concentration for each sample and display statistics for the replicates. Typically, the statistics displayed will include the average of the absorbance readings (or the average of the calculated protein concentrations), the standard deviation (SD), and the coefficient of variation (CV) for each standard or sample. If multiple dilutions of each sample have been run, average the results for the dilutions that fall in the most linear portion of the working range.

THE LOWRY PROTEIN ASSAY FOR DETERMINATION OF TOTAL PROTEINS

BASIC PROTOCOL 1

In 1951, Oliver H. Lowry introduced this colorimetric total protein assay method. It offered a significant improvement over previous protein assays, and his paper became one of the most cited references in the life-science literature (Lowry, 1951). The Lowry assay is easy to perform since the incubations are done at room temperature and the assay is sensitive enough to allow the detection of total protein in the low microgram per milliliter range. It is one of three copper chelation chemistry–based methods presented in this appendix. Essentially, the Lowry protein assay is an enhanced biuret assay (see Basic Protocol 3). After a short incubation, Lowry's reagent C (Folin phenol) is added for enhanced color development (Fig. A.3H.1). The Lowry assay requires fresh (daily) preparation of two reagents and a meticulously timed incubation step. The two reagents are combined just before use to make a buffered alkaline cupric sulfate working solution. The addition of sodium dodecyl sulfate (SDS) to Lowry's reagent D (i.e., Lowry's reagent D′) allows the method to be used with samples that contain detergents.

Materials

Standard protein: 2 mg/ml BSA (see recipe)
Sample buffer or solvent
Protein sample(s)
Lowry's Reagents C and D or D′ (see recipes)

1. Dispense 0 to 100 µl standard protein to appropriately labeled tubes and bring the total volume to 100 µl with sample buffer or solvent to prepare a dilution series from 10 to 100 µg.

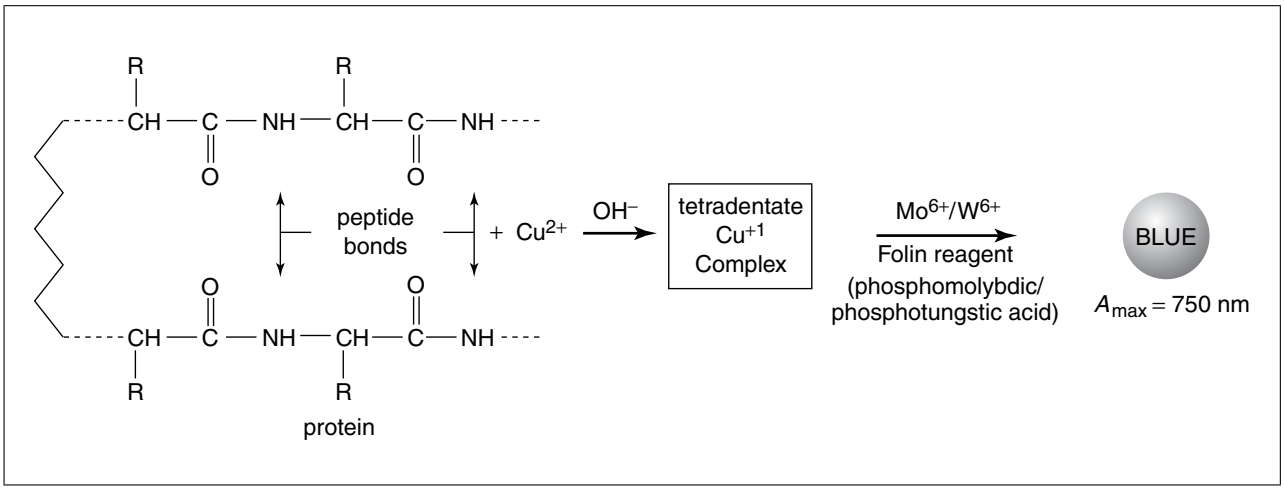

Figure A.3H.1 The reaction schematic for the Lowry Protein Assay.

These concentrations of albumin should produce A_{750} readings from ~0.10 to 1.0 AU in 1-cm cuvettes.

2. Dispense ≤100 µl protein sample(s) to separate labeled tubes and adjust the final volume to 100 µl using the same buffer or solvent used to prepare the sample.
3. Add 1 ml reagent D (or reagent D′) to each of the standards and unknown samples. Vortex the tubes immediately to develop optimum color. Incubate for precisely 10 min at room temperature.

   If samples contain detergent, use reagent D′ to eliminate the interference associated with up to 1% of various detergents. If a precipitate forms in reagent D′, warm the reagent and vortex before addition.

4. While mixing, add 0.1 ml reagent C. Vortex the tubes immediately. Incubate for 30 min at room temperature.
5. Measure the color at 750 nm (A_{750}) on a spectrophotometer zeroed with deionized water.

   Read samples within 10 min, as samples continue to develop color. Samples incubated >60 min should be discarded.

   If the absorbance reading of the sample is higher than that of the highest concentration of standard, dilute the sample with buffer and repeat the procedure on the diluted sample.

6. Plot a standard curve by graphing the average net or blank-corrected A_{750} values for each standard versus its protein concentration in milligrams per milliliter.
7. Determine sample protein concentration by interpolating from the standard curve (see Strategic Planning).

ALTERNATE PROTOCOL 1

MODIFIED LOWRY PROTEIN ASSAY FOR DETERMINATION OF TOTAL PROTEINS

Preformulated, stabilized, modified versions of the Lowry reagent are now commercially available from Pierce (the Modified Lowry Protein Assay Reagent) or from Bio-Rad (the DC Protein Assay). The assay can be performed in test tubes or a microtiter plate (see Alternate Protocol 2). The working range of this assay is 1 to 1500 µg/ml if the Pierce reagent is used, or 200 to 1400 µg/ml if the Bio-Rad reagent is used. Table A.3H.1 is a brief troubleshooting guide for this technique.

Table A.3H.1 Troubleshooting Guide for the Modified Lowry Protein Assay

Problem	Possible cause	Solution
No color in any tubes	Sample contains a chelating agent (e.g., EDTA, EGTA)	Dialyze or dilute the sample
		Precipitate the protein with TCA and dissolve the pellet in modified Lowry reagent
Blank A_{750} is normal, but standards show less color than expected	Sample changed the pH of the reagent	Dialyze or dilute the sample
	Color measured at the wrong wavelength	Measure the color at 750 nm
Precipitate forms in all tubes	Sample contains a surfactant (detergent)	Dialyze or dilute the sample
	Sample contains potassium ions	Precipitate the protein with TCA, dissolve the pellet in Modified Lowry Reagent
All tubes (including the blank) are dark purple	Sample contains a reducing agent	Dialyze or dilute the sample
	Sample contains a thiol	Precipitate the protein with TCA, dissolve pellet in Modified Lowry Reagent
Need to read color at a different wavelength	Colorimeter does not have 750-nm filter	Color may be read at any wavelength between 650 nm and 750 nm

Additional Materials *(also see Basic Protocol 1)*

Modified Lowry Protein Assay Kit (Pierce) containing:
2 mg/ml BSA in 0.9% (w/v) NaCl/0.05% (w/v) sodium azide
2 N Folin-Ciocalteu reagent: dilute fresh to 1 N
Modified Lowry's Reagent

1. Prepare a dilution series of 2 mg/ml BSA (e.g., the standard provided in the Modified Lowry Protein Assay Kit) in buffer to cover the range 2.0 to 1500 μg/ml.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

2. In duplicate, add 200 μl diluted standard, sample, or buffer (blank) into appropriately labeled test tubes.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

3. At 15-sec intervals, add 1.0 ml Modified Lowry Protein Assay Reagent to each of the tubes. Vortex 2 to 3 sec to mix the contents of the tube and incubate at room temperature for *exactly* 10 min.

4. At the end of the first tube's 10-min incubation, add 100 μl freshly diluted 1 N Folin-Ciocalteu reagent (freshly diluted from a 2 N stock). Immediately vortex the tube for 2 to 3 sec. Continue to maintain the 15-sec intervals from step 3 for addition of the reagent to the remaining tubes.

5. Allow each of the tubes to incubate for 30 min at room temperature.

6. Measure the color at 750 nm (A_{750}) on a spectrophotometer zeroed with deionized water.

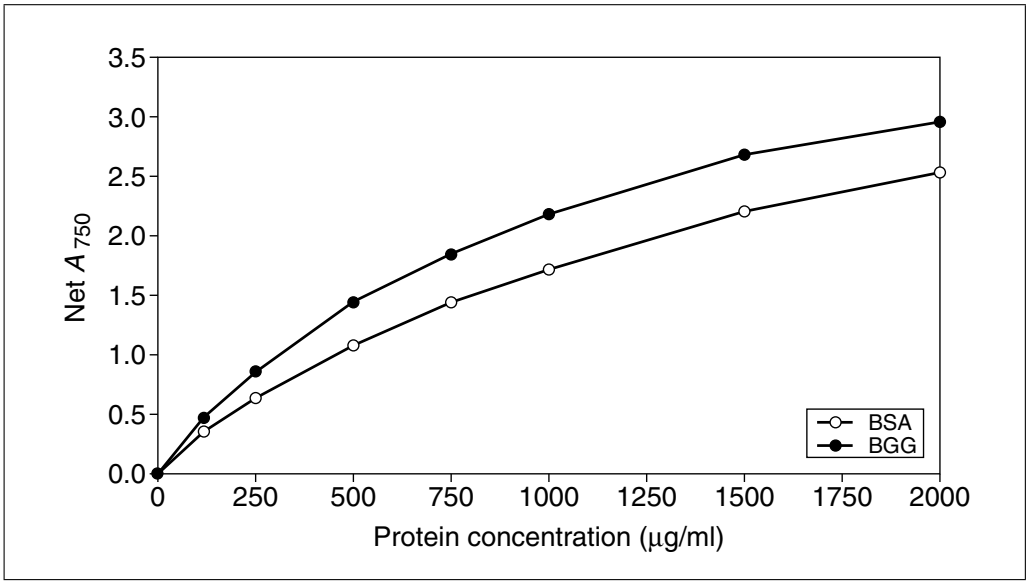

Figure A.3H.2 Graph of the color response curves obtained with Pierce's Modified Lowry Protein Assay Reagent using bovine serum albumin (BSA) and bovine gamma globulin (BGG). The standard tube protocol was performed and the color was measured at 750 nm in a Hitachi U-2000 spectrophotometer.

