INSTRUCTIONS

Pierce® BCA Protein Assay Kit

23225 23227

1296.5

Number	Description
23225	**Pierce BCA Protein Assay Kit**, sufficient reagents for 500 test-tube or 5,000 microplate assays
23227	**Pierce BCA Protein Assay Kit**, sufficient reagents for 250 test-tube or 2,500 microplate assays

Kit Contents:

BCA Reagent A, 1,000 ml (in Product No. 23225) or 500 ml (in Product No. 23227), containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide

BCA Reagent B, 25 ml, containing 4% cupric sulfate

Albumin Standard Ampules, 2 mg/ml, 10 × 1 ml ampules, containing bovine serum albumin (BSA) at 2.0 mg/ml in 0.9% saline and 0.05% sodium azide

Storage: Upon receipt store at room temperature. Product shipped at ambient temperature.

Note: If either Reagent A or Reagent B precipitates upon shipping in cold weather or during long-term storage, dissolve precipitates by gently warming and stirring solution. Discard any kit reagent that shows discoloration or evidence of microbial contamination.

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Introduction

The Pierce BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid.[1] The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2,000 µg/ml). The BCA method is not a true end-point method; that is, the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together.

The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA.[2] Studies with di-, tri- and tetrapeptides suggest that the extent of color formation caused by more than the mere sum of individual color-producing functional groups.[2] Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). A series of dilutions of known concentration are prepared from the protein and assayed alongside the unknown(s) before the concentration of each unknown is determined based on the standard curve. If precise quantitation of an unknown protein is required, it is advisable to select a protein

standard that is similar in quality to the unknown; for example, a bovine gamma globulin (BGG) standard (see Related Pierce Products) may be used when assaying immunoglobulin samples.

Two assay procedures are presented. Of these, the Test Tube Procedure requires a larger volume (0.1 ml) of protein sample; however, because it uses a sample to working reagent ratio of 1:20 (v/v), the effect of interfering substances is minimized. The Microplate Procedure affords the sample handling ease of a microplate and requires a smaller volume (10-25 µl) of protein sample; however, because the sample to working reagent ratio is 1:8 (v/v), it offers less flexibility in overcoming interfering substance concentrations and obtaining low levels of detection.

Preparation of Standards and Working Reagent (required for both assay procedures)

A. Preparation of Diluted Albumin (BSA) Standards

Use Table 1 as a guide to prepare a set of protein standards. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as the sample(s). Each 1 ml ampule of 2.0 mg/ml Albumin Standard is sufficient to prepare a set of diluted standards for either working range suggested in Table 1. There will be sufficient volume for three replications of each diluted standard.

Table 1. Preparation of Diluted Albumin (BSA) Standards

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20–2,000 µg/ml)			
Vial	**Volume of Diluent**	**Volume and Source of BSA**	**Final BSA Concentration**
A	0	300 µl of Stock	2,000 µg/ml
B	125 µl	375 µl of Stock	1,500 µg/ml
C	325 µl	325 µl of Stock	1,000 µg/ml
D	175 µl	175 µl of vial B dilution	750 µg/ml
E	325 µl	325 µl of vial C dilution	500 µg/ml
F	325 µl	325 µl of vial E dilution	250 µg/ml
G	325 µl	325 µl of vial F dilution	125 µg/ml
H	400 µl	100 µl of vial G dilution	25 µg/ml
I	400 µl	0	0 µg/ml = Blank
Dilution Scheme for Enhanced Test Tube Protocol (Working Range = 5–250 µg/ml)			
Vial	**Volume of Diluent**	**Volume and Source of BSA**	**Final BSA Concentration**
A	700 µl	100 µl of Stock	250 µg/ml
B	400 µl	400 µl of vial A dilution	125 µg/ml
C	450 µl	300 µl of vial B dilution	50 µg/ml
D	400 µl	400 µl of vial C dilution	25 µg/ml
E	400 µl	100 µl of vial D dilution	5 µg/ml
F	400 µl	0	0 µg/ml = Blank

B. Preparation of the BCA Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:

   (# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required

   Example: for the standard test-tube procedure with 3 unknowns and 2 replicates of each sample:

