

Assays for Determination of Protein Concentration

Accurate measurement of protein concentration is critical since the results are used in other calculations, such as determination of enzyme activity. Errors in protein concentration determination tend to amplify overall errors in these calculations. Furthermore, protein assays are often purchased as kits from commercial suppliers, which result in a poor understanding of the underlying chemistry. This unit presents a survey of the common protein assay methods and highlights their usefulness and limitations. Additionally, the chemistry underlying each assay is explained to aid in troubleshooting and assay selection.

There is no single protein assay method that yields absolutely accurate results. Each method has different advantages and limitations. The primary intention of this unit is to inform users how to select the most appropriate protein assay for a specific application. The Kjeldahl method (Ballentine, 1957) and the acid digestion–ninhydrin method (Lovrien and Matulis, 1995) are no longer in general use and are not included here. This unit discusses the following protein assay methods that are commonly used in biochemical laboratories: the Lowry assay (see Basic Protocol 1), the Bradford assay (see Basic Protocol 2 and Alternate Protocol 1), the BCA assay (see Basic Protocol 3 and Alternate Protocol 2), and UV spectroscopy to determine protein concentration (see Basic Protocol 4). Support Protocol 1 discusses standard curves and data processing in detail and is a good place for a novice to start before beginning any experimentation. Also included are two precipitation strategies for dealing with buffer incompatibility. Finally, many applications of SDS-PAGE require equal loading of samples based on total protein. Typically, this requires laborious extraction in a buffer compatible with a protein assay, before adding SDS-PAGE loading buffer. Alternate Protocol 3 details a method for alkylation of excess reducing agent in SDS-PAGE loading buffer for direct analysis in the BCA assay.

The protocols in this unit should produce valid results for most applications. There are also kits available for protein determination from Sigma, BioRad, and Pierce. Prior to employing an assay kit or protocol, the user is advised to consult the Strategic Planning section because it describes how to select the most appropriate assay for a particular task. The assays have different strengths and weaknesses, particularly regarding buffer compatibility. Since some investigators may have limited experience with biochemical techniques, the Commentary presents a discussion of the critical parameters for protein assays, focusing on the standard curve and a brief review of spectrophotometry. Finally, a Support Protocol provides suggestions for processing data using a spreadsheet.

STRATEGIC PLANNING

Assay choice

Two common applications of the protein assay are enzyme activity assays and equal protein loading of SDS-PAGE gels. The strategies and considerations for these applications are considerably different. In enzyme activity calculations, the primary concern is the accuracy of the results, whereas for equal loading of SDS-PAGE gels, precision is more important. There are many other applications for protein assays, e.g., in nutrition and pharmacology, and it is strongly recommend that the literature of the particular field

be consulted before employing one of the assays presented in this unit to ensure that these assays are applicable in the field. It is particularly important to realize that these assays are estimation procedures unless the individual assay has been standardized to total amino acid analysis (UNIT 3.2).

One of the most difficult aspects of assaying protein concentration is the selection of an assay compatible with the sample. All of the assays presented in this unit detect specific properties of a protein, not the entire protein itself; thus protein and buffer composition will be the primary determinants of the appropriate assay. Figure 3.4.1 presents a flow chart of the assay selection process. If selection of a routine estimation procedure for relatively pure protein samples is the goal, highest accuracy will be achieved by standardizing the assay to a total amino acid analysis of the unknown (UNIT 3.2). All of the presented methods have different sensitivities to different proteins such as bovine serum albumin and gamma-globulin (Stoscheck, 1990).

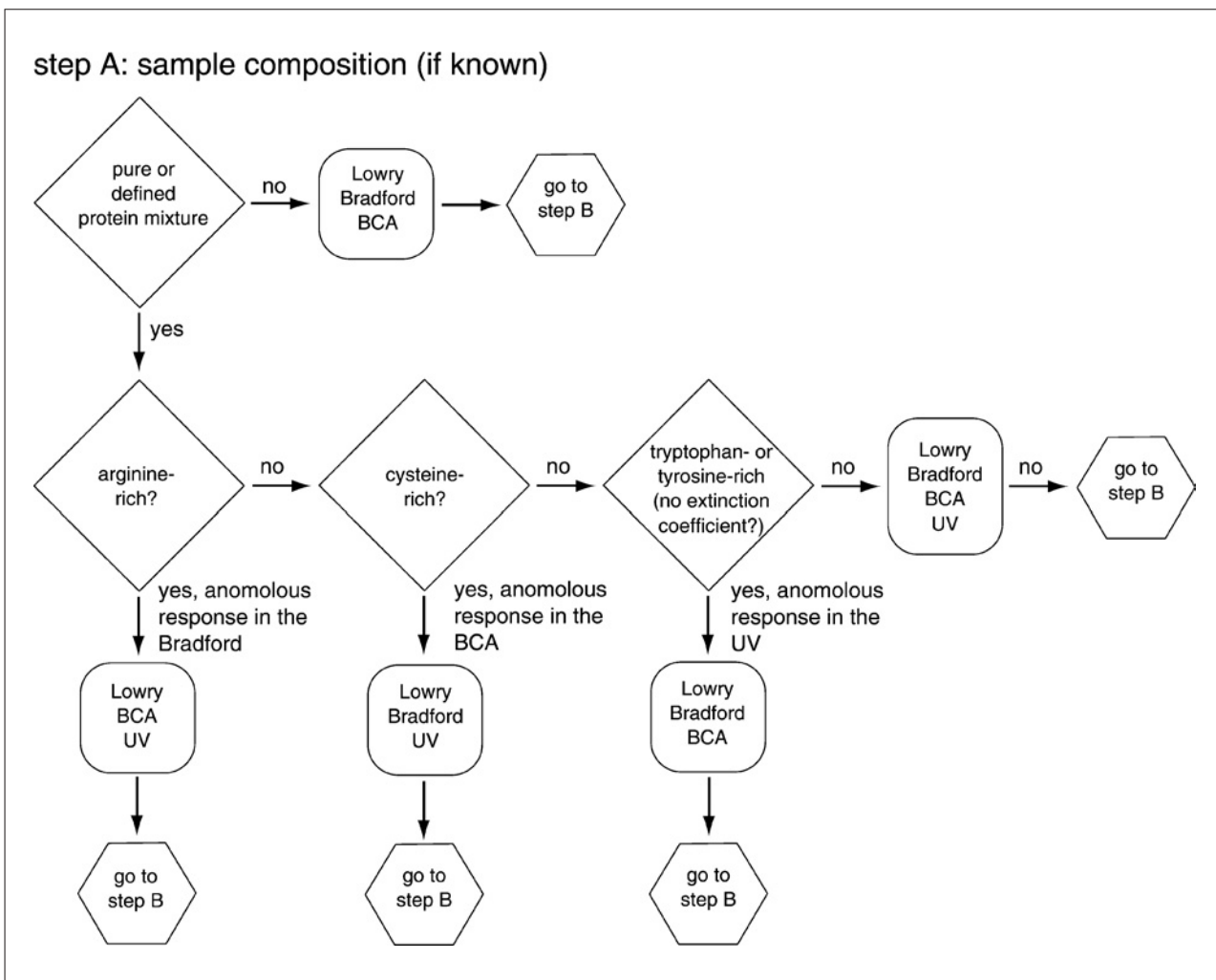


Figure 3.4.1 Flow chart for selecting a protein assay. To use the chart, begin with step A to find the protein assays that are most compatible with the sample.

**Assays for
Determination of
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3.4.2

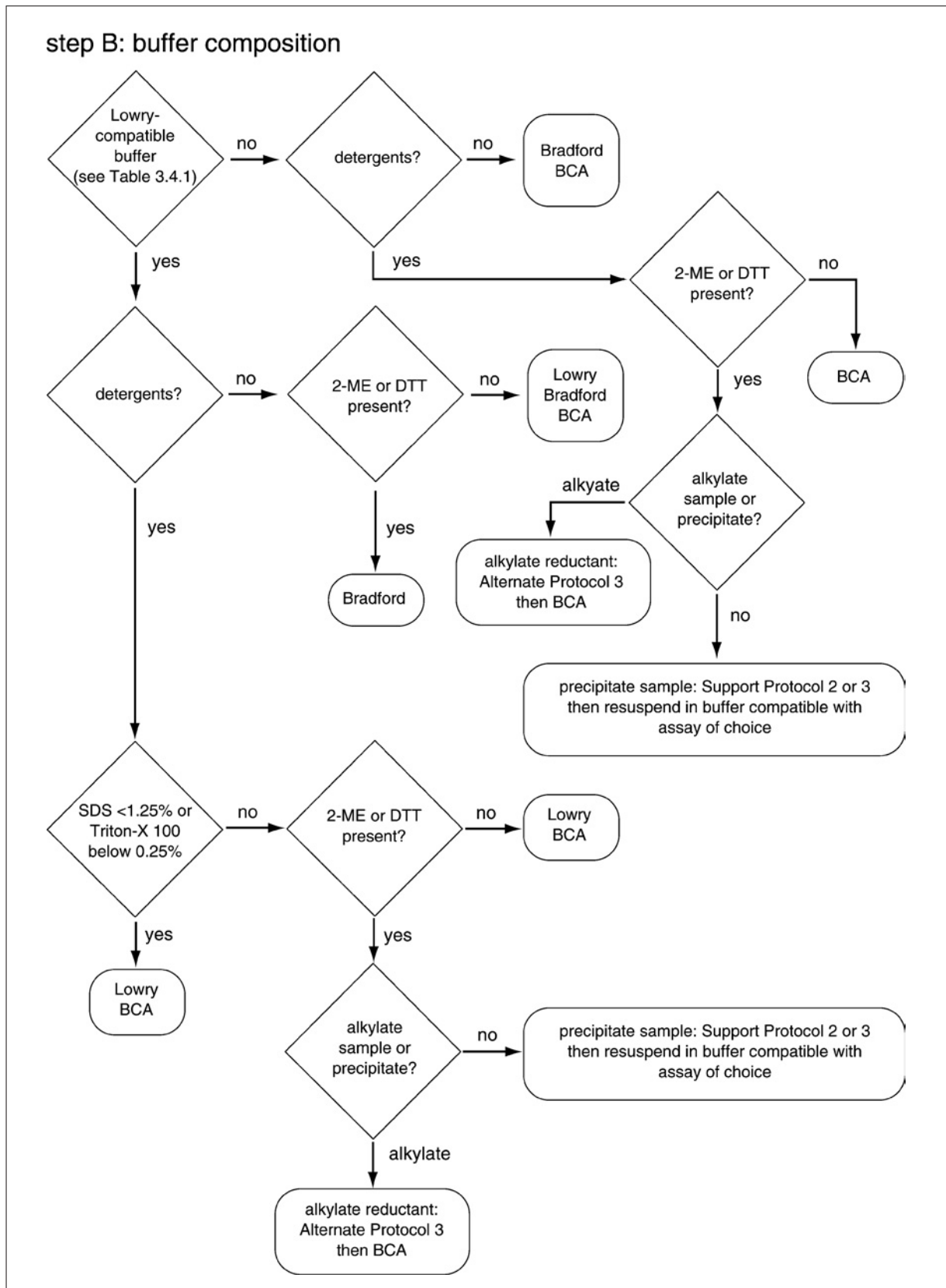


Figure 3.4.1 (continued) Then proceed to step B to obtain the list of assays compatible with the buffer system. Compare the results from each step to find the most compatible assay for the sample. Refer to the text for the assay and Table 3.4.1 to confirm assay compatibility before using a protein assay strategy. Abbreviations: 2-ME, 2-mercaptoethanol; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

Sample composition

Sample composition is critical when choosing a protein assay. For example, a protein rich in arginine residues will produce an artificially high result using a Bradford assay, whereas the same protein will most likely produce a more accurate result using either the Lowry or BCA assay. Conversely, a protein high in cysteine would produce an artificially high result using the BCA assay, but would likely produce better results with the Lowry or Bradford assay. In general, the BCA and Lowry assays perform better with complex protein mixtures.