7. Plot a standard curve by graphing the average net or blank-corrected A_{750} values for each BSA standard versus its concentration in micrograms per milliliter.

   Example color response curves for BSA and BGG are shown in Figure A.3H.2.

8. Determine the sample concentration by interpolating from the plot (see Strategic Planning). Determine the average total protein concentration for each sample from the average of its replicates.

ALTERNATE PROTOCOL 2

MICROTITER PLATE MODIFIED LOWRY ASSAY FOR TOTAL PROTEIN

The modified Lowry assay can also be done in a 96-well microtiter plate format. The assay has a working range of 1 to 1500 μg/ml.

Additional Materials *(also see Alternate Protocol 1)*

Microtiter plate and cover or tape seals
200-μl multichannel pipettor
Microtiter plate reader for 750 nm

1. Draw a template for placement of samples and standards on the microtiter plate.

   Blanks, standards, and samples should be prepared in triplicate.

2. Add 40 μl of each diluted BSA standard (see Alternate Protocol 1, step 1), sample, or diluent (blank) to the appropriate wells of a 96-well plate.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

3. Using a multichannel pipettor, quickly add 200 μl Modified Lowry Protein Assay Reagent to each of the wells. Mix immediately on a plate mixer for 30 sec.

4. Allow the plate to incubate at room temperature for *exactly* 10 min.

5. Using a multichannel pipettor, quickly add 20 μl freshly diluted 1 N Folin-Ciocalteu reagent to each well. Immediately mix on a plate mixer for 30 sec.

6. Cover the plate (to prevent evaporation) and incubate 30 min at room temperature.
7. Mix the plate again and measure the color (absorbance) of each well in a microtiter plate reader at 750 nm.
8. Plot a standard curve by graphing the average net or blank-corrected A_{750} values for each standard versus its protein concentration in micrograms per milliliter.
9. Determine the sample concentration by interpolating from the plot (see Strategic Planning). Calculate the average total protein concentration for each sample from the average of its replicates.

THE BICINCHONINIC ACID (BCA) ASSAY FOR DETERMINATION OF TOTAL PROTEIN

BASIC PROTOCOL 2

Smith et al. (1985) introduced the bicinchoninic acid (BCA) protein assay reagent. In one sense, it is a modification of the Lowry protein assay reagent. The mechanism of color formation with protein for the BCA protein assay reagent is similar to that of the Lowry reagent, but there are several significant differences. The BCA protein assay reagent combines the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (i.e., the biuret reaction; see Basic Protocol 3) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) by bicinchoninic acid.The purple-colored reaction product of this method is formed by the chelation of two molecules of BCA with one cuprous ion (Fig. A.3H.3). The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The primary advantage of the BCA protein assay reagent is that most surfactants, even if present in the sample at concentrations up to 5% (v/v), are compatible with this method. Table A.3H.2 is a brief troubleshooting guide for this technique.

Materials

Protein standard: 2 mg/ml BSA (see recipe)
Sample buffer or solvent
Protein sample
BCA working reagent: mix 100 parts BCA reagent A with 2 parts reagent B (see recipes for each reagent)

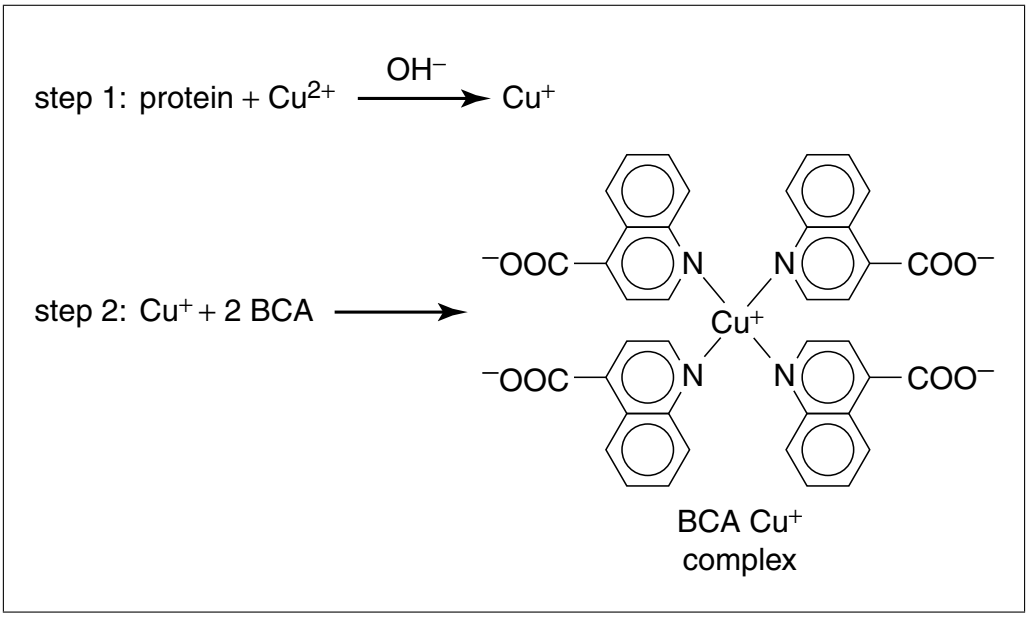

Figure A.3H.3 The reaction schematic for the BCA Protein Assay.

Table A.3H.2 Troubleshooting Guide for BCA Protein Assay

Problem	Possible cause	Solution
No color in any tubes	Sample contains a copper chelating agent	Dialyze or dilute the sample
		Increase the copper concentration in the working reagent (use 48 parts reagent A and 2 parts reagent B)
Blank A_{562} is normal, but standards and samples show less color than expected	Strong acid or alkaline buffer, alters working reagent pH	Dialyze or dilute the sample
	Color measured at the wrong wavelength	Measure the color at 562 nm
Color of samples appear darker than expected	Protein concentration is too high	Dilute the sample
	Sample contains lipids or lipoproteins	Add 2% (w/v) SDS to the sample to eliminate interference from lipids
All tubes (including the blank) are dark purple	Sample contains a reducing agent	Dialyze or dilute the sample
	Sample contains a thiol	Precipitate the protein with trichloroacetic acid (TCA) and deoxycholate (DOC), dissolve pellet in BCA working reagent
	Sample contains biogenic amines (catecholamines)	Treat the sample with iodoacetamide (for thiols)
Need to read color at a different wavelength	Colorimeter does not have 562-nm filter	Color may be read at any wavelength between 550 nm and 570 nm

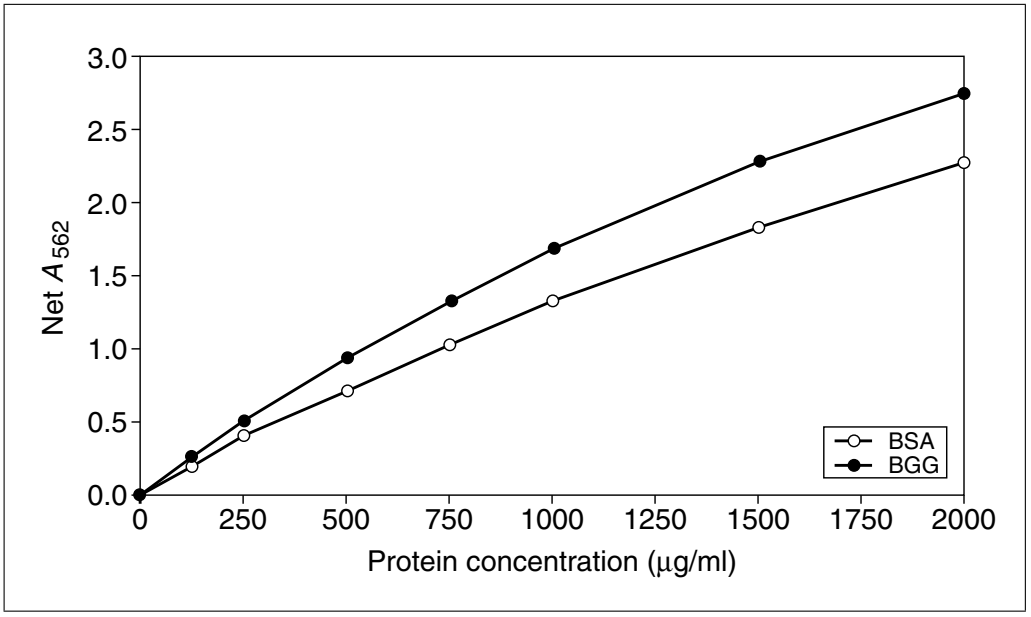

Figure A.3H.4 Graph of the color response curves obtained with Pierce's BCA Protein Assay Reagent using bovine serum albumin (BSA) and bovine gamma globulin (BGG). The standard tube protocol was performed and the color was measured at 562 nm in a Hitachi U-2000 spectrophotometer.