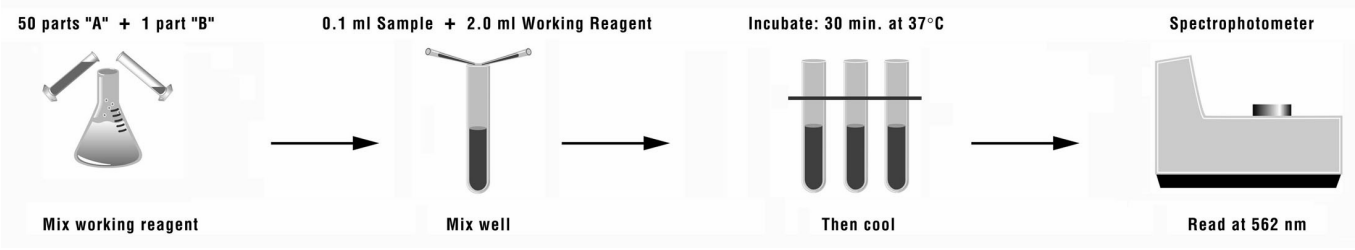
   (9 standards + 3 unknowns) × (2 replicates) × (2 ml) = 48 ml WR required

   Note: 2.0 ml of the WR is required for each sample in the test-tube procedure, while only 200 µl of WR reagent is required for each sample in the microplate procedure.

2. Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). For the above example, combine 50 ml of Reagent A with 1 ml of Reagent B.

   Note: When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).

Procedure Summary (Test-tube Procedure, Standard Protocol)



Test-tube Procedure (Sample to WR ratio = 1:20)

1. Pipette 0.1 ml of each standard and unknown sample replicate into an appropriately labeled test tube.
2. Add 2.0 ml of the WR to each tube and mix well.
3. Cover and incubate tubes at selected temperature and time:
   - Standard Protocol: 37°C for 30 minutes (working range = 20-2,000 µg/ml)
   - RT Protocol: RT for 2 hours (working range = 20-2,000 µg/ml)
   - Enhanced Protocol: 60°C for 30 minutes (working range = 5-250 µg/ml)

   Notes:
   - Increasing the incubation time or temperature increases the net 562 nm absorbance for each test and decreases both the minimum detection level of the reagent and the working range of the protocol.
   - Use a water bath to heat tubes for either Standard (37°C incubation) or Enhanced (60°C incubation) Protocol. Using a forced-air incubator can introduce significant error in color development because of uneven heat transfer.
4. Cool all tubes to RT.
5. With the spectrophotometer set to 562 nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples within 10 minutes.

   Note: Because the BCA assay does not reach a true end point, color development will continue even after cooling to RT. However, because the rate of color development is low at RT, no significant error will be introduced if the 562 nm absorbance measurements of all tubes are made within 10 minutes of each other.
6. Subtract the average 562 nm absorbance measurement of the Blank standard replicates from the 562 nm absorbance measurement of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

Microplate Procedure (Sample to WR ratio = 1:8)

1. Pipette 25 µl of each standard or unknown sample replicate into a microplate well (working range = 20-2,000 µg/ml).

   Note: If sample size is limited, 10 µl of each unknown sample and standard can be used (sample to WR ratio = 1:20). However, the working range of the assay in this case will be limited to 125-2,000 µg/ml.
2. Add 200 µl of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate and incubate at 37°C for 30 minutes.
4. Cool plate to RT.
5. Measure the absorbance at or near 562 nm on a plate reader.

   Notes:
   - Wavelengths from 540-590 nm have been used successfully with this method.
   - Because plate readers use a shorter light path length than cuvette spectrophotometers, the Microplate Procedure requires a greater sample to WR ratio to obtain the same sensitivity as the standard Test Tube Procedure. If higher 562 nm measurements are desired, increase the incubation time to 2 hours.

- Increasing the incubation time or ratio of sample volume to WR increases the net 562 nm measurement for each well and lowers both the minimum detection level of the reagent and the working range of the assay. As long as all standards and unknowns are treated identically, such modifications may be useful.

6. Subtract the average 562 nm absorbance measurement of the Blank standard replicates from the 562 nm measurements of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

   Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points.