Buffer composition

Buffer composition is an important consideration when selecting a protein assay. The Lowry assay is very sensitive for determining protein concentration; however, common buffer components such as ethylenediaminetetraacetic acid (EDTA) interfere with chromophore production. The BCA assay is compatible with a wide range of detergents, including sodium dodecylsulfate (SDS), but does not tolerate reducing agents such as dithiothreitol (DTT). The Bradford assay will not tolerate high concentrations of detergents but will work in the presence of reducing agents, such as DTT or 2-ME. See Table 3.4.1 for a comprehensive table listing interfering compounds and the limiting concentration of each for the different assay methods. If it is not possible to find an assay that is compatible with the buffer system, then the best strategy is to employ a precipitation step (see Support Protocols 2 and 3). After precipitation, the sample is resuspended in a buffer compatible with one of the protein assays.

High-throughput adaptations

The Bradford and BCA assays are easily adapted for high-throughput analysis using a microtiter plate format, as presented in Alternate Protocols 1 and 2, respectively. These protocols are carried out in flat-bottom 96-well microtiter plates and are the methods of choice when processing a large number of unknowns. If microtiter plate-based assays are performed often, it is recommended to use a multichannel repeating pipettor to increase accuracy and decrease user fatigue as samples are dispensed. A convenient approach is to set up a master plate with pre-diluted standards and unknowns that can then be dispensed onto multiple replicate plates. Another significant advantage of this format is that a larger number of standard curve points can be processed, thus increasing the overall accuracy of the results.

Selection of microtiter plate and plate reader

To adapt protein assays to high-throughput analyses, it is important to select a microtiter plate reader that will accommodate the rigors of analysis. An instrument that has two features: a tungsten and mercury or deuterium lamp as a light source, permitting assays at both UV and visible wavelengths is recommended. The instrument should also utilize interchangeable filter wheels or a diffraction grating to permit tuning of the light source. If a filter wheel is used, it is important to purchase a filter wheel with a fairly narrow bandpass (typically, a bandpass range of 10 to 20 nm; a wider bandpass may result in overlap with unbound Coomassie brilliant blue G-250 dye (CBBG) in the Bradford assay or the copper reagent in the BCA assay). Interference filters are more expensive than simple glass filters, but have a narrower bandpass. For the BCA assay, it is desirable to have a short time between each sample reading. Ideally, all 96 wells in the plate should be read in ≤ 2 min. Any polystyrene untreated flat-bottomed microplate should be acceptable. Suppliers include BD Falcon, Corning Costar, and Whatman.

Table 3.4.1 Concentration Limits of Chemicals in Protein Assays^a

Substance ^b	Concentration limits				
	Enhanced copper ^c	BCA ^d	Dye ^e	UV ^f	
				280 nm	205 nm
<i>Acids and bases</i>					
HCl		0.1 M	0.1 M	>1 M	0.5 M
NaOH		0.1 M	0.1 M	>1 M	25 mM
PCA	<1.25%	<1%		10%	1 M
TCA	<1.25%	<1%		10%	<1%
<i>Buffers</i>					
Acetate		0.2 M	0.6 M	0.1 M	10 mM
Ammonium sulfate	>28 mM	20%	1 M	>50%	9%
Borate		10 mM			>100 mM
Citrate	2.5 mM	<1 mM	50 mM	5%	<10 mM
Glycine	2.5 mM	1 M	0.1 M	1 M	5 mM
HEPES	2.5 μM	100 μM	100 mM		<20 mM
Phosphate	250 mM	250 mM	2 M	1 M	50 mM
Tris	250 mM	0.1 M	2 M	0.5 M	40 mM
<i>Detergents</i>					
Brij 35		1%		1%	1%
CHAPS		1%		10%	<0.1%
Deoxycholate	625 μg/ml		0.25%	0.30%	0.1%
Digitonin				10%	
Lubrol PX		1%		10%	
Octylglucoside		1%		10%	
SDS	1.25%	1%	0.10%	0.10%	0.10%
Triton X-100	0.25%	1%	0.10%	0.02%	<0.01%
Triton X-100(R)				>10%	2%
Tween 20	0.10%	1%		0.30%	0.1%
<i>Reductants</i>					
Dithiothreitol	50 μM	<1 mM	1 M	3 mM	0.1 mM
2-Mercaptoethanol	1.8 μM	<1%	1 M	10 mM	<10 mM
<i>Miscellaneous</i>					
DNA/RNA	0.2 mg	0.1 mg	0.25 mg	1 μg	
DMSO	>6.2%	5%		20%	<10%
EDTA	125 μM	10 mM	0.1 M	30 mM	0.2 mM
Glycerol	25%	10%	100%	40%	5%
KCl	30 mM	<10 mM	1 M	100 mM	50 mM

continued

Table 3.4.1 Concentration Limits of Chemicals in Protein Assays^a, *continued*

Substance ^b	Concentration limits				
	Enhanced copper ^c	BCA ^d	Dye ^e	UV ^f	
				280 nm	205 nm
NaCl	1.75 M	1 M	5 M	>1 M	0.6 M
Sucrose	50 mM	40%	1 M	2 M	0.5 M
Urea	>200 mM	3 M	6 M	>1 M	<0.1 M

^aThis table is a general guide. Figures preceded by (<) or (>) symbols indicate that the tolerable limit for the chemical is unknown but is, respectively, less than or greater than the amount shown. Blank spaces indicate that data were unavailable. Reproduced with permission from Stoscheck (1990).

^bAbbreviations: PCA, perchloric acid; TCA, trichloroacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EDTA, ethylenediamine tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate R, reduced; DMSO, dimethyl sulfoxide.

^cFigures indicate the final concentration of the chemical in the assay.

^dFigures indicate the concentration of the chemical in a 50- μ l sample.

^eFigures indicate the concentration of the chemical in a 25- μ l sample.

^fFigures indicate the concentration of the chemical that does not produce an absorbance of 0.5 over water.

BASIC PROTOCOL 1

THE LOWRY ASSAY

The Lowry method (Lowry et al., 1951) relies on two different reactions. The first reaction is the formation of a copper ion complex with amide bonds, forming reduced copper in alkaline solutions. This is called a Biuret chromophore and is commonly stabilized by the addition of tartrate (Gornall et al., 1949). The second reaction is reduction of the Folin-Ciocalteu reagent (phosphomolybdate and phosphotungstate), primarily by the reduced copper-amide bond complex as well as by tyrosine and tryptophan residues. The reduced Folin-Ciocalteu reagent is blue and thus detectable with a spectrophotometer in the range of 500 to 750 nm. The Biuret reaction itself is not very sensitive. Using the Folin-Ciocalteu reagent to detect reduced copper makes the Lowry assay nearly 100 times more sensitive than the Biuret reaction alone. Several useful modifications of the original Lowry assay have been developed to increase the dynamic range of the assay over a wider protein concentration (Hartree, 1972), to make the assay less sensitive to interference by detergents (Dulley and Grieve, 1975), and to first precipitate the proteins to remove interfering contaminants (Bensadoun and Weinstein, 1976).

The Lowry assay is relatively sensitive, but requires more time than other assays and is susceptible to many interfering compounds (Table 3.4.1). The following substances are known to interfere with the Lowry assay: detergents, carbohydrates, glycerol, Tricine, EDTA, Tris, potassium compounds, sulfhydryl compounds, disulfide compounds, most phenols, uric acid, guanine, xanthine, magnesium, and calcium. Many of these interfering substances are commonly used in buffers for preparing proteins or in cell extracts. This is one of the major limitations of the assay. The Lowry assay is also sensitive to variations in the content of tyrosine and tryptophan residues, a trait shared with the ultraviolet assay at 280 nm (see Basic Protocol 4). The assay is linear over the range of 1 to 100 μ g protein (Fig. 3.4.2). The absorbance can be read in the region of 500 to 750 nm, with 660 nm being the most commonly employed. Other wavelengths can also be used, however, and may reduce the effects of contamination (e.g., chlorophyll in plant samples interferes at 660 nm, but not at 750 nm). Also, if the A_{660} values are low, sensitivity can be increased by rereading the samples at 750 nm. A typical Lowry assay standard curve is depicted in Figure 3.4.2 and a typical assay spreadsheet is listed in Table 3.4.2.