1. Prepare a dilution series of 2 mg/ml BSA in sample buffer or diluent to cover a range from 125 to 2000 μg/ml.
2. Add 100 μl sample, diluted standard, or buffer (blank) into appropriately labeled tubes.
3. Add 2 ml BCA working reagent mix to each tube. Vortex immediately.
4. Incubate samples and standards for 30 min at 37°C, then cool to room temperature.
5. Measure the color at 562 nm (A_{562}) on a spectrophotometer zeroed with deionized water.
6. Plot a standard curve by graphing the average net or blank-corrected A_{750} values for the standards versus protein concentration in micrograms per milliliter.

   Example color response curves for BSA and BGG are shown in Fig. A.3H.4.

7. Determine the protein concentration of the sample by interpolation from the plot (see Strategic Planning).

USING KITS FOR BCA MEASUREMENTS OF TOTAL PROTEIN

ALTERNATE PROTOCOL 3

Preformulated versions of the BCA reagent are now commercially available from Pierce (BCA Protein Assay Reagent) or Sigma (Bicinchoninic Acid Kit for Protein Determination). This assay can be performed in test tubes or microtiter plates (see Alternate Protocol 4), and has a working range of 20 to 2000 μg/ml.

Additional Materials

BCA Protein Assay Reagent Kit (Pierce) or Bicinchoninic Acid Kit (Sigma) containing:
2 mg/ml BSA in 0.9% NaCl/0.05% sodium azide (also see recipe)
BCA reagent A (also see recipe)
BCA reagent B (also see recipe)
37°C water bath

1. Prepare a dilution series of BSA standard in buffer to cover the range 125 to 2000 μg/ml.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

2. Prepare sufficient BCA working reagent (a minimum of 2 ml/tube or 20 ml/96-well microtiter plate) by adding 2 parts BCA reagent B to 100 parts of BCA reagent A.

   After mixing, the BCA working reagent is clear and apple green in color.

3. In duplicate, add 100 μl standard, sample, or buffer (blank) into appropriately labeled test tubes.
4. Add 2.0 ml BCA working reagent to each tube. Mix well by vortexing each tube 2 to 3 sec.

5a. *Standard tube protocol:* Incubate all tubes in a 37°C water bath for 30 min.

5b. *Enhanced assay protocol:* Alternatively, incubate all tubes in a 60°C water bath for 30 min.

   Increasing the incubation temperature to 60°C lowers the minimum detection level to 5 μg/ml and narrows the working range of the assay to a maximum of 250 μg/ml. This is known as the enhanced BCA assay.

6. After incubation, cool all tubes to room temperature.

   Since the BCA reagent does not reach a true end point, color development will continue even after cooling to room temperature; however, the rate of color development is very slow after cooling to room temperature, so no significant error is introduced if the A_{562} readings of all the tubes can be read within ~10 min.

7. Before reading, mix each tube again and measure the amount of color produced in each tube with a spectrophotometer at 562 nm (A_{562}) versus deionized water.

8. Plot a standard curve by graphing the average net or blank-corrected A_{562} values for the standards versus protein concentration in micrograms per milliliter.

9. Determine the sample concentration by interpolating from the plot (see Strategic Planning). Calculate the average sample concentration from its replicates.

ALTERNATE PROTOCOL 4

MICROTITER PLATE ASSAY FOR BCA MEASUREMENT OF TOTAL PROTEIN

BCA assays can be run in 96-well microtiter plates. The assay has a working range of 125 to 2000 μg/ml.

Additional Materials *(also see Alternate Protocol 3)*

96-well microtiter plate with cover or tape seal
200-μl multichannel pipettor
Microtiter plate shaker
37°C dry-heat incubator
Microtiter plate reader

1. Draw a template for the placement of samples and standards on a microtiter plate.

   Blanks, standards, and samples should be prepared in triplicate.

2. Add 10 μl of each diluted BSA standard (see Alternate Protocol 3, step 1), sample, or diluent (blank) to the appropriate wells.

3. Using a multichannel pipettor, add 200 μl BCA working reagent (see Alternate Protocol 3, step 2) to each well. Mix well on a microtiter plate shaker for 30 sec.

4. Cover the plate and incubate in a 37°C dry-heat incubator for 30 min.

5. After incubation, allow the plate to cool to room temperature.

6. Mix the plate again, remove the plate cover and measure the color in each well of the plate at 562 nm (A_{562}) in a microtiter plate reader.

7. Plot a standard curve by graphing the average net or blank-corrected A_{562} values for each BSA standard versus its concentration in micrograms per milliliter.

8. Determine the sample concentration by interpolating from the plot (see Strategic Planning). Calculate the average total protein concentration for each sample from the average of its replicates.

BASIC PROTOCOL 3

THE BIURET ASSAY FOR DETERMINING TOTAL PROTEIN

All proteins are composed of amino acids joined by peptide bonds in a linear sequence. There are ~20 naturally occurring amino acids found in proteins. The amino acids are joined to each other by peptide bonds formed by a condensation reaction that occurs between the terminal amine of one amino acid and the carboxyl end of the next. Peptides containing three or more amino acid residues will form a colored chelate complex with

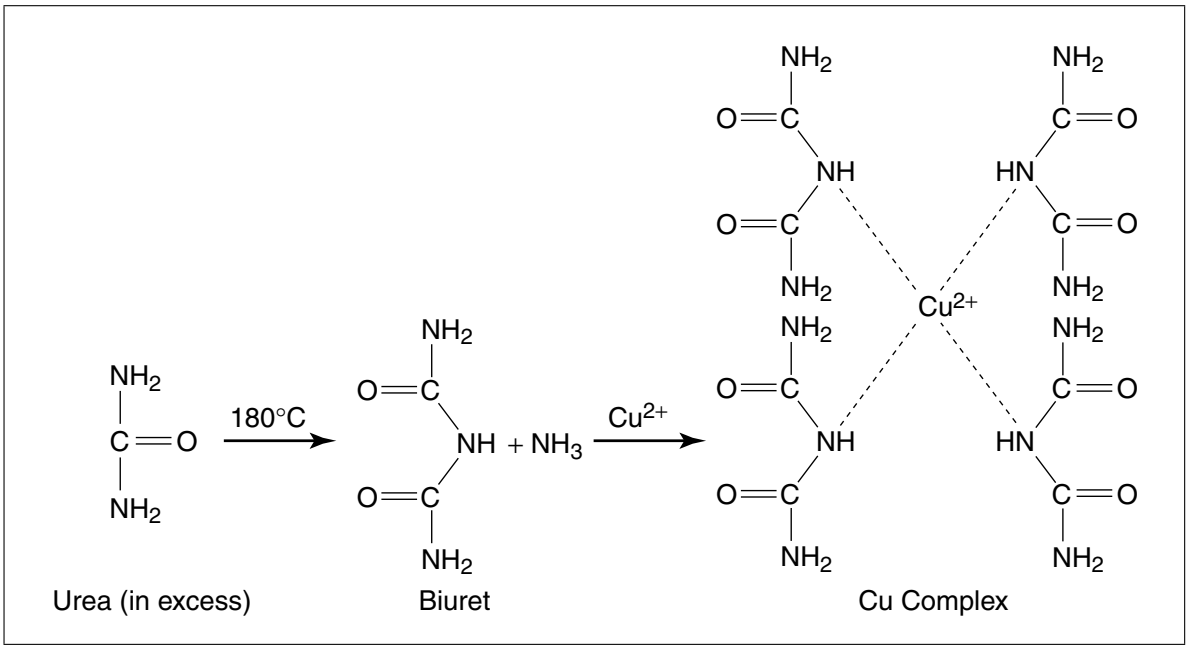

Figure A.3H.5 The schematic of the biuret reaction.

Table A.3H.3 Troubleshooting Guide for Biuret Protein Assay

Problem	Possible cause	Solution
No color in any tubes	Sample contains copper chelating agent	Dialyze or dilute the sample
Blank A_{540} is normal, but standards show less color than expected	Color measured at the wrong wavelength	Measure the color at 540 nm
All tubes (including the blank) are dark purple	Sample contains a reducing agent	Dialyze or dilute the sample

cupric ions in an alkaline environment containing sodium potassium tartrate. A similar colored chelate complex forms with the organic compound biuret (NH_2–CO–NH–CO–NH_2) and the cupric ion. The reaction in which a colored chelation complex is formed with peptide bonds in the presence of an alkaline cupric sulfate solution became known as the biuret reaction (Fig. A.3H.5). Thus, the biuret protein assay reagent gets its name from the above reaction even though it does not actually contain the organic compound biuret. Single amino acids or dipeptides do not give the biuret reaction, but tripeptides and larger polypeptides or proteins will react to produce the light-blue to violet complex that absorbs light at 540 nm. One cupric ion forms the colored coordinate complex with 4 to 6 nearby peptides bonds. The intensity of the color produced is proportional to the number of peptide bonds participating in the reaction. Thus, the biuret reaction is the basis for a simple and rapid colorimetric method of quantitatively determining total protein concentration.

Because the working range for the biuret assay is from 5 to 160 mg/ml, the biuret reagent has found utility in the clinical laboratories for the quantitation of total protein in serum. The formulation employed in the biuret total protein reagent (Sigma Diagnostics) was developed by Doumas et al. (1981) as a candidate reference method for the determination of serum total protein in the clinical lab. Using Sigma's biuret reagent, the expected range for total protein in serum is from 63 to 83 mg/ml. Bilirubin, lipids, hemoglobin, and dextran are known to interfere in the biuret assay for total serum protein.