Troubleshooting

Problem	Possible Cause	Solution
No color in any tubes	Sample contains a copper chelating agent	Dialyze, desalt, or dilute sample Increase copper concentration in working reagent (e.g., use 50:2, Reagent A:B) Remove interfering substances from sample using Product No. 23215
Blank absorbance is OK, but standards and samples show less color than expected	Strong acid or alkaline buffer, alters working reagent pH	Dialyze, desalt, or dilute sample
	Color measured at the wrong wavelength	Measure the absorbance at 562 nm
Color of samples appears darker than expected	Protein concentration is too high	Dilute sample
	Sample contains lipids or lipoproteins	Add 2% SDS to the sample to eliminate interference from lipids[3] Remove interfering substances from sample using Product No. 23215
All tubes (including blank) are dark purple	Buffer contains a reducing agent	Dialyze or dilute sample Remove interfering substances from sample using Product No. 23215
	Buffer contains a thiol	
	Buffer contains biogenic amines (catecholamines)	
Need to measure color at a different wavelength	Spectrophotometer or plate reader does not have 562 nm filter	Color may be measure at any wavelength between 540 nm and 590 nm, although the slope of standard curve and overall assay sensitivity will be reduced

A. Interfering substances

Certain substances are known to interfere with the BCA assay including those with reducing potential, chelating agents, and strong acids or bases. Because they are known to interfere with protein estimation at even minute concentrations, avoid the following substances as components of the sample buffer:

Ascorbic Acid	EGTA	Iron	Impure Sucrose
Catecholamines	Impure Glycerol	Lipids	Tryptophan
Creatinine	Hydrogen Peroxide	Melibiose	Tyrosine
Cysteine	Hydrazides	Phenol Red	Uric Acid

Other substances interfere to a lesser extent with protein estimation using the BCA assay, and these have only minor (tolerable) effects below a certain concentration in the original sample. Maximum compatible concentrations for many substances in the Standard Test Tube Protocol are listed in Table 2 (see last page of Instructions). Substances were compatible at the indicated concentration in the Standard Test Tube Protocol if the error in protein concentration estimation caused by the presence of the substance in the sample was less than or equal to 10%. The substances were tested using WR prepared immediately before each experiment. Blank-corrected 562 nm absorbance measurements (for a 1,000 µg/ml BSA standard + substance) were compared to the net 562 nm measurements of the same standard prepared in 0.9% saline. In the Microplate Procedure, where the sample to WR ratio is 1:8 (v/v), maximum compatible concentrations will be lower.

B. Strategies for eliminating or minimizing the effects of interfering substances

The effects of interfering substances in the Pierce BCA Protein Assay may be eliminated or overcome by one of several methods.

- Remove the interfering substance by dialysis or gel filtration.
- Dilute the sample until the substance no longer interferes. This strategy is effective only if the starting protein concentration is sufficient to remain in the working range of the assay upon dilution.
- Precipitate the proteins in the sample with acetone or trichloroacetic acid (TCA). The liquid containing the substance that interfered is discarded and the protein pellet is easily solubilized in ultrapure water or directly in the alkaline BCA WR.[4] A protocol detailing this procedure is available from our website. Alternatively, Product No. 23215 may be used (see Related Pierce Products).
- Increase the amount of copper in the WR (prepare WR as 50:2 or 50:3, Reagent A:B), which may eliminate interference by copper chelating agents.

Note: For greatest accuracy, the protein standards must be treated identically to the sample(s).

Related Products

23209	**Albumin Standard Ampules, 2 mg/ml**, 10 × 1 ml ampules, containing bovine serum albumin (BSA)
23208	**Pre-Diluted Protein Assay Standards: Bovine Serum Albumin (BSA) Set**, 7 × 3.5 ml
23212	**Bovine Gamma Globulin Standard, 2 mg/ml**, 10 × 1 ml ampules
23213	**Pre-Diluted Protein Assay Standards**, Bovine Gamma Globulin Fraction II (BGG) Set, 7 × 3.5 ml aliquots in the range of 125-2,000 µg/ml
23235	**Pierce Micro BCA Protein Assay Kit**, working range of 0.5-20 µg/ml
23236	**Coomassie Plus - The Better Bradford™ Assay Kit**, working range of 1-1,500 µg/ml
23215	**Compat-Able™ Protein Assay Preparation Reagent Set**, sufficient reagents to pre-treat 500 samples to remove interfering substances before total protein quantitation
23250	**Pierce BCA Protein Assay Kit–Reducing Agent Compatible**

Additional Information

A. Please visit our website for additional information including the following items:

- Frequently Asked Questions
- Tech Tip protocol: Eliminate interfering substances from samples for BCA Protein Assay

B. Alternative Total Protein Assay Reagents

If interference by a reducing substance or metal-chelating substance contained in the sample cannot be overcome, try the Coomassie Plus - The Better Bradford Assay Kit (Product No. 23236), which is less sensitive to such substances.