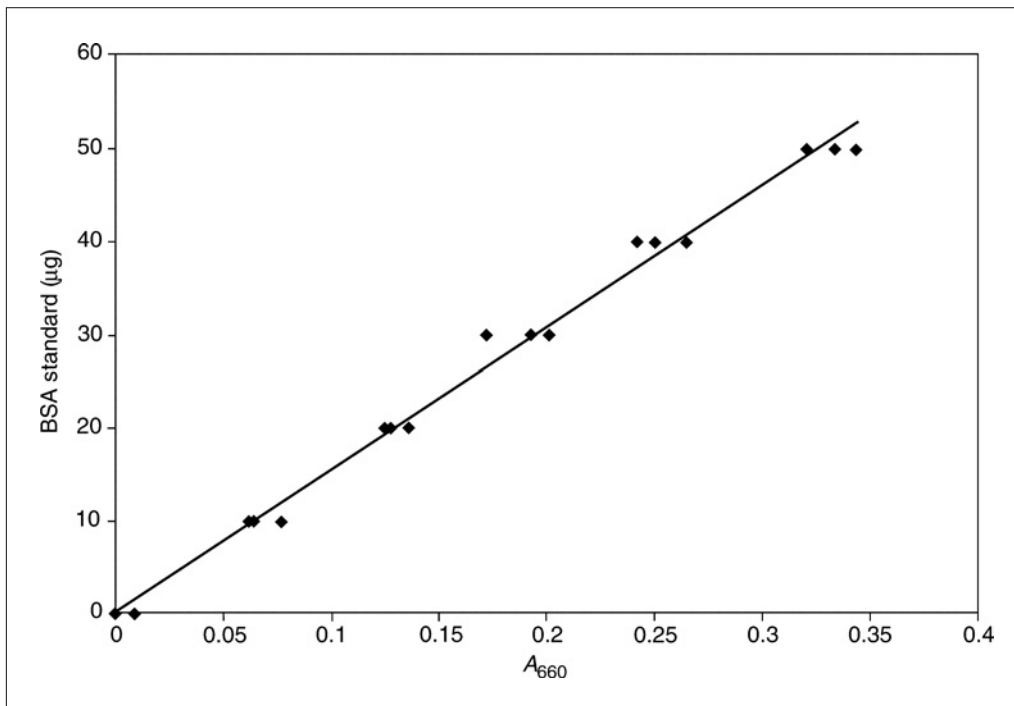


Figure 3.4.2 A sample Lowry protein assay standard curve produced using BSA at triplicate points of 0, 10, 20, 30, 40, and 50 μg . The data are fit with a linear regression by the line $y = 153.06x + 0.179$ with an R^2 value of 0.992. The data table used to generate the figure and depiction of a typical Lowry assay is shown in Table 3.4.2.

Materials

1 mg/ml protein standard (e.g., BSA, albumin, or γ -globulin; see Support Protocol 1)

Sample

Lowry assay mix (see recipe)

Freshly prepared diluted Folin-Ciocalteu reagent (see recipe)

Test tubes (e.g., 16×125 -mm)

Spectrophotometer warmed up and set to 660 nm (or other appropriate wavelength) and cuvette

1. Prepare standards containing a range of 1 to 100 μg protein (e.g., albumin or γ -globulin) to a volume of 1 ml (see Support Protocol 1 for information on standard curve preparation).
2. Add sample to water in a 16×125 -mm test tube to yield a final volume of 1 ml. Also include two separate tubes containing water for water blanks. Additionally, use buffer blanks if necessary (see Spectrophotometry for more detail).
3. To each tube, add 5 ml of freshly prepared Lowry assay mix and thoroughly vortex.
4. Incubate tubes 10 min at room temperature.
5. Add 0.5 ml of diluted Folin-Ciocalteu reagent to each tube and vortex immediately.
6. Incubate 30 min at room temperature.

Table 3.4.2 Typical Layout of a Lowry Assay and Data^a*Standard*

1 mg/ml standard (μl)	H ₂ O (μl)	Assay mix (ml)	Diluted Folin-Ciocalteu reagent (ml)	BSA (μg)	A ₆₆₀
0	1000	5	0.5	0	0
0	1000	5	0.5	0	0.009
0	1000	5	0.5	0	-0.003
10	990	5	0.5	10	0.062
10	990	5	0.5	10	0.064
10	990	5	0.5	10	0.077
20	980	5	0.5	20	0.125
20	980	5	0.5	20	0.127
20	980	5	0.5	20	0.136
30	970	5	0.5	30	0.172
30	970	5	0.5	30	0.193
30	970	5	0.5	30	0.201
40	960	5	0.5	40	0.25
40	960	5	0.5	40	0.265
40	960	5	0.5	40	0.242
50	950	5	0.5	50	0.344
50	950	5	0.5	50	0.321
50	950	5	0.5	50	0.334

Equation 1: $[\mu\text{g protein}] = (153.06 \times A_{660}) + 0.179$

Unknowns

Unknown (μl)	H ₂ O (μl)	Assay mix (ml)	Diluted Folin-Ciocalteu reagent (ml)	A ₆₆₀	Protein (μg) from Eqn. 1
1	999	5	0.5		
1	999	5	0.5		
5	995	5	0.5		
5	995	5	0.5		
20	980	5	0.5		
20	980	5	0.5		

^aTypical layout of a Lowry assay and data used to create the sample standard curve shown in Figures 3.4.2 and 3.4.6. The volumes of unknown used are typical amounts required to be within the dynamic range of the assay.

- Vortex the tubes, zero the spectrophotometer with the blank, and measure absorbance at 660 nm (or other appropriate wavelength).

The absorbance will not change significantly if all the samples are read at the same time. The Lowry assay is not an endpoint assay, so samples will change in absorbance if too much time elapses between sample readings. The typical time that elapses during the reading of samples (<10 min) does not usually result in significant changes in absorbance.

- Refer to Support Protocol 1 for data analysis.

THE BRADFORD ASSAY

The Bradford assay (Bradford et al., 1976) is a popular protein assay because it is simple, rapid, inexpensive, and sensitive. The Bradford assay is based on the direct binding of Coomassie brilliant blue G-250 dye (CBBG) to proteins at arginine, tryptophan, tyrosine, histidine, and phenylalanine residues. The assay primarily responds to arginine residues (eight times as much as the other listed residues), therefore, if the sample is rich in arginines (e.g., histone), it may be necessary to use an arginine-rich standard as well. Anionic CBBG binds to these residues producing an absorbance maximum at 595 nm, whereas the free dye in solution has an absorbance maximum at 470 nm (Fig. 3.4.3). The assay is monitored at 595 nm in a spectrophotometer, and thus measures the CBBG complex with the protein, which results in an absorption peak shift.

The absorption spectra of the bound and free dye overlap and cause the Bradford assay to respond nonlinearly to protein concentration, conflicting with a common misconception that the response is linear with respect to protein concentration. However, when a standard curve is performed, a second-order curve will fit the data more accurately than a linear fit (Fig. 3.4.4). Since the data will be analyzed with software that is capable of fitting second-order curves, there is no reason not to use an appropriate curve fit to the standards.

One crucial aspect of this assay is the buffer blank (see Spectrophotometry). Since the assay responds nonlinearly to protein concentration, it is important to be certain about the zero point. Because this point is so important to the curve fit, it is highly recommended that at least two buffer blanks be included. If it is determined that interference is not occurring, as is the usual case, proceed to use the water blank. The water and buffer blanks will have a relatively high absorbance, therefore, zeroing the spectrophotometer with air is recommended. This will produce an A_{595} of ~ 0.5 for the blank. However, to zero the spectrophotometer with the blank would force the spectrophotometer to subtract

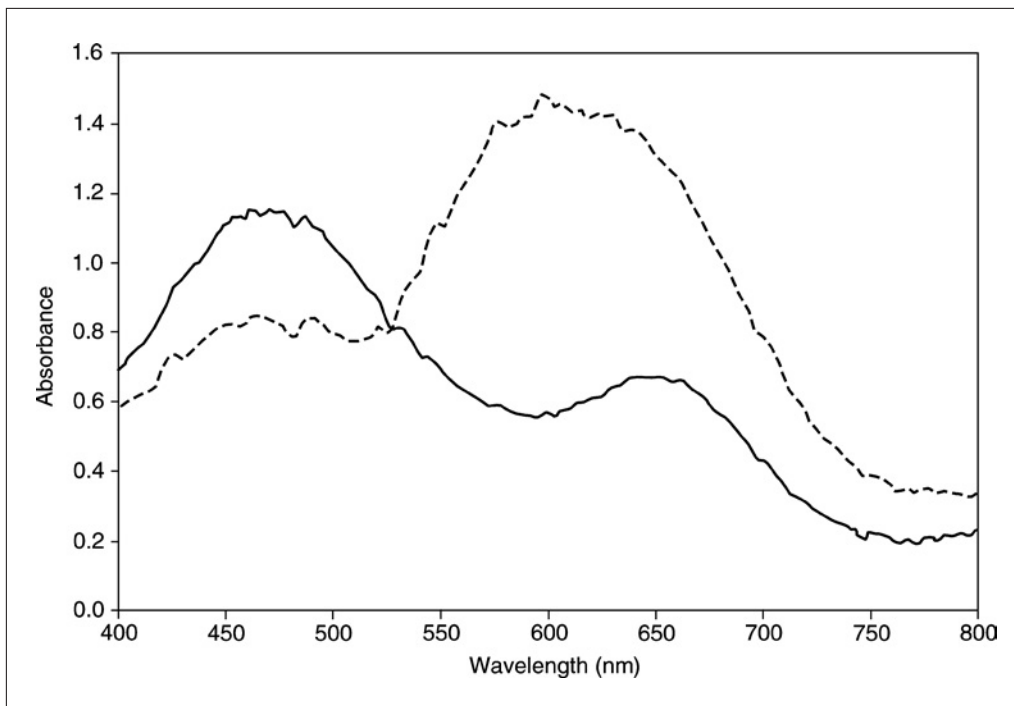


Figure 3.4.3 Absorbance spectra of the Bradford reagent and the Bradford reagent bound to 20 μg of BSA standard. The free reagent (solid line) has an absorbance peak of 470 nm whereas the Bradford reagent complexed with protein (dashed line) has an absorbance peak near 600 nm. Note that the unbound dye partially overlaps with the bound form of the reagent and thus leads to the nonlinear response of the Bradford assay.