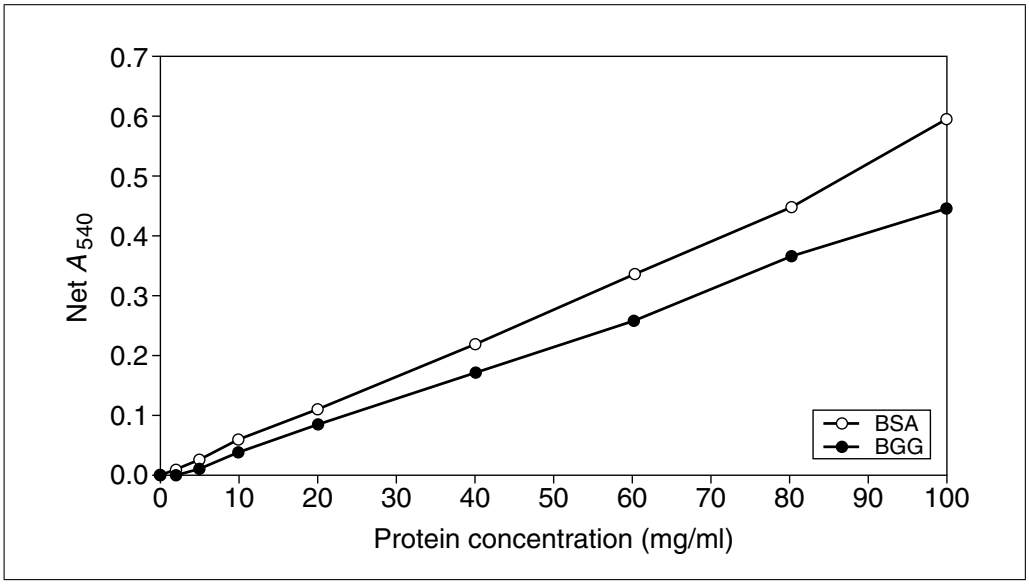

Figure A.3H.6 Graph of the color response curves obtained with Sigma's biuret Total Protein Reagent using bovine serum albumin (BSA) and bovine gamma globulin (BGG). The standard tube protocol was performed and the color was measured at 540 nm in a Hitachi U-2000 spectrophotometer.

Outside of this application, other copper chelating agents such as EDTA, EGTA, citrate, Tris, iminodiacetic acid, and nitrilotriacetic acid will interfere. The working range of this assay is 5 to 160 mg/ml. Table A.3H.3 is a brief troubleshooting guide for this technique.

Materials

Standard protein (also see Commentary)
Sample (unknown) protein
Biuret total protein reagent (Sigma Diagnostics; also see recipe)

1. Select a protein to use as the standard (see Strategic Planning) and prepare a dilution series with buffer to cover the range 10 to 160 mg/ml.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the standard that was used with the samples.

2. In duplicate, add 20 µl standard, sample, or diluent (blank) to appropriately labeled test tubes.

3. Add 1.0 ml biuret reagent to each tube. Mix well by vortexing 2 to 3 sec.

4. Incubate tubes at ambient room temperature (18° to 26°C) for 10 min.

5. Measure the color of each tube with a spectrophotometer at 540 nm (A_{540}). Compare to the blank.

6. Plot a standard curve by graphing the blank-corrected A_{540} values for the standards versus protein concentration in milligrams per milliliter.

   Example color response curves for BSA and BGG are shown in Figure A.3H.6.

7. Determine the sample concentration by interpolation from the standard curve (see Strategic Planning).

MICROTITER PLATE BIURET ASSAY FOR TOTAL PROTEIN

ALTERNATE PROTOCOL 5

The biuret assay can be performed in a 96-well microtiter plate and has a working concentration of 10 to 160 mg/ml.

Additional Materials *(also see Basic Protocol 3)*

96-well microtiter plate and cover or tape sealer
250-μl multichannel pipettor and appropriate tips
Microtiter plate mixer
Microtiter plate reader

1. Draw a template for placement of samples and standards on the microtiter plate.
2. Select a protein to use as the standard (see Strategic Planning). Prepare a dilution series with the same buffer used to dilute the samples to cover the range of 10 to 160 mg/ml.
3. In triplicate, add 5.0 μl of each diluted standard or sample into the appropriate microtiter plate wells. Use the buffer or diluent that was used to dilute the standard and samples for the blank wells.
4. Using a multichannel pipettor, add 250 μl biuret reagent to each well. Mix well on a plate shaker for 30 sec.
5. Cover the plate and incubate at room temperature for 10 min.
6. Mix again. Remove the plate cover and measure the color in each well of the plate at 540 nm (A_{540}) in a microtiter plate reader.
7. Prepare a standard curve by graphing the average net or blank-corrected A_{540} values for each standard versus its concentration in milligrams per milliliter.
8. Determine the sample concentration by interpolating from the plot (see Strategic Planning).

THE COOMASSIE DYE–BINDING (BRADFORD) ASSAY FOR DETERMINING TOTAL PROTEIN

BASIC PROTOCOL 4

The Coomassie dye–based protein-binding assays have the advantage of being the fastest and the easiest to perform (Fig. A.3H.7). In addition, the assay is performed at room temperature and no special equipment, other than a spectrophotometer, is required. Briefly, the sample is added to the ready-to-use reagent and, following a short incubation, the resultant blue color is measured at 595 nm versus deionized water.

In 1976, Marion Bradford introduced the first Coomassie dye–based reagent for the rapid colorimetric detection and quantitation of total protein. The Coomassie dye (Bradford) protein assay reagents have the advantage of being compatible with most salts, solvents, buffers, thiols, reducing substances, and metal chelating agents encountered in protein samples.

Materials

Sample buffer or solvent
Protein standard (e.g., 2 mg/ml BSA; see recipe)
Protein sample
Coomassie dye reagent (Pierce or Bio-Rad; also see recipe)

1. Prepare a dilution series from protein standard (e.g., 2 mg/ml BSA) and sample buffer to cover the range 100 to 1000 μg/ml.

   Bovine serum albumin (BSA) is often used as a calibration standard, but it has greater general dye-binding capacity than most proteins (Bradford, 1976).

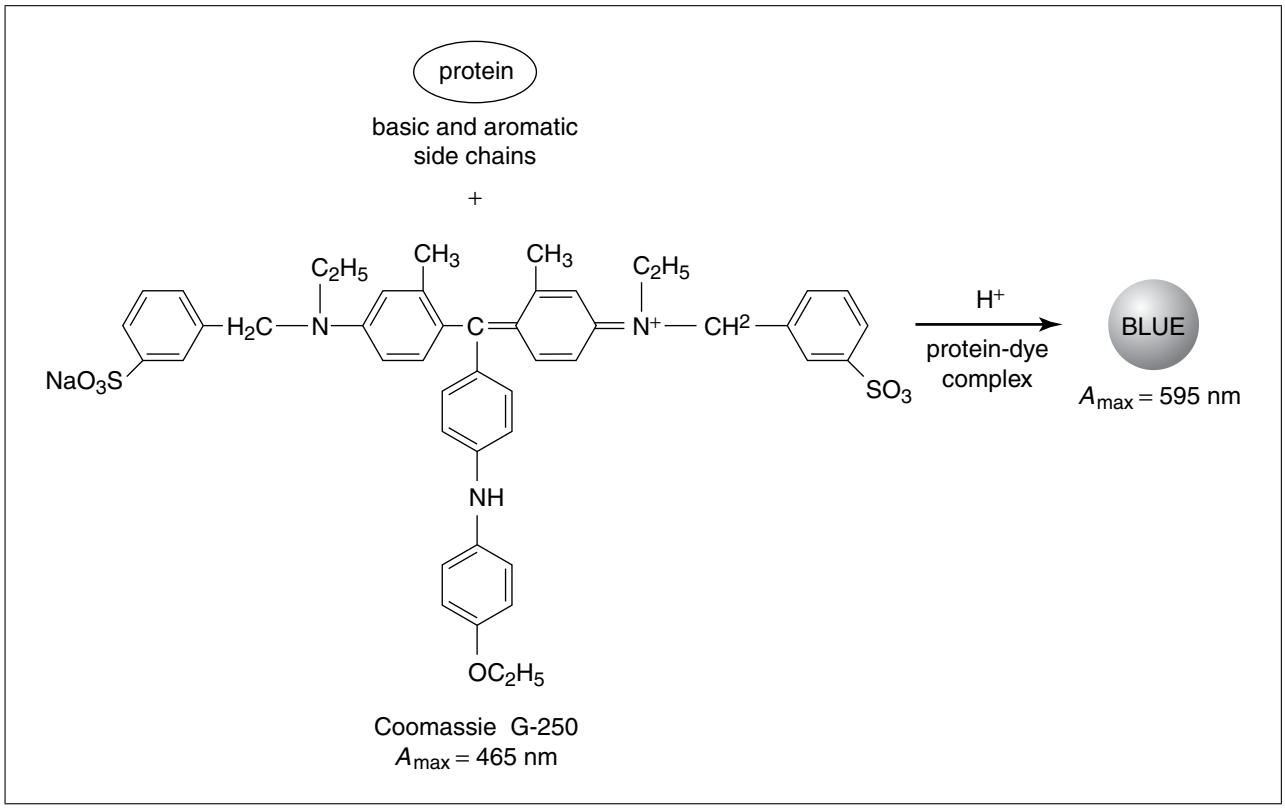

Figure A.3H.7 The reaction schematic for the Coomassie Protein Assay.

2. Dispense 0.1 ml standard, sample, or buffer to appropriately labeled tubes.

   If commercially prepared Coomassie reagent is used, follow the manufacturer's instructions. Commercial reagents tend to produce less turbidity and more consistent results.

3. Add 5 ml Coomassie dye reagent. Vortex immediately.
4. Incubate 10 min at room temperature.
5. Vortex each tube just before measuring the absorbance at 595 nm (A_{595}).
6. Plot a standard curve by graphing the average net or blank-corrected A_{595} values for the standard versus its protein concentration in micrograms per milliliter.
7. Determine the sample concentration by interpolation from the standard curve (see Strategic Planning).