C. Cleaning and Re-using Glassware

Exercise care when re-using glassware. All glassware must be cleaned and given a thorough final rinse with ultrapure water.

D. Response characteristics for different proteins

Each of the commonly used total protein assay methods exhibits some degree of varying response toward different proteins. These differences relate to amino acid sequence, pI, structure and the presence of certain side chains or prosthetic groups that can dramatically alter the protein's color response. Most protein assay methods use BSA or immunoglobulin (IgG) as the standard against which the concentration of protein in the sample is determined (Figure 1). However, if great accuracy is required, the standard curve should be prepared from a pure sample of the target protein to be measured.

Typical BCA Protein Assay protein-to-protein variation in color response is listed in Table 3. All proteins were tested at a concentration of 1,000 µg/ml using the 30-minute/37°C Test Tube Protocol. The average net color response for BSA was normalized to 1.00 and the average net color response of the other proteins is expressed as a ratio to the response of BSA.

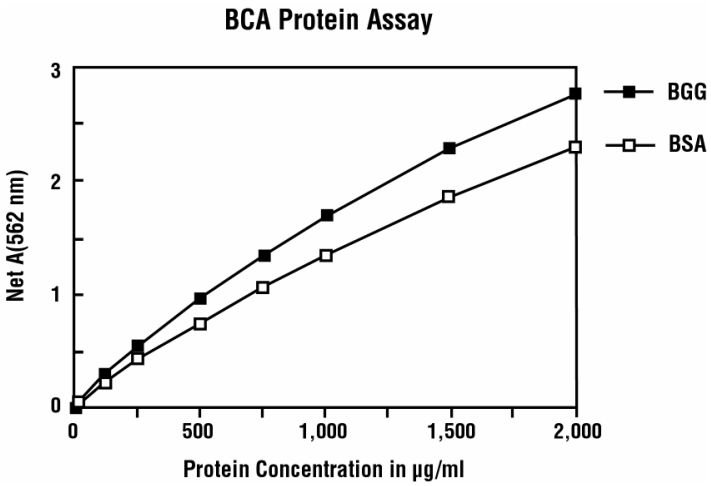


Figure 1: Typical color response curves for BSA and BGG using the Standard Test Tube Protocol (37°C/30-minute incubation).

Table 3. Protein-to-protein variation. Absorbance ratios (562 nm) for proteins relative to BSA using the Standard Test Tube Protocol.

Ratio = (Avg "test" net Abs.) / (avg. BSA net Abs.)

Protein Tested	Ratio
Albumin, bovine serum	1.00
Aldolase, rabbit muscle	0.85
α-Chymotrypsinogen, bovine	1.14
Cytochrome C, horse heart	0.83
Gamma globulin, bovine	1.11
IgG, bovine	1.21
IgG, human	1.09
IgG, mouse	1.18
IgG, rabbit	1.12
IgG, sheep	1.17
Insulin, bovine pancreas	1.08
Myoglobin, horse heart	0.74
Ovalbumin	0.93
Transferrin, human	0.89
	1.02
Standard Deviation	**0.15**
Coefficient of Variation	**14.7%**

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1. Smith, P.K., *et al.* (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem*. **150**:76-85.
2. Wiechelman, K., *et al.* (1988). Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal Biochem*. **175**:231-7.
3. Kessler, R. and Fanestil, D. (1986). Interference by lipids in the determination of protein using bicinchoninic acid. *Anal. Biochem*. **159**:138-42.
4. Brown, R., *et al.* (1989). Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal. Biochem*. **180**:136-9.

Product References

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Prozialeck, W.C., *et al.* (2002). Chlamydia trachomatis disrupts N-cadherin-dependent cell-cell junctions and sequester β-catenin in human cervical epithelial cells. *Infection and Immunity* **70:**2605-13.

Roberts, K.P., *et al.* (2002). A comparative analysis of expression and processing of the rat epididymal fluid and sperm-bound forms of proteins D and E. *Biology of Reproduction* **67:**525-33.

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Table 2. Compatible substance concentrations in the BCA Protein Assay (see text for details).