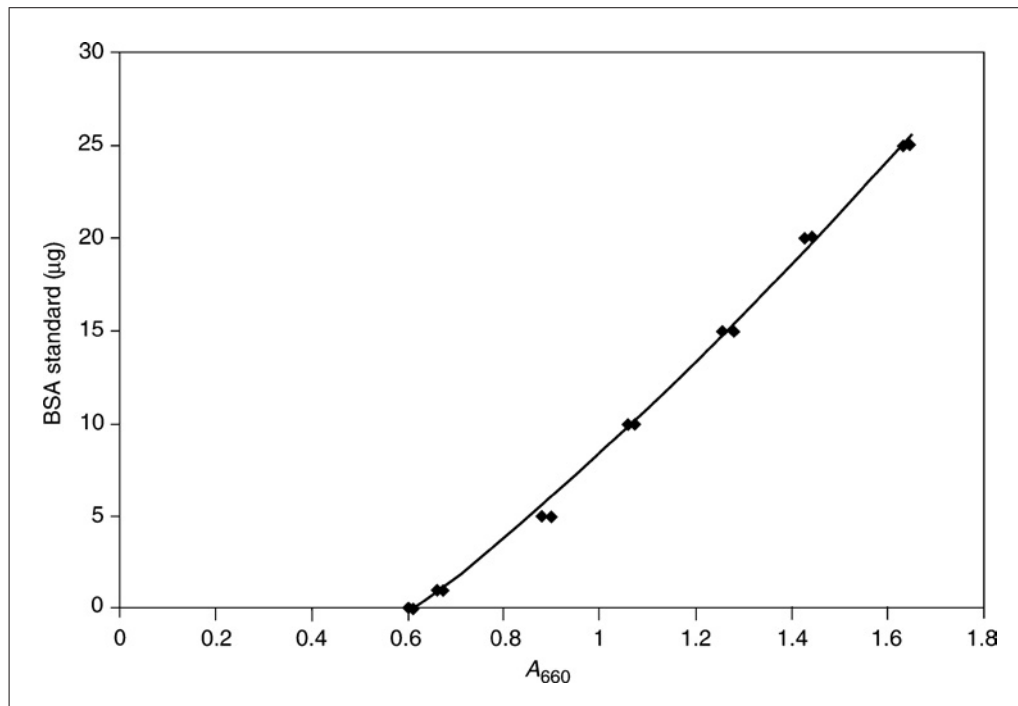


Figure 3.4.4 A sample micro-Bradford assay standard curve. BSA standard was added in triplicate at 0, 0.25, 1.25, 2.5, 5, 7.5, and 10 µg. Note that the assay does not respond linearly to the concentration of standard. The data is fit with the equation $y = 4.5898x^2 + 14.424x - 10.694$ and has an R^2 value of 0.9979. The data table used to generate the figure and depiction of a typical micro-Bradford assay is shown in Table 3.4.3.

a very large amount from every sample read. An absorbance of 0.5 is equivalent to only 31% of the light being transmitted by the reagent blank. This will tend to produce error and imprecision into the sample readings especially at high protein concentration. By zeroing against air, less noise occurs in the readings and A_{595} values stay below 2.0, a common upper limit for absorbance values (see Spectrophotometry).

The choice of standard protein for this assay is important to the success of the assay. Many investigators have noted abnormalities of using various standards with the Bradford assay. Bovine serum albumin (BSA) was the original standard of choice, and is the standard that most likely comes with the assay if purchased as a kit. However, it has been noted that BSA has a significantly higher than “normal” response in the assay (e.g., Stoscheck, 1990) and may not always be suitable. Several researchers, therefore, use immunoglobulin G (IgG) or lysozyme as the preferred standard for the assay, but other standards may be appropriate depending on the particular sample protein.

The CBBG dye used in the assay strongly binds to quartz cuvettes. Therefore, glass or plastic cuvettes should be utilized. It is convenient to use disposable plastic cuvettes, making cleanup much easier. Using a single cuvette for all the samples is recommended, as plastic cuvettes are not constructed with the same precision as quartz or glass cuvettes. By using a single microcuvette and maintaining its orientation in the sample holder, this eliminates one potential source error from the system. For the 1-ml micro-Bradford assay, after taking an A_{595} reading, the contents are aspirated and 0.1 ml of the next sample is used to wash the cuvette. The cuvette is again aspirated and the remaining 0.9 ml of the next sample is added to the cuvette for A_{595} determination.

There are two major formats of this assay, each with a different detection range. The micro-assay format is designed for protein concentrations between 1 and 20 µg. The macro-assay format is designed for protein concentrations in the range of 20 to 100 µg.

It is generally more convenient to use the micro-assay format because it uses less of the sample. The micro-assay format also results in less chemical waste and allows the concentrate to be used for more assays. The assay can also be carried out in a microwell plate, which is a very convenient way to process a large number of samples rapidly (see High-Throughput Adaptations).

Some proteins, especially hydrophobic, membrane or “sticky” proteins tend to precipitate in the presence of CBBG and will appear as dark-blue clumps after the Bradford reagent has been added. Precipitation of the sample causes light scattering and incorrect reporting of absorbance. If there is formation of precipitate when the dye reagent is added to the sample, add a small amount of sodium hydroxide to help solubilize the protein (see steps 3a and 3b below).

It is commonly stated that detergent or surfactant-containing samples are not compatible with the Bradford assay. It is common to lyse bacterial cultures in a proprietary surfactant solution such as BugBuster (Novagen). The authors have found that the amount of surfactant in extracts prepared with 100 μ l BugBuster for pelleted cells from a 5-ml culture is sufficiently small that it does not interfere with the Bradford assay. Extracts containing 0.5 to 4 mg/ml protein require <10 μ l of sample in a 1-ml total assay volume. A typical standard curve for the micro-Bradford assay is depicted in Figure 3.4.4 and a typical micro-Bradford assay spreadsheet is shown in Table 3.4.3.

Materials

1 mg/ml protein standard (e.g., BSA, albumin, or γ -globulin; see Support Protocol 1)

Protein sample

1 M NaOH (optional)

Bradford reagent (see recipe)

Spectrophotometer warmed up at least 15 min before use and set to 595 nm, and cuvette

Test tubes (e.g., 10 \times 75-mm for the micro assay or 17 \times 100-mm for the macro assay)

Micro-Bradford assay

- 1a. Prepare standards containing a range of 1 to 20 μ g protein (e.g., albumin or γ -globulin) to a volume of 800 μ l (to a volume of 700 μ l if adding 1 M NaOH). See Support Protocol 1 for information on standard curve preparation.

Table 3.4.3 contains detailed information for preparing a typical Bradford assay with the stand curve results depicted in Figure 3.4.4.

- 2a. Add sample to water in a final volume of 800 μ l (700 μ l if adding 1 M NaOH).

- 3a. Optional: Add 100 μ l of 1 M NaOH to each sample and vortex.

NaOH is added to ensure that the sample does not precipitate upon addition of Bradford reagent in step 4a. If a sample (not containing NaOH) precipitates upon addition of Bradford reagent then a new sample must be prepared since addition of NaOH after precipitation will not cause it to dissolve. NaOH should thus be added to the new sample prior to addition of Bradford reagent.

- 4a. Add 200 μ l Bradford reagent, vortex, and incubate 5 min at room temperature.

The Bradford assay is an endpoint assay, therefore, absorbance readings will not change significantly over time.

- 5a. Record blank, vortex samples again, and measure the absorbance at 595 nm.

- 6a. Refer to Support Protocol 1 for information on data processing.

Table 3.4.3 Typical Layout for a Micro-Bradford Assay and Data^a

<i>Standard</i>					
1 mg/ml standard (μl)	H ₂ O (μl)	1 M NaOH (μl)	Bradford reagent (μl)	BSA (μg)	A ₅₉₅
0	700	100	200	0	0.603
0	700	100	200	0	0.603
0	700	100	200	0	0.613
1	699	100	200	1	0.675
1	699	100	200	1	0.664
1	699	100	200	1	0.662
5	695	100	200	5	0.9
5	695	100	200	5	0.878
5	695	100	200	5	0.883
10	690	100	200	10	1.069
10	690	100	200	10	1.062
10	690	100	200	10	1.062
15	685	100	200	15	1.254
15	685	100	200	15	1.257
15	685	100	200	15	1.279
20	680	100	200	20	1.443
20	680	100	200	20	1.444
20	680	100	200	20	1.43
25	675	100	200	25	1.651
25	675	100	200	25	1.631
25	675	100	200	25	1.638

$$\text{Equation 2: } [\mu\text{g protein}] = (4.5898 \times A_{595}^2) + (14.424 \times A_{595}) - 10.694$$

Unknowns

Unknown (μl)	H ₂ O (μl)	1 M NaOH (μl)	Bradford reagent (μl)	A ₅₉₅	Protein (μg) from Eqn. 2
1	699	100	200		
1	699	100	200		
5	695	100	200		
5	695	100	200		
20	680	100	200		
20	680	100	200		

^aTypical layout for a micro-Bradford assay and the data used to create the sample standard curve in Figure 3.4.4. The volumes of unknown used are typical amounts required to be within the dynamic range of the assay.

Macro-Bradford assay

- 1b. Prepare standards containing a range of 20 to 200 μg protein (e.g., albumin or γ-globulin) in a standard volume (generally ≤1 ml). Refer to Support Protocol 1 for information on standard curves.
- 2b. Dilute unknowns to an estimated concentration of 20 to 200 μg protein per tube in a same volume as the standards.

- 3b. Optional: Add 0.25 ml of 1 M NaOH to each sample and vortex.

NaOH is added to ensure that the sample does not precipitate upon addition of Bradford reagent in step 4a. If a sample (not containing NaOH) precipitates upon addition of Bradford reagent then a new sample must be prepared since addition of NaOH after precipitation will not cause it to dissolve. NaOH should thus be added to the new sample prior to addition of Bradford reagent.

- 4b. Add 5 ml Bradford reagent, vortex, and incubate 5 min at room temperature.
5b. Record blank, vortex samples again, and measure the absorbance at 595 nm.
6b. Refer to Support Protocol 1 for information on data processing.

MICROTITER PLATE BRADFORD

For microtiter plate–based Bradford assays, it is recommended that a commercial reagent be considered. The authors find that, due to the small volume, any precipitates that may be in a laboratory prepared reagent will interfere with the determination. A laboratory prepared reagent must be filtered before use. The reagent should be pipetted from the top of the solution to avoid interference from precipitates that may be found at the bottom. The microtiter plate assay version is more prone to protein precipitation due to the higher concentration of dye. Even if the protein sample does not precipitate in the regular Bradford assay, it should again be verified that the protein is not precipitating in this assay format. If protein sample precipitation is observed, add a small volume of 1 M NaOH (see step 2) to solubilize the protein.

Materials

- Sample
 - 1 mg/ml protein standard (e.g., BSA; see Support Protocol 1)
 - Bradford reagent (see recipe)
 - 1 M NaOH
- Microtiter plate (see Strategic Planning)
- Multichannel pipettor or repeating pipettor, optional
- Microtiter plate reader (see Strategic Planning)

1. Prepare unknowns and standards in a final volume of 160 μ l.

If protein solubility is an issue in the presence of the Bradford reagent reduce sample volume to 140 μ l to accommodate addition of NaOH in step 2.

2. Optional: Add 20 μ l of 1 M NaOH.

NaOH is added to ensure that the sample does not precipitate upon addition of Bradford reagent in step 4a. If a sample (not containing NaOH) precipitates upon addition of Bradford reagent then a new sample must be prepared since addition of NaOH after precipitation will not cause it to dissolve. NaOH should thus be added to the new sample prior to addition of Bradford reagent.

3. Add 40 μ l of Bradford reagent to each well of a microtiter plate and carefully mix.

Using a multichannel pipettor facilitates mixing. If one is not available, carefully tap the side of the microtiter plate to avoid cross-contaminating wells or use a minishaker.

4. Incubate 5 min at room temperature.

If this is the first time using the microtiter plate format, carefully inspect the microtiter plate wells on a lightbox for signs of precipitate formation. If protein precipitation is observed, add 20 μ l of 1 M NaOH and reduce the final sample volume as indicated above.