ALTERNATE PROTOCOL 6

THE COOMASSIE PLUS PROTEIN ASSAY FOR DETERMINATION OF TOTAL PROTEIN

Several companies offer modified Bradford Coomassie dye–based protein assay reagents. Perhaps the most popular such reagent is the Protein Assay Reagent available from Bio-Rad. The Coomassie Plus Protein Assay Reagent available from Pierce is another modification of the Bradford formulation. In addition to the attributes cited above, the Coomassie Plus Protein Assay Reagent has the unique advantage of producing a linear response curve within a portion of its working range. For BSA, the response curve is linear from 125 to 1000 μg/ml and for bovine gamma globulin (BGG), the response curve is linear from 125 to 1500 μg/ml. The complete working range of the assay covers the concentration range from 100 to 2000 μg/ml for the tube protocol and from 1 to 25 μg/ml for the microtiter protocol (see Alternate Protocol 7).

Table A.3H.4 Troubleshooting Guide for Coomassie Plus Protein

Problem	Possible cause	Solution
Blank A_{595} is normal, but standards show less color than expected	Improper reagent storage	Store reagent refrigerated
	Reagent still cold	Warm to room temperature before use
	Color measured at the wrong wavelength	Measure the color at 595 nm
Blank and standards are normal, but samples show little color	Low molecular weight of sample protein (<3000 kDa)	Use the BCA (see Basic Protocol 2) or Lowry protein assay (see Basic Protocol 1)
A precipitate forms in all tubes	Sample contains a surfactant (detergent)	Dialyze or dilute the sample
		Precipitate the protein with TCA, dissolve pellet in 50 mM NaOH
All tubes (including the blank) are dark blue	Strong alkaline buffer or reagent raises reagent's pH	Dialyze or dilute the sample
	Sample volume too large, reagent pH raised	Maximum of 1 part sample and 1 part reagent
Need to read color at a different wavelength	Colorimeter does not have 595 nm filter	Color may be read at any wavelength between 575 nm and 615 nm

The main disadvantage of all Bradford-type protein assay reagents is that they are not compatible with surfactants at concentrations routinely used to solubilize membrane proteins. With some exceptions, the presence of a surfactant in the sample, even at low concentrations, causes precipitation of the reagent. Table A.3H.4 is a brief troubleshooting guide for this technique.

Additional Materials *(also see Basic Protocol 4)*

Coomassie Plus Protein Assay Reagent Kit (Pierce) containing Coomassie Plus Protein Assay Reagent

1. Prepare a dilution series with 2 mg/ml BSA and buffer to cover a range from 100 to 2000 μg/ml.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

2. Allow the Coomassie Plus Protein Assay reagent to come to room temperature. Mix the assay reagent well by gentle inversion before use.

3. In duplicate, dispense 50 μl standard, sample, or diluent (blank) into appropriately labeled test tubes.

4. Add 1.5 ml Coomassie Plus Protein Assay Reagent to each tube. Mix each tube well by vortexing 2 to 3 sec.

5. Let the tubes stand 10 min at room temperature.

6. Mix each tube again just before measuring the absorbance at 595 nm (A_{595}).

   Disposable polystyrene cuvettes eliminate the job of cleaning dye-stained quartz or glass cuvettes.

7. Plot a standard curve by graphing the average net or blank-corrected A_{595} reading for each standard versus its concentration in micrograms per milliliter.

   Example color response curves for BSA and BGG are shown in Figure A.3H.8.

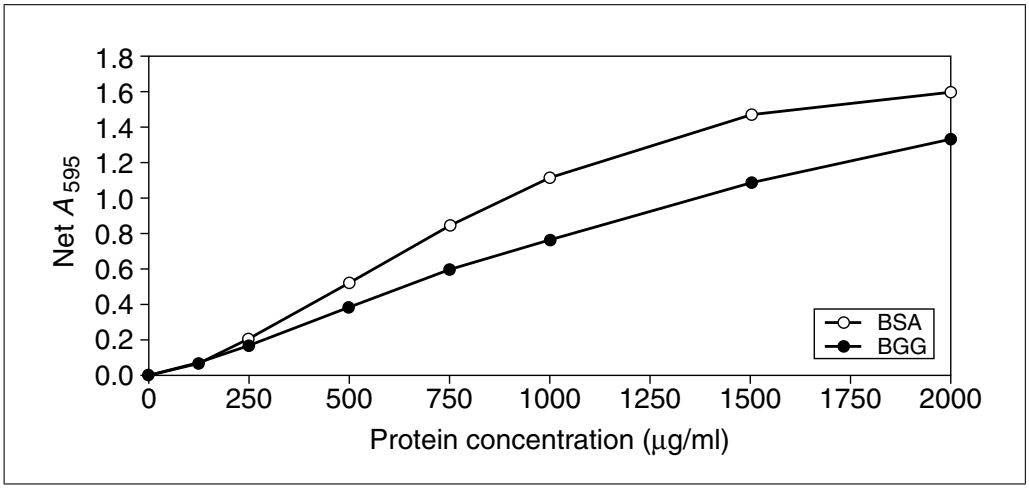

Figure A.3H.8 Graph of the color response curves obtained with Pierce's Coomassie Plus Protein Assay Reagent using bovine serum albumin (BSA) and bovine gamma globulin (BGG). The standard tube protocol was performed and the color was measured at 595 nm in a Hitachi U-2000 spectrophotometer.

8. Determine the protein concentration for each sample by interpolating from the standard curve (see Strategic Planning). Calculate the average total protein concentration for each sample from the replicates.

ALTERNATE PROTOCOL 7

MICROTITER PLATE COOMASSIE ASSAY FOR TOTAL PROTEIN

This microtiter plate assay has a working range of 1 to 25 μg/ml.

Additional Materials *(see Basic Protocol 4)*

Coomassie Plus Protein Assay Reagent Kit (Pierce) containing Coomassie Plus Protein Assay reagent
96-well microtiter plate
300-μl multichannel pipettor
Microtiter plate mixer
Microtiter plate reader

1. Allow the Coomassie Plus Protein Assay Reagent to come to room temperature. Once the reagent is at room temperature, mix the reagent well by gentle inversion of the bottle.
2. Draw a template for placement of samples and standards on a 96-well microtiter plate.
3. In triplicate, dispense 10 μl of each diluted BSA standard, sample, or diluent (blank) into the appropriate wells of a 96-well microtiter plate.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock standard that was used with the samples.

4. Dispense 300 μl Coomassie Plus Protein Assay reagent into each well with a multichannel pipettor. Mix the plate well on a plate shaker for 30 sec.
5. Let the plate incubate 10 min at room temperature.
6. Just before reading, mix the plate again, then measure the absorbance at 595 nm (A_{595}) on a plate reader.

7. Prepare a standard curve by graphing the average net or blank-corrected A_{595} values for each standard versus its concentration in micrograms per milliliter.

8. Determine the sample concentration by interpolating from the standard curve (see Strategic Planning). Calculate the average total protein concentration for each sample from the replicates.

REAGENTS AND SOLUTIONS

Use Milli-Q water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BCA reagent A

1 g 4,4′-dicarboxy-2,2′-biquinoline, disodium salt (Na_2BCA; Pierce or Sigma; 1% w/v final)
2 g $Na_2CO_3 \cdot H_2O$ (2% w/v final)
160 mg sodium tartrate dihydrate (0.16% w/v final)
0.4 g NaOH (0.4% w/v final)
0.95 g $NaHCO_3$ (0.95% w/v final)

Dissolve all of the above chemicals except the sodium bicarbonate in deionized water and adjust the final volume to 100 ml. Adjust the pH to 11.25 by adding the sodium bicarbonate a little at a time. Store this alkaline reagent in a plastic container 1 to 3 weeks at room temperature, longer at 4°C.

Only the disodium salt of BCA is soluble at neutral pH; the free acid is not readily soluble.

BCA reagent B

4 g $CuSO_4 \cdot 5H_2O$ (4% w/v final)
100 ml H_2O
Store up to 6 months at room temperature

Biuret total protein reagent

0.6 mol/liter sodium hydroxide
12.0 mmol/liter copper sulfate
31.9 mmol/liter sodium potassium tartrate
30.1 mmol/liter potassium iodide
Store up to 6 months at room temperature

This reagent is based upon the candidate reference method for the determination of total protein in serum developed by Doumas et al. (1981). It is available from Sigma Diagnostics.

BSA, 2 mg/ml (w/v)

200 mg BSA (crystallized or lyophilized or one of the Cohn Fraction V preparations which are 96% to 98% protein and 3% to 4% water) in 100 ml of 0.9% saline containing 0.05% (w/v) sodium azide. Store up to 6 months at 4°C.

Coomassie dye reagent

100 mg Coomassie Brilliant Blue G-250 (0.01% w/v final)
50 ml 95% ethanol (4.7% (w/v) final)

100 ml 85% (w/v) phosphoric acid (8.5% w/v final)

In a small container, dissolve the dye in ~25 ml ethanol, add the dye/ethanol solution to 800 ml water. Use the remaining ethanol to rinse the dye/ethanol container and add the rinses to the formulation. While mixing, slowly add the acid to the formulation and adjust the final volume to 1000 ml with deionized water. Filter the reagent through a single pad of Whatman no. 2 filter paper. Store up to 1 month at room temperature in a glass container.

continued

Coomassie Brilliant Blue G-250 (color index 42655) is available from a number of different suppliers (e.g., ACROS, Aldrich, AMRESCO, Bio-Rad, Fisher Biotech, Fluka, ICN Biomedicals, J.T. Baker, Research Organics, Serva, Sigma, or USB).

Lowry's reagent A

21.2 g sodium carbonate (2% w/v)
40 ml 1 N NaOH (or 4.0 g NaOH; 0.1 N final)
H_2O to 1 liter
Make fresh daily

Lowry's reagent B

0.5 g $CuSO_4 \cdot 5H_2O$ (0.5% final)
1 g sodium tartrate (1% final)
Make fresh daily H_2O to 100 ml

Sodium tartrate may be replaced with disodium tartrate, potassium sodium tartrate, or sodium citrate for better solubility.