Substance	Compatible Concentration
Salts/Buffers	
ACES, pH 7.8	25 mM
Ammonium sulfate	1.5 M
Asparagine	1 mM
Bicine, pH 8.4	20 mM
Bis-Tris, pH 6.5	33 mM
Borate (50 mM), pH 8.5 (# 28384)	undiluted
B-PER® Reagent (#78248)	undiluted
Calcium chloride in TBS, pH 7.2	10 mM
Na-Carbonate/Na-Bicarbonate (0.2 M), pH 9.4 (#28382)	undiluted
Cesium bicarbonate	100 mM
CHES, pH 9.0	100 mM
Na-Citrate (0.6 M), Na-Carbonate (0.1 M), pH 9.0 (#28388)	1:8 dilution*
Na-Citrate (0.6 M), MOPS (0.1 M), pH 7.5 (#28386)	1:8 dilution*
Cobalt chloride in TBS, pH 7.2	0.8 mM
EPPS, pH 8.0	100 mM
Ferric chloride in TBS, pH 7.2	10 mM
Glycine•HCl, pH 2.8	100 mM
Guanidine•HCl	4 M
HEPES, pH 7.5	100 mM
Imidazole, pH 7.0	50 mM
MES, pH 6.1	100 mM
MES (0.1 M), NaCl (0.9%), pH 4.7 (#28390)	undiluted
MOPS, pH 7.2	100 mM
Modified Dulbecco's PBS, pH 7.4 (#28374)	undiluted
Nickel chloride in TBS, pH 7.2	10 mM
PBS; Phosphate (0.1 M), NaCl (0.15 M), pH 7.2 (#28372)	undiluted
PIPES, pH 6.8	100 mM
RIPA lysis buffer; 50 mM Tris, 150 mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS, pH 8.0	undiluted
Sodium acetate, pH 4.8	200 mM
Sodium azide	0.2%
Sodium bicarbonate	100 mM
Sodium chloride	1 M
Sodium citrate, pH 4.8 or pH 6.4	200 mM
Sodium phosphate	100 mM
Tricine, pH 8.0	25 mM
Triethanolamine, pH 7.8	25 mM
Tris	250 mM
TBS; Tris (25 mM), NaCl (0.15 M), pH 7.6 (#28376)	undiluted
Tris (25 mM), Glycine (192 mM), pH 8.0 (#28380)	1:3 dilution*
Tris (25 mM), Glycine (192 mM), SDS (0.1%), pH 8.3 (#28378)	undiluted
Zinc chloride in TBS, pH 7.2	10 mM

Substance	Compatible Concentration
Detergents**	
Brij®-35	5.0%
Brij-56, Brij-58	1.0%
CHAPS, CHAPSO	5.0%
Deoxycholic acid	5.0%
Octyl β-glucoside	5.0%
Nonidet P-40 (NP-40)	5.0%
Octyl β-thioglucopyranoside	5.0%
SDS	5.0%
Span® 20	1.0%
Triton® X-100	5.0%
Triton X-114, X-305, X-405	1.0%
Tween®-20, Tween-60, Tween-80	5.0%
Zwittergent® 3-14	1.0%
Chelating agents	
EDTA	10 mM
EGTA	--------
Sodium citrate	200 mM
Reducing & Thiol-Containing Agents	
N-acetylglucosamine in PBS, pH 7.2	10 mM
Ascorbic acid	--------
Cysteine	--------
Dithioerythritol (DTE)	1 mM
Dithiothreitol (DTT)	1 mM
Glucose	10 mM
Melibiose	--------
2-Mercaptoethanol	0.01%
Potassium thiocyanate	3.0 M
Thimerosal	0.01%
Misc. Reagents & Solvents	
Acetone	10%
Acetonitrile	10%
Aprotinin	10 mg/L
DMF, DMSO	10%
DMSO	10%
Ethanol	10%
Glycerol (Fresh)	10%
Hydrazides	--------
Hydrides (Na_2BH_4 or $NaCNBH_3$)	--------
Hydrochloric Acid	100 mM
Leupeptin	10 mg/L
Methanol	10%
Phenol Red	--------
PMSF	1 mM
Sodium Hydroxide	100 mM
Sucrose	40%
TLCK	0.1 mg/L
TPCK	0.1 mg/L
Urea	3 M
o-Vanadate (sodium salt), in PBS, pH 7.2	1 mM

* Diluted with ultrapure water; ** Detergents were tested using Pierce high-purity Surfact-Amps® Products, which have low peroxide content;
-- Dashed-line entry indicates that the material is incompatible with the assay.