5. Read samples at an appropriate wavelength (commonly 595 nm).

6. See Support Protocol 1 for information on data processing.

ALTERNATE PROTOCOL 1

Detection and Assay Methods

3.4.13

BCA ASSAY

The BCA assay (Smith et al., 1985) measures the formation of Cu^+ from Cu^{2+} by the Biuret complex in alkaline solutions of protein using bicinchoninic acid (BCA). Originally, the mechanism of the BCA assay was thought to be similar to the Lowry assay, but it is now known that there are two distinct reactions that take place with copper ions unique to the BCA assay (Wiechelman et al., 1988). The first reaction occurs at lower temperatures and is the result of the interaction of copper and BCA with cysteine, cystine, tryptophan, and tyrosine residues in the protein. At elevated temperatures, the peptide bond also is responsible for color development. Hence, performing the assay at 37°C or 60°C versus room temperature increases the sensitivity and reduces the variation in the response of the assay as a function of protein composition. When possible, the assay should be incubated at 60°C since, after the reaction is complete, the absorbance does not increase appreciably, whereas after cooling samples incubated at 37°C to room temperature, the blank continues to increase in absorbance at $\sim 2.3\%$ every 10 min.

The BCA reagent replaces the Folin-Ciocalteu reagent in the Lowry assay. The BCA reagent forms a complex with Cu^+ , which has a strong absorbance at 562 nm. BCA is advantageous in that it does not interact with as many contaminants and buffer components as the Folin-Ciocalteu reagent, especially detergents. Components that interfere with the BCA assay either lead to reduction of Cu^{2+} (e.g., DTT) or copper chelators (e.g., EGTA). Generally, these are not critical components of buffers and can be easily removed or omitted prior to the assay.

Note that there are two variations of the assay. The micro-based assay requires three reagents, whereas the test tube protocol requires only two reagents. The micro-based assay reagents are at a significantly higher concentration than the macro-based assay reagents. To prevent precipitation of BCA, it is prepared as a separate reagent in the micro-based format. It is important to use the correct reagent mixtures and ratios for the

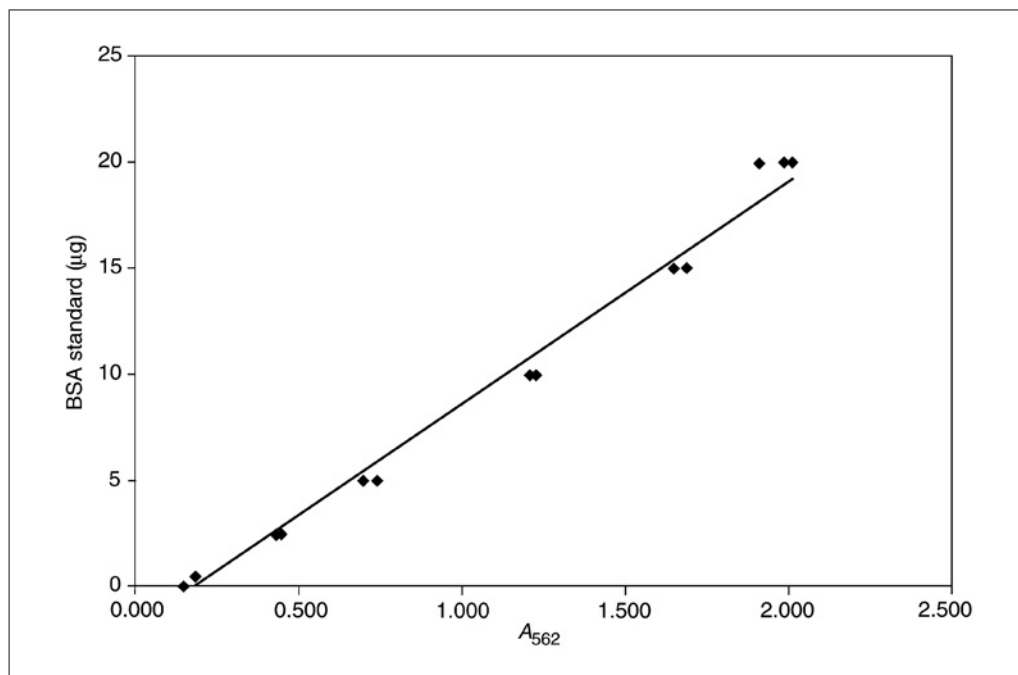


Figure 3.4.5 A sample micro-BCA assay standard curve with triplicate BSA standard points of 0, 0.625, 3.125, 6.25, 12.5, and 18.75 µg. Note that the curve is linear across a wide range and is fit with the equation $y = 10.571x - 2.0254$ and has a R^2 value of 0.9893. The data table used to generate the figure and depiction of a typical micro-BCA assay is shown in Table 3.4.4.

chosen format. In Strategic Planning, a microtiter plate adaptation of the micro-based assay is described. A typical standard curve for the micro-BCA assay is depicted in Figure 3.4.5 and assay setup is listed in Table 3.4.4.

Table 3.4.4 Typical Layout of a Micro-BCA Assay and Data^a

<i>Standard</i>				
1 mg/ml standard (μl)	H ₂ O (μl)	Micro-BCA solution (μl)	BSA total (μg)	A ₅₆₂
0	500	500	0	0.149
0	500	500	0	0.153
0	500	500	0	0.151
0.5	499.5	500	0.5	0.179
0.5	499.5	500	0.5	0.187
0.5	499.5	500	0.5	0.179
2.5	497.5	500	2.5	0.433
2.5	497.5	500	2.5	0.451
2.5	497.5	500	2.5	0.445
5	495	500	5	0.744
5	495	500	5	0.700
5	495	500	5	0.738
10	490	500	10	1.228
10	490	500	10	1.209
10	490	500	10	1.226
15	485	500	15	1.689
15	485	500	15	1.642
15	485	500	15	1.651
20	480	500	20	1.988
20	480	500	20	1.913
20	480	500	20	2.009

Equation 3: $[\mu\text{g protein}] = (10.571 \times A_{562}) - 2.0254$

Unknowns

Unknown (μl)	H ₂ O (μl)	Assay mix (ml)	A ₅₆₂	Protein (μg) from Eqn. 3
1	499	500		
1	499	500		
5	495	500		
5	495	500		
20	480	500		
20	480	500		

^aTypical layout of a micro-BCA assay and the data used to create the sample standard curve in Figure 3.4.5. The volumes of unknown used are typical amounts required to be within the dynamic range of the assay.

Materials

Macro-BCA or micro-BCA assay solution (see recipes)

Samples

1 mg/ml standard proteins (e.g., BSA, albumin, or γ -globulin; see Support Protocol 1)

Test tubes (e.g., 10 \times 75–mm for micro-BCA assay or 13 \times 100–mm for macro-BCA assay)

Spectrophotometer warmed up and set to 562 nm, and cuvette
60°C (or 37°C) heating block or water bath

Micro-BCA assay (1-ml)

- 1a. Prepare fresh micro-BCA assay solution. Set up test tubes (e.g., 10 \times 75–mm) containing samples and known amounts of standard protein in the range of 0.5 to 20 μ g with each final sample volume of 500 μ l. Refer to Support Protocol 1 for information on preparation of a standard curve.
- 2a. Add 500 μ l of micro-BCA assay solution to each tube, vortex, and incubate the sample 15 min at 60°C.

Alternatively, incubate 1 hr at 37°C, although, in practice, a 60°C incubation will yield more consistent results.

- 3a. Cool samples to room temperature.

Note that this is extremely important. The simplest method is to put the test-tube rack into a pan containing room temperature water.

- 4a. Vortex samples and read absorbance at 562 nm.
- 5a. Refer to Support Protocol 1 for information on data processing.

Macro-BCA assay (2.1-ml)

- 1b. Prepare fresh macro-BCA assay solution.
- 2b. Set up 13 \times 100–mm test tubes containing samples and known amounts of standard protein in the range of 0 to 100 μ g in a total tube volume of 0.1 ml.
- 3b. Add 2.0 ml macro-BCA assay solution to each tube and vortex.
- 4b. Incubate tubes 15 min at 60°C.

Alternatively, incubate tubes 30 min at 37°C.

- 5b. Cool the tubes to room temperature.
- 6b. Vortex samples and read the absorbance at 562 nm.
- 7b. Refer to Support Protocol 1 for information on data processing.

ALTERNATE PROTOCOL 2

MICROTITER PLATE BCA ASSAY

The microtiter plate BCA assay is based on the micro-BCA assay (see Basic Protocol 3) and utilizes the same reagents. This protocol results in a final volume of 250 μ l and in the authors' experience, works with most commercially available microtiter plates, but occasionally, some plates cannot accommodate 250 μ l without well-to-well contamination. In this case, reducing the volume of reagents may help, but may result in a narrowing of the detection range. For the microtiter plate–based assay, incubating for 15 min at 60°C and cooling the plate to room temperature prior to reading the plate is recommended. Some microtiter plate readers can do this entire operation automatically.

Materials

Micro-BCA working solution (see recipe)

1 mg/ml protein standard (e.g., BSA; see Support Protocol 1)

Microtiter plate(s) (BD Falcon 353915; Costar 3370; Whatman 7701-1350)

Microtiter plate reader, warmed up and ready to read at 562 nm

60°C (or 37°C) oven or water bath

1. Prepare sufficient micro-BCA working solution for all assays—prepare ~15% to 20% extra to account for waste and to avoid running out.
2. Prepare standards and unknowns each in a 125- μ l final volume in individual wells of the microtiter plate.
3. Add 125 μ l of micro-BCA working solution to each well and gently mix by tapping the side of the microtiter plate or using a minishaker
4. Incubate 15 min at 60°C.
Alternatively, incubate 30 min at 37°C, but the absorbance will continue to increase at 2.3% per 10 min
5. Cool plate to room temperature.
6. Gently mix by tapping the side of the plate or by using a minishaker and read absorbance at 562 nm.

UV ABSORBANCE TO MEASURE PROTEIN CONCENTRATION

The use of ultraviolet absorbance to measure protein concentration is perhaps the most simple and quick method, but also the most likely to produce inaccurate results. Determination of protein concentration by ultraviolet absorption, usually at 280 nm, depends on the presence of aromatic amino acids in proteins. Tyrosine and tryptophan absorb at ~280 nm, whereas phenylalanine has a peak of absorbance at ~260 nm. Higher orders of protein structure may also modify the molar absorptivities of tyrosine and tryptophan and thus the UV detection is sensitive to pH and ionic strength.

Unless the protein sample is pure and its extinction coefficient is known, UV determination of protein concentration will invariably be significantly in error. This protocol is included here because it is extremely useful for making quick and rough estimates of protein concentration before proceeding to a more accurate assay such as those listed above. A useful equation (Layne, 1957) for approximate concentration determination using UV absorbance is as follows:

$$[\text{protein}] \text{ (mg/ml)} = (1.55 \times A_{280}) - (0.76 \times A_{260})$$

This equation is not sufficiently accurate for routine biochemical calculations.