Lowry's reagent C (1 N Folin phenol reagent)

Dilute 2 N Folin phenol (Sigma, Fisher, or VWR) with an equal volume water. Prepare immediately before use.

Lowry's reagent D (reagent A and B mix)

Mix 1 vol Lowry's reagent B and 50 vol Lowry's reagent A (see recipes). Prepare imsmediately before use.

Lowry's reagent D′

Add 2 ml of 10% (w/v) sodium dodecyl sulfate (SDS; *APPENDIX 2A* for 20% solution) in water to each 100 ml Lowry's reagent D (see recipe). Prepare immediately before use.

COMMENTARY

Background Information

The Modified Lowry Protein Assay

Although the exact mechanism of color formation reaction in the Lowry protein assay remains poorly understood, it is known that the color producing reaction with protein occurs in two distinct steps. As seen in Figure A.3H.1, protein is first reacted with alkaline cupric sulfate in the presence of tartrate during a 10-min incubation at room temperature. During this incubation, a tetradentate copper complex forms from four peptide bonds and one atom of copper. The tetradentate copper complex is light blue in color (this is the "biuret reaction"). Following the 10-min incubation, Folin phenol reagent is added. It is believed that color enhancement occurs when the tetradentate copper complex transfers electrons to the phosphomolybdic/phosphotungstic acid complex (i.e., the Folin phenol reagent).

The reduced phosphomolybdic/phosphotungstic acid complex produced by this reaction is intensely blue in color. The Folin phenol reagent loses its reactivity almost immediately upon addition to the alkaline working reagent/sample solution. The blue color continues to intensify during a 30-min room temperature incubation. It has been suggested by Lowry et al. (1951) and by Legler et al. (1985) that during the 30-min incubation, a rearrangement of the initial unstable blue complex leads to the stable final blue colored complex, which has higher absorbance.

For small peptides, the amount of color increases with the size of the peptide. The presence of any of five amino acid residues (tyrosine, tryptophan, cysteine, histidine, and asparagine) in the peptide or protein backbone further enhance the amount of color produced, because they contribute additional reducing equivalents for further reduction of the phosphomolybdic/phosphotungstic acid complex. With the exception of tyrosine and tryptophan, free amino acids will not produce a colored product with the Lowry reagent; however, most dipeptides can be detected. In the absence

of any of the five amino acids listed above, proteins containing proline residues have a lower color response with the Lowry reagent because it interferes with complex formation.

The final blue color is optimally measured at 750 nm, but it can be measured at any wavelength between 650 nm and 750 nm with little loss of color intensity. Dr. Lowry (1951) recommended reading the color at 750 nm because few other substances absorb light at that wavelength. The amount of light absorbed at 750 nm is directly proportional to the amount of protein in the sample, but the color response curve produced is nonlinear.

The sensitivity of the Modified Lowry Protein Assay Reagent is greatly enhanced over that of the biuret total protein reagent. The working range of the method covers the total protein range from 1 to 1500 μg/ml. In comparison, the working range for the biuret assay is from 5 to 160 mg/ml.

The Modified Lowry Protein Assay Reagent will form precipitates in the presence of surfactants or potassium ions. The problem of precipitation that is caused by the presence of potassium ions in the sample can sometimes be overcome by centrifuging the tube and reading the color in the supernatant. Most surfactants will cause precipitation of the reagent even at very low concentrations. One exception is sodium dodecyl sulfate (SDS), which is compatible with the reagent at concentrations up to 1% (w/v) in the sample. Chelating agents interfere because they bind copper and thus prevent formation of the copper-peptide bond complex. Reducing agents and free thiols interfere, as they reduce the phosphotungstate-phosphomolybdate complex, immediately forming an intensely blue colored product upon their addition to the Modified Lowry Protein Assay Reagent.

The Coomassie Plus Protein Assay

The primary advantage of the Coomassie Plus Protein Assay is that it is generally compatible with most of the buffers and reagents found in samples and is unaffected by the presence of chelating agents, reducing agents, or free sulfhydryls in the sample.

The development of color in the Coomassie dye-binding methods has been associated with the presence of certain basic amino acids (primarily arginine, lysine, and histidine) in the protein. Van der Waals forces and hydrophobic interactions also participate in the binding of the dye by protein. The number of Coomassie dye ligands bound to each protein molecule is approximately proportional to the number of positive charges found on the protein. In the acidic environment of the Coomassie Plus Protein Assay Reagent, protein binds to the Coomassie dye. This results in a spectral shift of the reagent from the reddish/brown form of the dye, with an absorbance maximum at 465 nm, to the blue form of the dye, with an absorbance maximum at 610 nm (see Fig. A.3H.7).

The difference between the two forms is greatest at 595 nm; therefore, this is the optimal wavelength to measure the blue color from the Coomassie dye–protein complex. If desired, the blue color can be read at any wavelength between 575 and 615 nm. At the two extremes (575 and 615 nm) there is a loss of ~10% in the measured amount of color (absorbance) compared to the value obtained at 595 nm.

Free amino acids, peptides, and low molecular weight proteins do not produce color with the Coomassie Plus Protein Assay Reagent. In general the molecular weight of the peptide or protein must be at least 3000 Da to be assayed with this reagent. In some applications this can be an advantage. The reagent has been used to measure "high molecular weight proteins" during fermentation in the beer brewing industry.

One disadvantage of any Coomassie dye–based protein assay is that surfactants in the sample will cause precipitation of the reagent. Another disadvantage is that the Coomassie Plus Protein Assay Reagent shows almost twice as much protein-to-protein variation as that obtained with the protein-copper chelation–based assay reagents (the Lowry Protein Assay Reagent or the BCA Protein Assay Reagent). While this is true, the reagent exhibits the least protein-to-protein variation of all Coomassie dye–based (Bradford) reagents. Since the Coomassie dye reagent is highly acidic, a small number of proteins cannot be assayed with this reagent due to poor solubility. In addition, the glass or quartz cuvettes routinely used to hold the solution in the spectrophotometer while the color intensity is being measured are stained by Coomassie dye–based reagents.

The BCA Protein Assay

The BCA Protein Assay is a modification of the Lowry protein assay in which enhanced color production is due to the reaction of reduced copper with bicinchoninic acid (BCA). This reagent has a unique advantage over the Lowry Protein Assay Reagent and the Coomassie Plus Protein Assay Reagent

because it is compatible with samples that contain up to 5% (v/v) surfactants (detergents). Unlike the Lowry protein assay, all reactants needed are present in the BCA working reagent. Large numbers of tubes can be run without regard to the limits imposed in the Lowry assay by the need to add a second reagent at a precise time interval.

The working range of the BCA Protein Assay Reagent is from 20 to 2000 μg/ml for both the standard tube and the standard microtiter plate protocols. Since the color reaction is not a true end-point reaction, this allows more protocol flexibility. By increasing the incubation temperature, the sensitivity of the reagent can be increased. When using the enhanced tube protocol (incubating at 60°C for 30 min), the working range for the assay shifts to 5 to 250 μg/ml and the minimum detection level becomes 5 μg/ml.

Although the mechanism of color formation with protein for the BCA Protein Assay Reagent is similar to that of the Lowry reagent, there are several significant differences. The BCA Protein Assay combines the well-known reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (the so-called biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) by bicinchoninic acid.The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion (see Fig. A.3H.3). The resultant BCA/Cu^{+} complex is water-soluble and exhibits a strong linear absorbance maximum at 562 nm with increasing protein concentrations. If desired, the purple color may be measured at any wavelength between 550 nm and 570 nm with minimum (<10%) loss of signal.

The reaction that leads to BCA color formation as a result of the reduction of Cu^{2+} is also strongly influenced by the presence of any of four amino acid residues (tyrosine, tryptophan, cysteine, or cystine) in the amino acid sequence of the protein. Unlike the Coomassie dye–binding (Bradford) methods, which require a minimum mass of protein to be present for the dye to bind, the presence of only a single amino acid residue in the sample may result in the formation of a colored BCA-Cu^{+} chelate. This is true for any of the four amino acids cited above. Studies done with di- and tripeptides indicate that the total amount of color produced is greater than can be accounted for by the simple addition of the color produced with each BCA-reactive amino acid, so the peptide backbone must contribute to the reduction of copper as well.

The rate of color formation is dependent on the incubation temperature, the types of protein present in the sample, and the relative amounts of reactive amino acids contained in the proteins. The recommended protocols do not result in end-point determinations, so the incubation periods were chosen to yield maximal color response in a reasonable time frame.

The protein-to-protein variation in the amount of color produced with the BCA Protein Assay Reagent (CV = 15% for the group of 14 proteins at 1000 μg/ml in the standard tube protocol) is similar to that observed for the Modified Lowry Protein Assay Reagent.

The Biuret Total Protein Assay

Riegler (1914) introduced the biuret reaction as a method for the estimation of albumin in urine in 1914. Modifications and improvements to the biuret method were made by Autenrieth and Mink (1915), Hiller (1926), and Fine (1935). All of these early methods required the separate addition of a sodium hydroxide solution and the separate addition of a copper sulfate solution to the sample. The methods suffered from poor precision due to ineffective mixing and variation in reaction time during mixing. The first "one solution" biuret reagent was introduced by Kingsley (1942) for use in measuring total protein, albumin, and globulin in human serum. Weichselbaum (1946) and Gornall et al. (1949) modified Kingsley's biuret reagent by decreasing the sodium hydroxide concentration to prevent the formation of precipitates and by adding sodium potassium tartrate to stabilize the reagent. Potassium iodide was added to prevent the autoreduction of Cu^{2+}. Goa (1953) published on the use of the biuret reagent for determining total protein in human cerebrospinal fluid.