Many other cellular components, particularly nucleic acids, also absorb UV light. Although different proteins will have different amino acid compositions and thus different molar absorptivities, this method can be very accurate when comparing different solutions of the same protein.

Glass and polystyrene cuvettes absorb UV light, therefore, quartz cuvettes should be used. Methacrylate plastic cuvettes are also available, which transmit ~60% of 280-nm light and it is possible to zero spectrophotometers with them and acquire absorbance readings. Methacrylate cuvettes are significantly less expensive than quartz cuvettes and may be a good alternative in environments where cuvette breakage is a concern (e.g., teaching laboratories).

BASIC PROTOCOL 4

Detection and Assay Methods

3.4.17

This method is the least sensitive of the methods presented in this unit. For increased sensitivity, the wavelength can be altered to a range of 210 to 225 nm (Stoscheck, 1990). This measures the amide bonds in proteins and is often the wavelength range used for measuring absorbance of HPLC column fractions. However, it is much more subject to interference from many more biological components and compounds used to make buffer solutions.

If using a pure protein with a known extinction coefficient (ϵ), it is possible to directly determine the concentration from the measured A_{280} . The extinction coefficients for many proteins have been published (Fasman, 1989). Because of the different mole fractions of tyrosine and tryptophan residues in different proteins, their extinction coefficients can be very different. An example of the difference in extinction coefficient at 280 nm between proteins having different amino acid compositions is as follows: consider the following $\epsilon^{1\%}$ values (Fasman, 1989) for horse liver alcohol dehydrogenase, 4.2 to 4.5; bovine serum albumin, 6.3 to 6.8; rabbit IgG, 13.5 to 14.6; and egg white lysozyme, 24.7 to 27.2. Most protein extinction coefficients are not expressed as ϵ_{molar} ($\text{M}^{-1} \text{cm}^{-1}$) as is usual for most biomolecules, but rather as $\epsilon^{1\%}$ ($\%^{-1} \text{cm}^{-1}$). The $\epsilon^{1\%}$ is defined as the A_{280} value of a 1% solution (1 g/100 ml) of the protein. It is necessary to be aware of this difference when calculating the amount of protein from A_{280} values. Using the molar absorptivity, $A_{280}/\epsilon_{\text{molar}}$ produces a concentration in molar terms. Using the percent absorptivity, $A_{280}/\epsilon^{1\%}$ produces a concentration in percent. When using the percent absorptivity, it is common to use the equation $(A_{280}/\epsilon^{1\%}) \times 10$ to yield the concentration in units of mg/ml. If the amino acid sequence of the sample protein is known, it is possible to calculate the theoretical extinction coefficient (Gill and von Hippel, 1989).

A procedure for determining the concentration of an unknown using UV absorbance and a standard curve is presented here. In general, the results from this approach will also not be accurate enough to use in downstream calculations.

Materials

Sample
Protein standard prepared in the same buffer as unknown
Sample buffer
Spectrophotometer and quartz or UV-transparent cuvettes

1. To perform an ultraviolet reading at 280 nm, warm up the spectrophotometer and set it to 280 nm.
2. Zero the spectrophotometer with a water blank.
3. Construct a standard curve (0 to 3 mg/ml) using a protein standard of choice in the expected concentration range at 280 nm in a quartz or UV-transparent cuvette.
4. Measure the A_{280} of the buffer used to prepare the sample.
5. Measure the A_{280} of the sample. If the A_{280} is >2 , dilute an aliquot of the sample in the sample buffer and remeasure.
6. Calculate the protein concentration from the standard curve.
7. Refer to Support Protocol 1 for information on data processing.

Standard Curves and Standard Selection

Two details must be understood about standard curves. First, they are called standard “curves” because the response of absorbance to the amount of added protein is not necessarily linear. Second, the range of protein concentrations chosen for the standard curve (e.g., 0 to 25 μg or 0 to 100 μg) will depend on the assay employed. The A_{280} method (see Basic Protocol 4) is the least sensitive and requires the highest amounts of protein in the standard curve, whereas the Bradford method (see Basic Protocol 2) is sensitive, but not linear over a very wide range, necessitating the use of a relatively narrow range of standard protein.

Another factor to keep in mind is that the standard curve is based on one protein (usually BSA). The mixture of proteins in the sample may not yield identical results as the standard, in terms of a signal as a function of protein concentration directly corresponding to the standard chosen. While BSA, in general, is a suitable choice as a standard, it does have limitations as a standard (see Standard Selection) and this should be kept in mind when assaying protein samples.

One of the most often overlooked assay parameters is the generation of a standard curve with each assay, which is known as an internal standard curve. Internal standard curves are essential for obtaining accurate results, especially for the Lowry (see Basic Protocol 1) and BCA (see Basic Protocol 3 and Alternate Protocol 2) assays, which are non-endpoint reactions. In the case of the Bradford assay, many procedures state that it is not necessary to run internal standard curves with each assay. However, analysis of years of compiled data for various Bradford assays has shown that standard curves can exhibit significant amounts of drift, concluding that neglecting to perform an internal standard curve may yield inaccurate results. Typically, standard curves should be run at least in duplicate.

Sample dilution series

In addition to having unknown samples fall within the linear response range of the standard curve, it is good practice to perform a dilution series of the unknown(s). Expect to observe a change in the response of the unknown that follows the slope of the standard curve. For example, if 5 and 10 μl of the sample produce responses equivalent to 7.5 and 15 μg of protein, respectively, confidence may be placed in the results. If the unknowns do not change when diluted, or the response curve does not follow that of the standard curve, assay selection and buffer compatibility may need to be re-evaluated.

Standard selection

Standard selection for protein assays is critical. If the selected standard does not respond to the assay in a manner similar to the unknowns, the magnitude of error in the assay may be significant. The most common standards are BSA or purified-IgG proteins. A review by Stoscheck (1990) describes how differently these two proteins respond in all of the assays presented in this unit. While these have become the de facto standards of choice, there are experiments where these may not be the most appropriate standards. Unfortunately, the only way to ensure total accuracy is by standardizing any assay procedure to quantitative amino acid analysis (*UNIT 3.2*).

Bovine serum albumin (BSA) is the most commonly used standard for protein assays. BSA in no way is the perfect standard, but it has become the arbitrary standard of choice. Serum albumins are “sticky” proteins to the extent that drug adsorption to them is a critical factor in pharmacokinetic calculations (Mandula et al., 2006). This means that BSA tends to bind to other proteins and molecules and, thus, it is very difficult to purchase

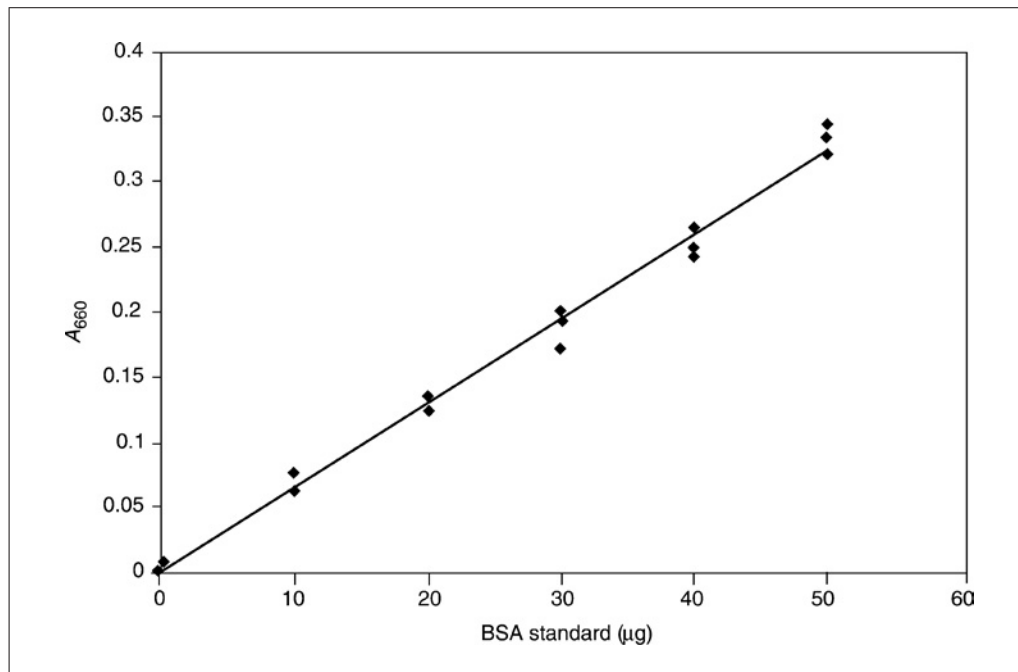


Figure 3.4.6 Data for a sample Lowry protein assay standard curve produced using BSA standard at triplicate points of 0, 10, 20, 30, 40, and 50 µg. The data are identical to those in Figure 3.4.2 except that BSA (µg) is plotted on the x axis and A_{660} is plotted on the y axis. The data are fit with a linear regression by the line $y = 0.0065x + 0.0001$ with an R^2 value of 0.992. Note that to use the equation to return protein concentration, the equation must be rearranged.

in a 100% pure form. These contaminants can lead to problems with any of the listed assays due to side reactions or blocking interaction sites.

Protein standards can be purchased from most chemical suppliers in liquid form or they may be prepared in the laboratory. Usually, a 1 to 5 mg/ml stock solution is prepared in bulk (50 to 100 ml) to avoid variation in the concentration of standards from day to day. When the solution is prepared, do not shake it because this will lead to denaturation of the protein (observed as bubbles on the top of the solution), but rather gently swirl to dissolve the BSA in water. This solution is then dispensed into smaller aliquots (1- to 5-ml) and stored at -20°C . As with any solution, it must be mixed upon thawing because melting solutions form an internal concentration gradient.

The best strategy for preparing a standard curve dilution series is to prepare the standard as a 1 to 5 mg/ml stock in water and prior to the assay, dilute the stock in the same buffer as the unknowns—including a buffer blank. This strategy will provide the most accurate result, because the effects of the buffer are accounted for in the standard curve. It is also possible to prepare a standard curve dilution series in water and use a buffer blank, however, the buffer used in the unknowns should be fully compatible with the assay.

Placing absorbance on the x axis during data processing

Presented here is a hypothetical example that illustrates why it is more convenient to use the y axis for protein concentration. Consider the data used to generate Figure 3.4.2 and listed in Table 3.4.2. If this data is plotted with the protein (µg) data on the x axis, the standard curve has a curve fit line with the equation $y = 0.0065x + 0.0001$ (Fig. 3.4.6). The x values are micrograms of protein and the y values are A_{590} . Thus, the equation can be rewritten as follows to be more intuitive:

$$[A_{590}] = 0.0065x[\mu\text{g protein}] + 0.0001$$

The measurement taken is the absorbance of the sample, therefore, to calculate the micrograms of protein in the unknown sample, the equation is rearranged as follows:

$$[\mu\text{g protein}] = ([A_{590}] - 0.0001)/0.0065$$

While not a difficult rearrangement, it could introduce errors into the calculation of the amount of protein by an inexperienced laboratory worker.

Now consider the same data, rearranged so that the protein (μg) data is on the y axis as in Figure 3.4.2. A linear fit of this data produces the equation $y = 153.06x + 0.179$. The x values this time are A_{590} , whereas the y values are micrograms of protein. Thus, the equation becomes

$$[\mu\text{g protein}] = (153.06 \times [A_{590}]) + 0.179$$

Now the equation need not be rearranged before use. The advantage of plotting standard curves with the protein amounts on the y axis is especially apparent when using a nonlinear fit as with the Bradford assay (Fig. 3.4.4). In the case where a spreadsheet is being used to calculate standard curve and experimental data, the line-fit equation can be directly copied from the graph and pasted into the spreadsheet formula, thus simplifying the entire process of analyzing data.

Considerations for Processing Data with Spreadsheet Software

There are numerous spreadsheet software packages that can be used for processing data from a standard curve. Some general considerations are presented here for preparing the data using these software packages.

Standard curve generation

Standard curves should be produced using a scatter plot. When possible, plot each data point individually (see examples in Figs. 3.4.2, 3.4.4, and 3.4.5). This will allow the curve-fitting algorithm to fit each data point and for the curve fit to reflect the real variation in the data points. As mentioned above, the y axis should be concentration values and the x axis should be absorbance values, as this will allow the curve fit to directly return a function without having to rearrange the equation. Note that many spreadsheet programs allow calculation of a curve fit with a function in the spreadsheet from the set of standard data. For experienced users, this is a convenient shortcut and allows the production of template spreadsheets for data analysis. For inexperienced protein assay users, it is recommended to produce a graphical plot with a curve fit to avoid errors in data analysis and to have a visual check that there has not been an error in entering the data.

If performing the Bradford assay, be sure to use a polynomial curve fit as the Bradford assay is a nonlinear assay (see Fig. 3.4.4). The BCA and Lowry assays both perform linearly, therefore, a linear curve fit is acceptable (Figs. 3.4.2 and 3.4.5).

Unknowns within standard curve range

A common error made by novices is the utilization of sample data points that do not fit within their standard curves to extrapolate results. To make valid use of a standard curve, data points must be within the linear range of the spectrophotometer (see Spectrophotometry). When unknowns are compared to a standard curve in this way, it is termed interpolation. Avoid extrapolating results in cases where no standard curve data points are in the range of the unknown to support the value that has been determined.

Furthermore, the response range of the assay (which is listed with each assay) is considerably narrower than the range of the spectrophotometer. It is generally best to have at least two standard curve data points on either side of the results for the unknowns. This

**SUPPORT
PROTOCOL 2**

may require pilot assays to ensure the addition of the proper amounts or to properly dilute the sample into the correct range. To find the proper range, Basic Protocol 4 describes a crude, but rapid, estimation of the protein concentration by UV spectrophotometry.

ACETONE PRECIPITATION OF PROTEIN

Often, the easiest way to remove interfering non-proteinaceous substances from protein preparations is to precipitate the protein prior to the assay. In this case, two standard curves should be performed. The first standard curve is prepared from a dilution series of standards that are precipitated under similar conditions as the unknowns, and a second standard curve is prepared without precipitation to allow estimation of the amount of sample lost during the precipitation. It is best to precipitate identical points on the standard curve dilution, however, this is time consuming. Alternatively, a few select points covering the entire range of the standard curve can be precipitated and then compared to the results of the nonprecipitated standard points. For example, in the Lowry assay described in Table 3.4.2 points at 10, 30, and 50 μg of BSA would be precipitated and compared to nonprecipitated standards in a compatible buffer. Acetone precipitation is the easiest method and is the most compatible with downstream assays.

Materials

100% acetone, -20°C
Protein sample
Protein assay-compatible buffer
 -20°C freezer
1.5-ml microcentrifuge tubes (acetone-compatible, not polycarbonate)
Microcentrifuge

1. Add cold 100% acetone to the protein sample to a final concentration of 80% (v/v) (e.g., 0.2 ml diluted protein and 0.8 ml acetone) in acetone-compatible microcentrifuge tubes.
2. Vortex the sample vigorously.
3. Incubate 1 hr at -20°C in an explosion-proof freezer.
4. Pellet the protein by centrifuging 20 min at $15,000 \times g$, 4°C .

CAUTION: Acetone is flammable. Do not scale this precipitation up to volumes >1.5 ml.

5. Carefully decant the supernatant and dispose as organic waste, being careful not to dislodge the pellet.
6. Dry the pellet 20 to 30 min at room temperature by inverting the tube onto a clean paper towel or lint-free wipe. Care should be taken not to dislodge the pellet. Do not over-dry the pellet as this may make it difficult to resuspend.
7. Resuspend the pellet in a protein assay-compatible buffer by vortexing.

TCA PRECIPITATION OF PROTEIN

Acetone precipitation (see Support Protocol 2) is the easiest method and is most compatible with downstream assays, however, a large protein sample may require an impractically large volume to bring the concentration of acetone up to 80%. In these cases, a second protocol utilizing trichloroacetic acid (TCA) precipitation of protein is provided. In TCA precipitation, it is critical that excess TCA be removed before assaying the protein.

CAUTION: TCA is caustic and will burn the skin, therefore, proper precautions must be taken when handling this reagent.

**SUPPORT
PROTOCOL 3**

**Assays for
Determination of
Protein
Concentration**

3.4.22

Materials

80% (w/v) TCA
Protein sample
80% acetone, -20°C
Protein assay-compatible buffer
 -20°C freezer
1.5-ml centrifuge tubes (acetone-compatible, not polycarbonate)
Microcentrifuge

1. Wearing gloves, add TCA to the protein sample to a final concentration of 4%.
2. Incubate 30 min on ice.
3. Centrifuge 10 min at $15,000 \times g$, 4°C .
4. Decant the supernatant and dispose as organic waste, being careful not to dislodge the pellet.
5. Add an equal volume of 80% cold acetone to the pellet to wash away residual TCA. Vortex vigorously to release the pellet from the bottom of the centrifuge tube.
6. Centrifuge 10 min at $15,000 \times g$, 4°C and repeat 80% acetone wash four additional times.
7. Dry the pellet inverted on a paper towel or lint-free wipe for 30 min being careful not to dislodge or over-dry the pellet.
8. Resuspend the pellet in a protein assay-compatible buffer by vigorous vortexing.

ALKYLATION OF REDUCTANT AND BCA ASSAY OF PROTEIN SAMPLES IN SDS-PAGE LOADING BUFFER

ALTERNATE PROTOCOL 3

One of the most common problems encountered when performing polyacrylamide gel electrophoresis (PAGE; Chapter 10) and immunoblot analysis (*UNITS 10.7 & 10.8*) of protein extracts is how to load equal amounts of samples for semi-quantitative comparison. All too often researchers load SDS-PAGE gels based on tissue weight before homogenization, but this can lead to significant sample-to-sample variation. Also, some researchers will stain a duplicate gel and compare one or two bands between the gels and state that they are equally loaded. When investigating changes in protein composition between two samples, it is preferable to load based on total protein concentration.

Many vendors now offer detergent- and reductant-compatible protein assays to quantitate samples prior to loading of gels for electrophoretic fractionation. These assays are usually more expensive than normal assay kits and either rely on a method similar to what follows, or on protein precipitation prior to an assay such as the Lowry assay (e.g., BioRad RC/DC). One critical factor to this assay is that bromophenol blue should not be added to the SDS-PAGE sample buffer as it may interfere. It is preferable to prepare SDS-PAGE loading buffer as a $6\times$ stock without bromophenol blue and then, if desired, add bromophenol blue from a 10 mg/ml stock to the sample prior to gel loading. Once the amount of sample that is required from each unknown for equal loading is determined, it is critical that all of the samples are diluted to the same volume. If this is not done, band broadening (for high volume samples) and band narrowing (for small volume samples) during the stacking phase of electrophoretic fractionation may be observed.

This assay (Hill and Straka, 1988) is a two-step procedure and takes advantage of the fact that the BCA assay is compatible with SDS up to concentrations of 5%. The $6\times$ SDS-PAGE loading buffer described in *UNIT 10.1* results in a 1% final concentration of SDS

Detection and Assay Methods

3.4.23

in the sample. However, it also contains DTT or 2-mercaptoethanol (2-ME) at greater than millimolar concentrations, which is not compatible with the assay. Thus, prior to the assay, the thiol groups of DTT (or 2-ME) must be alkylated with iodoacetamide to form an S-carboxymethyl derivative of DTT or 2-ME. Iodoacetamide does not interfere with the BCA assay, nor does alkylated DTT or 2-ME, therefore, after the iodoacetamide treatment, samples may be assayed directly in the BCA assay (see Basic Protocol 3 or Alternate Protocol 2).

Finally, two standard curves should be performed, especially when starting out with this procedure. Samples for one standard curve will be suspended in SDS-PAGE sample buffer while the others will be in water and will not be treated with iodoacetamide. This will ensure that the addition of the SDS-PAGE loading buffer has not interfered with the BCA assay, and it will also confirm a negligible difference in BCA assay response in the presence of SDS-PAGE loading buffer. In the authors' experience, little detectable difference has been found between samples prepared in SDS-PAGE sample buffer compared to those in water. It is appropriate to include both water blanks and buffer blanks in this assay. The spectrophotometer should be allowed to warm up and then zeroed with the water blank before recording the A_{562} of the buffer blank so it can be subtracted from the standard curve containing SDS-PAGE loading buffer.

Materials

Stock protein standards

6× SDS-PAGE loading dye without bromophenol blue (*UNIT 10.1*; see recipe)

Unknowns

20 mM iodoacetamide solution (see recipe)

Additional reagents and equipment for BCA assay (see Basic Protocol 3)

1. Dilute stock protein standard to 100 μ l final volume of water and 100 μ l final volume of 1× SDS-PAGE loading buffer (without bromophenol blue)
2. Prepare unknowns in a final volume of 100 μ l with 1× SDS-PAGE loading buffer without bromophenol blue.

Make sure samples have been homogenized, heated, and centrifuged prior to assaying for total protein.

3. Add 100 μ l of 20 mM iodoacetamide solution to the sample.
4. Incubate 20 min at 37°C.
5. Proceed to Basic Protocol 3 by adding working reagent directly to the sample.
6. See Support Protocol 1 for data analysis.