Sigma Diagnostic's biuret total protein reagent is based upon the candidate reference method formulation used for the determination of total protein in serum developed by Doumas et al. (1981). Because the primary application for this reagent is the determination of total protein in serum, most of the studies regarding assay precision, assay linearity, and interfering substances have been done on clinical samples. On samples with total protein concentrations in the range of 26 to 121 mg/ml, within-run and between-run precision was found to be excellent (CV's ranged from 4.2% to 1.4%). Lipids, bilirubin, hemoglobin, dextran, and

certain drugs have been shown to interfere with the total protein results obtained with the biuret Total Protein Reagent. Interference caused by the presence of lipid in the sample is due to turbidity. Interference caused by the presence of bilirubin or hemoglobin is small, almost negligible. Dextran causes precipitation in the reaction mixture during color development; however, centrifuging the reaction mixture before reading the color can minimize this. The biuret Total Protein Reagent contains 0.6 mol/liter sodium hydroxide, 12.0 mmol/liter copper sulfate, 31.9 mmol/liter sodium potassium tartrate, and 30.1 mmol/liter potassium iodide. It should be stored at ambient room temperature until the expiration date shown on the label. Certain drugs and other substances are known to influence circulating levels of total protein (Young, 1990).

Critical Parameters

The Modified Lowry Protein Assay Reagent

The Modified Lowry Protein Assay Reagent must be refrigerated for long-term storage. If the entire bottle of reagent will be used within a reasonable time, the reagent may be stored at ambient room temperature (18° to 26°C) for up to 1 month. Reagent that has been left at room temperature for more than a month may show lower color response with protein, especially at the higher end of the working range. If the reagent has been stored refrigerated, it must be warmed to room temperature before use. The use of cold Modified Lowry Protein Assay Reagent will result in low A_{750} values.

The protocol requires that the Folin phenol reagent be added to each tube precisely at the end of the 10-min incubation. At the alkaline pH of the Lowry reagent, the Folin phenol reagent is almost immediately inactivated; therefore, it is best to add the Folin phenol reagent at the precise time while simultaneously mixing each tube. Because it is somewhat cumbersome, it requires some practice to do the assay well. From a practical point of view, it also limits the total number of tubes that can be done in a single run. If a 10-sec interval between tubes is used, the maximum number of tubes that can be done within 10 min is 60 (10 sec/tube × 60 tubes = 600 sec or 10 min).

The Coomassie Plus Protein Assay Reagent

The Coomassie Plus Protein Assay Reagent must be refrigerated for long-term storage. If the entire bottle of reagent will be used within a reasonable time, the reagent may be stored at ambient room temperature (18° to 26°C) for up to 1 month. Reagent that has been left at room temperature for more than a month may show lower color response with protein, especially at the higher end of the working range. If the reagent has been stored refrigerated, it must be warmed to room temperature before use. The use of cold Coomassie Plus Protein Assay Reagent will result in low A_{595} values.

The Coomassie Plus Protein Assay Reagent must be mixed gently by inversion just before use. The Coomassie dye in the reagent spontaneously forms loosely associated dye-dye aggregates upon standing. These aggregates may become visible after the reagent has been standing for as little as 60 min. Gentle mixing of the reagent by inversion of the bottle will uniformly disperse the dye-dye aggregates. After binding to protein, the dye also forms protein-dye-protein-dye aggregates. Fortunately, these aggregates can be dispersed easily by mixing the reaction tube. This is common to all Coomassie dye–based (Bradford) protein assay reagents. Since these aggregates form relatively quickly, it is a good idea to routinely mix (vortex for 2 to 3 sec) each sample just before measuring the color.

Bradford-type reagents containing Coomassie dye will leave a blue stain on glass or quartz cuvettes. The stain can be removed by washing the cuvettes in a dilute detergent solution in hot tap water, rinsing with water, then washing with methanol or ethanol, and finally rinsing with deionized water. Disposable plastic (polystyrene) cuvettes are strongly recommended because they eliminate the need to clean; however, these cuvettes are not compatible with samples containing organic solvents (e.g., acetone, DMF, acetonitrile).

The BCA Protein Assay Reagent

Since the BCA Protein Assay is not a true end-point assay, the amount of color produced varies with the incubation time and the incubation temperature. While this allows considerable flexibility in optimizing the BCA assay for each application, it also requires that the optimized procedure be followed exactly every time the assay is done.

At room temperature, following an initial lag phase, the rate of color formation remains relatively constant for hours. If the incubation time alone is increased, the total amount of color produced by a given mass of protein increases.

If the incubation temperature is increased, the rate of color formation increases and the total amount of color produced by a given mass of protein increases. After cooling the reaction mixture back to room temperature, the rate of color development slows from its initial rate. As both time and temperature are increased, the total amount of color produced by a given mass of protein approaches a maximum. This is apparent from the dramatic decrease in the rate of color formation upon cooling to room temperature following incubation at 60°C for 30 min.

Above 75°C, a black precipitate forms in the BCA reaction mixture and the absorbance at 562 nm of the blank increases dramatically. This appears to be caused by the formation of copper oxide at high temperature.

For greatest accuracy and precision when comparing sets of data from multiple runs, a set of standards must be included with each run, and the standards and the samples must be treated exactly the same.

The Biuret Total Protein Reagent

The biuret total protein reagent is considerably less sensitive to total protein than the other three protein assay reagents discussed in this unit. This limits the applications in which the biuret reagent can be used. Since the primary use of the biuret reagent has been for serum total protein in the clinical laboratory, there is little published information about its compatibility with substances and reagents common to nonclinical samples.

Anticipated Results

Standard Curves

Typical standard curves are shown in Figures A.3H.2, A.3H.4, A.3H.6, and A.3H.8 for each of the four assay methods. In each case, the tube protocols were performed in duplicate on diluted BSA or BGG standard. The color in each tube was measured at the appropriate wavelength in a dual-beam spectrophotometer. The net absorbance for each sample was plotted versus its protein concentration.

Figure A.3H.2 shows the color response curves obtained with the Modified Lowry Protein Assay Reagent using BSA and BGG. The graph shows the net absorbance at 750 nm versus the protein concentration at seven concentrations from 125 to 2000 μg/ml for each protein. Note that the response curve for BGG is higher than the response curve for BSA.

Figure A.3H.4 shows the color response curves obtained with the BCA Protein Assay Reagent using BSA and BGG. The graph shows the net absorbance at 562 nm versus the protein concentration at seven concentrations from 125 to 2000 μg/ml for each protein. Note that the response curve for BGG is higher than the response curve for BSA.

Figure A.3H.6 shows the color response curves obtained with the biuret total protein reagent using BSA and BGG. The graph shows the net absorbance at 540 nm versus the protein at eight concentrations from 5 to 100 mg/ml.

Figure A.3H.8 shows the color response curves obtained with the Coomassie Plus Protein Assay Reagent using BSA and BGG. The graph shows the net absorbance at 595 nm versus the protein concentration at seven concentrations from 125 to 2000 μg/ml for each protein. Note that the color response curve for BGG is lower than the response curve for BSA.

Protein-to-Protein Variation

Each protein in a sample is unique and can demonstrate that individuality in protein assays as variation in the color response. Such protein-to-protein variation refers to differences in the amount of color (absorbance) that are obtained when the same mass (microgram or milligram) of various proteins are assayed concurrently (i.e., in the same run) by the same method. These differences in color response relate to differences among proteins due to amino acid sequence, isoelectric point (pI), secondary structure, and the presence of certain side chains or prosthetic groups.

To analyze protein-to-protein variation for each method, a group of fourteen proteins was assayed in duplicate using the standard tube protocol in a single run. The net (blank-corrected) average absorbance for each protein was calculated. To make it easier to interpret, the net absorbance for each protein was expressed as a ratio to the net absorbance for BSA. If a protein has a ratio of 0.80, it means that the protein produces ~80% of the color that is obtained for an equivalent mass of BSA.

Table A.3H.5 demonstrates the relative degree of protein-to-protein variation that can be expected with the different protein assay methods. This differential may be a consideration in selecting a protein assay method, especially if the relative color response ratio of the protein in the samples is unknown. As expected, the protein assay methods that share the same basic chemistry show similar protein-to-protein variation.

The protein-to-protein variation observed with the various protein assay methods makes it obvious why the largest source of error for

Table A.3H.5 Protein-to-Protein Variation[a]

Protein tested	Ratios obtained with the BCA method	Ratios obtained with the Coomassie plus method	Ratios obtained with the modified Lowry method
Albumin, bovine (BSA)	1.00	1.00	1.00
Aldolase, rabbit	0.85	0.74	0.94
α-Chymotrypsinogen, bovine	1.14	0.52	1.17
Cytochrome C, horse	0.83	1.03	0.94
Gamma globulin, bovine	1.11	0.58	1.14
IgG, bovine	1.21	0.63	1.29
IgG, human	1.09	0.66	1.13
IgG, mouse	1.18	0.62	1.20
IgG, rabbit	1.12	0.43	1.19
IgG, sheep	1.17	0.57	1.28
Insulin, bovine pancreas	1.08	0.67	1.12
Myoglobin	0.74	1.15	0.90
Ovalbumin	0.93	0.68	1.02
Transferrin, human	0.89	0.90	0.92
Average ratio	1.02	0.73	1.09
Standard Deviation (SD)	0.15	0.12	0.13
Coefficient of Variation	0.15	0.29	0.12

[a]The protein-to-protein variation in color response was measured at 1000 μg/ml for each protein in duplicate using the standard tube protocol. Within each assay, the average net or blank-corrected absorbance was determined for each protein. The average net absorbance for each protein was divided by the average net absorbance obtained with BSA and expressed as a ratio. The standard deviation (SD) and the coefficient of variation (CV) is presented for the fourteen proteins assayed on the three methods. By comparing the CV's, the relative degree of protein-to-protein variation to be expected with the three methods can be assessed.

protein assays is the choice of protein for the standard curve. If the sample contained IgG as the major protein and BSA was used for the standard curve, the estimated total protein concentration of the sample will be inaccurate. Whether the concentration was underestimated or overestimated depends upon which total protein assay method was used. If the Coomassie Plus Protein Assay Reagent was used, the total protein (IgG) concentration in the sample would be underestimated by ~40%. (From Table A.3H.5, the response ratio for IgG is ~0.58 for IgG compared to 1.00 for BSA.) If the BCA Protein Assay Reagent was used, the total protein (IgG) concentration in the sample would be overestimated by ~15%. (From Table A.3H.5, the response ratio for IgG is ~1.15 for IgG compared to 1.00 for BSA.) On the other hand, if BGG had been used for both standard curves, the total protein estimates for the sample would have been in much greater agreement between the two methods.