REAGENTS AND SOLUTIONS

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

Bradford reagent

To 50 ml of 95% ethanol, add 100 mg Coomassie Brilliant Blue G-250 (CBBG).

When the CBBG is partially dissolved, add 100 ml 85% (w/v) phosphoric acid and stir until CBBG is completely dissolved. Let the solution stir overnight to assure maximal dissolution. Dilute to 1 liter with water. Store up to 1 month at room temperature or 3 months at 4°C.

continued

Prior to use, filter enough reagent for the day's work through Whatman no. 1 filter paper.

While it is possible to make the reagent in the laboratory, due to the use of phosphoric acid, it is more cost effective to purchase a commercial reagent. Commercial reagents also do not need to be filtered prior to use. Note that different brands of commercial reagent (e.g., BioRad or Sigma) have different concentrations and thus different volumes are required.

Bromophenol blue stock solution

10 mg/ml bromophenol blue in water

Bromophenol blue is not very soluble in water, therefore, allow the solution to settle prior to use. Store up to 1 year at room temperature.

Folin-Ciocalteu reagent

Dilute purchased reagent (e.g., Sigma F9252) with an equal volume of water to prepare the desired volume. Prepare diluted reagent fresh daily.

Iodoacetamide solution

20 mM iodoacetamide (2-iodoacetamide) in 100 mM Tris·Cl, pH 8.0

Prepare fresh daily

Any buffer compatible with the BCA assay may be used in lieu of Tris·Cl, pH 8.0, as long as the pH is near physiological pH.

Lowry alkaline reagent

2 g NaOH (0.1 M)

10 g Na₂CO₃

2.5 g sodium tartrate (use of potassium salt will cause SDS to be insoluble)

12.5 g sodium dodecylsulfate

Bring to a final volume of 500 ml with water

Store in a plastic bottle up to 3 months at room temperature and check that it is optically clear before using. If the solution is cloudy or particulates are visible, prepare a new batch.

Lowry assay mix

50 ml Lowry alkaline reagent (see recipe) and 0.5 ml Lowry copper reagent (see recipe)

Prepare fresh daily

Lowry copper reagent

1 g CuSO₄·5H₂O

Bring to a final volume of 100 ml with water

Store up to 1 year at room temperature

Macro-BCA assay reagent A

1 g sodium bicinchoninate (BCA)

2 g sodium carbonate

0.16 g sodium tartrate

0.4 g NaOH

0.95 g sodium bicarbonate

Adjust the pH to 11.25 with 10 M NaOH.

Bring to 100 ml with distilled water

Store up to 1 year at room temperature

Macro-BCA assay reagent B

0.4 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
Bring to 10 ml with distilled water
Store for 1 year at room temperature

Macro-BCA assay solution

50:1 (v/v) macro-BCA assay reagent A/macro-BCA assay reagent B (see recipes)
Make fresh daily

Micro-BCA assay reagent A

8 g sodium carbonate monohydrate
1.6 g sodium tartrate
Adjust the pH to 11.25 with 10 M NaOH
Bring to 100 ml with distilled water
Store up to 1 year at room temperature

Micro-BCA assay reagent B

4 g BCA in 100 ml distilled water
Store up to 1 year at room temperature

Micro-BCA assay reagent C

0.4 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 10 ml water.
Store up to 1 year at room temperature

Micro-BCA assay solution

25:25:1 (v/v/v) micro-assay reagent A/micro-assay reagent B/micro-assay reagent C (see recipes)
Prepare fresh daily

SDS-PAGE loading buffer without bromophenol blue, 6×

7 ml 0.5 M Tris·Cl, 0.4% SDS, pH 6.8
3.0 ml glycerol
1 g SDS
0.93 g DTT
Add water to 10 ml
Store in small aliquots at -70°C , do not re-freeze

Do not add bromophenol blue until after the BCA assay has been performed.

COMMENTARY**Critical Parameters and Troubleshooting**

In addition to sample composition (see Strategic Planning) and proper standard curve production (see Support Protocol 1) there are several other elements for a successful protein assay. Be sure to use clean glassware since it is difficult to get accurate results using dirty glassware. For the methods described in this unit, it is recommended to use new glass tubes and discard them after use. High-quality detergents and distilled water should be used to clean glassware. In some cases, such as the Bradford assay, it may be more convenient

to use disposable plastic cuvettes, rather than those made from Pyrex or quartz, because the assay reagents may leave a residue. If a cuvette does not look clean, it should be first washed with soap and water. If the residue remains, a pipe cleaner works well for cleaning cuvettes. Bend the pipe cleaner in the middle and use the bent end to scrub the inside of the cuvette, being careful not to use the other end of the pipe cleaner as the wires will scratch the optical surfaces of the cuvette. If this is not sufficient to remove residues from cuvettes, try soaking in 50% nitric acid overnight. Also, a wash with ethanol or methanol often works well

to remove contaminating materials. Cuvettes should be thoroughly washed with distilled water between determinations. While carrying out absorbance determinations, hold the filled cuvette up and look through it to determine that the solution is optically clear and without precipitates or floaters.

Spectrophotometry

Spectrophotometry used in the protein assays presented in this unit either relies on the presence of a metal (e.g., Cu^{2+} in the BCA and Lowry assays) or involves a system of conjugated double bonds (e.g., CBBG in the Bradford assay) to absorb photons produced by the spectrophotometer. Spectrophotometry is advantageous in that it is a nondestructive method. Because different molecules have different absorption characteristics, a particular compound in a mixture can often be singled out for observation. Most importantly, highly accurate measurements can be made in a short period of time.

Lambert-Beer (or Beer-Lambert) equation

The Beer-Lambert law of absorption states that the fraction of light absorbed is (1) proportional to the thickness of the absorbing solution, (2) proportional to the concentration of absorbing species, and (3) independent of the light intensity. Mathematically presented as follows:

The Beer-Lambert equation:
$$A = -\log(I/I_0) = \epsilon cl$$

where ϵ is the molar extinction coefficient or molar absorptivity and usually has units of $\text{M}^{-1} \text{cm}^{-1}$, except as noted above (see Basic Protocol 4 introduction) for proteins based on a mass percent. If I_0 = intensity of the light entering a colored solution; I = intensity of light leaving the solution; c = concentration; l = length of the light path (pathlength) through the solution; then $-\log(I/I_0)$ is proportional to both the pathlength and the concentration. The term $-\log(I/I_0)$ is called the absorbance (A) of the solution.

Extinction coefficients

The extinction coefficient is a proportionality constant that relates absorbance to the concentration and pathlength.

$$\epsilon = A/(cl)$$

Absorbance is a dimensionless quantity (essentially a ratio). Pathlength is usually 1 cm. Notice that ϵ is a value true for only the wavelength at which it is defined. The same

molecule at different wavelengths has different molar absorptivities.

Percent transmittance

Many spectrophotometers are capable of measuring both absorbance, A , and percent transmittance, $\%T$.

Transmittance is defined as I/I_0 and is not directly proportional to the absorbance. Absorbance is the $-\log$ of the transmittance. Values of transmittance normally vary from 0 to 1.0.

Percent transmittance is defined as:
$$\%T = (I/I_0) \times 100.$$

Percent transmittance is helpful for zeroing the spectrophotometer prior to use. With a buffer blank, the $\%T$ should read 100%, and with the light beam blocked, it should read 0%.

There is a practical limit on the absorbance that can be measured in a spectrophotometer. An absorbance of 1 means that the transmittance is 0.10 and that only 10% of the light is reaching the light-measuring photodiode. An absorbance of 2 means that the transmittance is 0.01 and only 1% of the light is passing through the sample. An absorbance of 3 means a transmittance of 0.001 and 0.1% of the light is passing through the sample. Good spectrophotometers can measure absorbance values up to 3. Less sophisticated spectrophotometers that are more commonly found in laboratories can generally only measure absorbance to 2 or 2.5 before there is so little light being measured that the signal-to-noise ratio deteriorates. For the purposes of protein assays, keeping the absorbance range between 0.05 and 2.0 is recommended.

Zeroing and blanks

The use of the jargon “zero” and “blank” is often confusing and sometimes used interchangeably. Here, “zero” is used as a verb to mean the act of setting the absorbance of a spectrophotometer readout to a value of 0.0. Obviously, one could zero the spectrophotometer without a cuvette in the sample holder; this is not normally done and would be referred to as zeroing with air. It is much more common to zero the spectrophotometer with a cuvette in the sample holder. The cuvette used to zero the spectrophotometer is called a blank. If a cuvette containing only water is used, it is called a water blank. If a cuvette containing water instead of sample, plus the assay reagents, is used to zero the spectrophotometer, it is referred to as a reagent blank.

If a cuvette containing the buffer components found in the sample to be measured plus the assay reagents is used, this is called a buffer blank. It is important to keep these differences in mind, since they are not equivalent and can lead to misinterpretation and different results.

Quantitative amino acid analysis

The assays described in this unit have different sensitivities for proteins with different amino acid compositions. This is often not a significant issue for complex protein extracts, but becomes significant for purified proteins. Typically, amino acid analysis is performed by a service facility. An increasing number of companies will perform this service at a reasonable cost. Most service companies will be able to provide the precise protein concentration, and these results can be compared to results from one or all of the assays presented in this unit.

Anticipated Results

Tables 3.4.2 to 3.4.4 demonstrate typical results for the Lowry, Bradford, and BCA assays, respectively. These tables can also be used as assay and spreadsheet templates and are of particular value to new assay users.

Time Considerations

Basic Protocols 1 and 3 (the Lowry and BCA assays) can easily be completed and data collected in 1 to 1.5 hr. The Bradford assay (see Basic Protocol 2) typically can be completed in <1 hr. UV absorbance (see Basic Protocol 1) is one of the quickest methods and data can be acquired in <30 min. Users unfamiliar with data analysis of protein assay data can anticipate requiring ~30 min to 1 hr to analyze data. Once familiar with the data analysis process, it is typically completed in 10 to 20 min, especially if using the spreadsheet templates in Tables 3.4.2 to 3.4.4. If sample preparation such as precipitation is required prior to assay, this will typically add 1 hr to analysis time.

The high-throughput adaptations can be completed in a similar amount of time as the regular assays for approximately five microtiter plates. Additional microtiter plates only add a few minutes to analysis time. These times can be significantly shortened and the number of samples scaled up by the utilization of multichannel and repeating pipettors. For large-scale operations, these assays will scale nicely with robotics. Many microtiter plate readers also come with software that can

easily automate both data acquisition and analysis. Typically, the high-throughput methods are the best strategies to use if the assay is compatible with the sample, as they are quick, reliable, and allow acquisition of more data points per sample.

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