While Table A.3H.5 is useful because it provides an estimate of the protein-to-protein variation in color response that can be expected with each method, it does not tell the whole story. Because the comparisons were done at a single protein concentration, it is not apparent that the color response ratio also varies with changes in protein concentration.

Compatible and Incompatible Substances

An extensive list of substances that have been found to be compatible with each of the reagents is shown in Table A.3H.6 Each substance was assayed in duplicate using the standard tube protocol for each reagent. In addition to adding the substance to a sample containing 1000 μg/ml BSA, a blank sample containing only the substance was tested. When added to the sample, a substance was deemed to be compatible with a reagent if the blank-corrected absorbance for the sample containing the substance was within 10% of the blank-corrected absorbance for the sample containing only BSA (also at 1000 μg/ml).

Time Considerations

The amount of time required to complete a total protein assay will vary for the four colorimetric total protein assay methods described.

Table A.3H.6 Maximum Compatible Sample Concentration of 92 Substances[a]

Substance tested	BCA method	Coomassie plus method	Modified Lowry method
Detergents			
Brij 35	5.0%	0.062%	0.031%
Brij 56	1.0%	0.031%	0.062%
Brij 58	1.0%	0.016%	0.062%
CHAPS	5.0%	5.0%	0.062%
CHAPSO	5.0%	5.0%	0.031%
Deoxycholic acid	5.0%	0.04%	Not tested
Lubrol PX	1.0%	0.031%	0.031%
Nonidet P-40	5.0%	0.5%	0.016%
Octyl glucoside	5.0%	0.5%	0.031%
Octyl β-thioglucoside	5.0%	3.0%	Not tested
SDS (lauryl)	5.0%	0.016%	1.0%
SPAN 20	1.0%	0.5%	0.25%
Triton X-100	5.0%	0.062%	0.031%
Triton X-114	1.0%	0.062%	0.031%
Triton X-305	1.0%	0.125%	0.031%
Triton X-405	1.0%	0.25%	0.031%
Tween 20	5.0%	0.031%	0.062%
Tween 60	5.0%	0.025%	Not tested
Tween 80	5.0%	0.016%	0.031%
Zwittergent 3-14	1.0%	0.025%	Not tested
Salts and buffers			
ACES, pH 7.8	25 mM	100 mM	Not tested
Ammonium sulfate	1.5 M	1 M	Not compatible
Asparagine	1 mM	10 mM	5 mM
Bicine, pH 8.4	20 mM	100 mM	Not tested
Bis-Tris, pH 6.5	33 mM	100 mM	Not tested
Borate (50 mM), pH 8.5 (BupH pack)	Undiluted	Undiluted	Not tested
B-PER cell lysis reagent	Undiluted	Diluted 1:2	Not tested
Calcium chloride in TBS	10 mM	10 mM	Not tested
Carbonate/bicarbonate, Na (0.2 M), pH 9.4	Undiluted	Undiluted	Not tested
Cesium bicarbonate	100 mM	100 mM	50 mM
CHES, pH 9.0	100 mM	100 mM	Not tested
Cobalt chloride in TBS	0.8 mM	10 mM	Not tested
EPPS, pH 8.0	100 mM	100 mM	Not tested
Ferric chloride in TBS	10 mM	10 mM	Not tested
Glycine	1 mM	100 mM	100 mM

continued

Table A.3H.6 Maximum Compatible Sample Concentration of 92 Substances,[a] *continued*

Substance tested	BCA method	Coomassie plus method	Modified Lowry method
HEPES	100 mM	100 mM	1 mM
Imidazole, pH 10.2	50 mM	200 mM	25 mM
MES, pH 6.1	100 mM	100 mM	100 mM
0.1 M MES/0.9% NaCl, pH 4.7	Undiluted	Undiluted	Not tested
Salts and bufferes (continued)			
MOPS, pH 7.2	100 mM	100 mM	Not tested
Modified Dulbecco's PBS	Undiluted	Undiluted	Not tested
Nickel chloride in TBS	10 mM	10 mM	Not tested
Phosphate buffered saline (PBS), pH 7.2	Undiluted	Undiluted	Not tested
PIPES, pH 6.8	100 mM	100 mM	Not tested
RIPA lysis buffer, pH 8.0	Undiluted	Diluted 1:40	Not tested
Sodium acetate	200 mM	180 mM	200 mM
Sodium azide	0.2%	0.5%	0.2%
Sodium bicarbonate	100 mM	100 mM	100 mM
Sodium chloride	1.0 M	1 M	1 M
Sodium citrate, pH 4.8	200 mM	200 mM	Not tested
Sodium phosphate	100 mM	100 mM	100 mM
Tricine, pH 8.0	25 mM	100 mM	Not tested
Triethanolamine, pH 7.8	25 mM	100 mM	Not tested
Tris	250 mM	2 M	10 mM
TBS, pH 7.6	Undiluted	Undiluted	Not tested
25 mM Tris/192 mM glycine, pH 8.0	Diluted 1:3	Undiluted	Not tested
25 mM Tris/192 mM glycine/0.1% SDS, pH 8.0	Undiluted	Diluted 1:4	Not tested
Zinc chloride in TBS	10 mM	10 mM	Not tested
Reducing agents			
N-acetylglucosamine in PBS	10 mM	100 mM	Not tested
Ascorbic acid	Not compatible	50 mM	1 mM
Catecholamines	Not compatible	Not tested	Not tested
Creatinine	Not compatible	Not tested	Not tested
Glucose	10 mM	1 M	0.1 mM
Melibiose	Not compatible		
Potassium thiocyanate	3 M		

continued

Table A.3H.6 Maximum Compatible Sample Concentration of 92 Substances,[a] *continued*

Substance tested	BCA method	Coomassie plus method	Modified Lowry method
Thiol-containing agents			
Cysteine	Not compatible	10 mM	1 mM
Dithioerythritol (DTE)	1 mM	1 mM	Not compatible
Dithiothreitol (DTT)	1 mM	5 mM	Not compatible
2-Mercaptoethanol	0.01%	1 M	1 mM
Thimerosal	0.01%	0.01%	0.01%
Chelating agents			
EDTA	10 mM	100 mM	1 mM
EGTA	Not compatible	2 mM	1 mM
Sodium citrate, pH 4.8	200 mM	200 mM	0.1 mM
Solvents/miscellaneous			
Acetone	10%	10%	10%
Acetonitrile	10%	10%	10%
Aprotinin	10 mg/liter	10 mg/liter	10 mg/liter
DMF	10%	10%	10%
DMSO	10%	10%	10%
Ethanol	10%	10%	10%
Glycerol (fresh)	10%	10%	10%
Guanidine·HCl	4 M	3.5 M	100 mM
Hydrochloric acid	100 mM	100 mM	100 mM
Leupeptin	10 mg/liter	10 mg/liter	10 mg/liter
Methanol	10%	10%	10%
Phenol Red	Not compatible	0.5 mg/liter	0.1 mg/liter
PMSF	1 mM	1 mM	1 mM
Sodium hydroxide	100 mM	100 mM	100 mM
Sucrose	40%	10%	7.5%
TLCK	0.1 mg/liter	0.1 mg/liter	0.01 mg/liter
TPCK	0.1 mg/liter	0.1 mg/liter	0.1 mg/liter
Urea	3 M	3 M	3 M
o-vanadate in PBS	1 mM	1 mM	Not tested

[a]Taken from the Protein Assay Technical Handbook, Pierce Chemical, 1999.

For the purpose of providing an estimate of the amount of time required to perform a run by each method, it was assumed that the run included twenty samples and eight standards (including the blank) and that each sample or standard was assayed in duplicate using the standard tube protocol. The estimates do not include the time spent obtaining the samples or the time it takes to prepare the samples for analysis, but they do include the incubation time(s) plus an estimate of the time it takes to do the following:

1. Prepare (dilute) the standard protein in the diluent buffer (10 min).
2. Organize the run and label the tubes (5 min).
3. Pipet the samples and reagents into the tubes (10 min).
4. Mix or incubate the tubes or plates (varies).

Table A.3H.7 Estimated Time Requirements

Method	Incubation time (s)	Estimated total assay time
Modified Lowry Reagent	10 and 30 min	110 min (1 hr, 50 min)
Coomassie Plus Reagent	10 min	80 min (1 hr, 20 min)
BCA Reagent	30 min	100 min (1 hr, 40 min)
Biuret Reagent	10 min	80 min (1 hr, 20 min)

5. Measure the color produced in the tubes (15 min).

6. Graph the standard curve, calculate, record, and report the results (30 min).

For each of the four methods, a run of 20 samples (unknowns) and the standard curve (each done in duplicate) can be completed in the time estimated in Table A.3H.7

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