INSTRUCTIONS

BCA Protein Assay Kit

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23225

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Product Description

Number	Description
23225	**BCA Protein Assay Kit** Contains sufficient reagents for 500 test tube or 5,000 microwell plate assays. **BCA Reagent A**, *1,000 ml* of reagent containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide. Available separately as Product No. 23223. **BCA Reagent B**, *25 ml* of a solution containing 4% cupric sulfate. Available separately as Product No. 23224. **Albumin Standard**, *10 x 1 ml* ampules containing bovine serum albumin (BSA) at a concentration of 2.0 mg/ml in a solution of 0.9% saline and 0.05% sodium azide. Available separately as Product No. 23209.

When stored at room temperature, the kit has a shelf life of at least 12 months from the date of receipt. Should either Reagent A or Reagent B precipitate upon shipping in cold weather or during long-term storage, dissolve the precipitates by gently warming the solutions while stirring. If any kit reagent shows discoloration or evidence of microbial contamination, discard the reagent.

Note: If you are working with IgG samples, you may prefer our Bovine Gamma Globulin (BGG) Standard (Prod. No. 23212, *10 x 1 ml ampules, 2 mg/ml*). This BGG standard may be diluted and used as described for the BSA standard included in this kit (Table 1).

If you would like to avoid the tedious and time-consuming effort of preparing the diluted protein standards, Pierce also offers both the BSA and BGG standards in sets containing *7 x 3.5 ml bottles* of pre-diluted protein standards covering the range from 125 µg/ml to 2,000 µg/ml. The Pre-Diluted Protein Standards are available separately as Prod. No. 23208 (BSA) or 23213 (BGG).

The BCA-Protein Reaction Scheme

1). **Protein (peptide bonds) + Cu^{+2}** $\xrightarrow{OH-}$ **tetradentate-Cu^{+1} complex**

2). **Cu^{+1} + 2 Bicinchoninic Acid (BCA)** $\longrightarrow$ **BCA-Cu^{+1} complex (purple colored, read at A562)**

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 linear with increasing protein concentrations over a broad working range of 20 µg/ml to 2,000 µg/ml. The BCA method is not a true end-point method – the final color continues to develop but, following incubation, the rate of color development is slowed sufficiently to allow large numbers of samples to be done in a single run. The macromolecular structure of protein, the number of peptide bonds and the presence of four 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 is not simply due to the sum of the contributions of the individual color-producing functional groups.[2]

Preparation

1. Preparation of Diluted BSA Standards

Prepare a fresh set of protein standards by diluting the 2.0 mg/ml BSA stock standard (Stock), preferably in the same diluent as your sample. For a list of standard dilutions, see Table 1. The BSA Standard (1 ml ampule of the 2.0 mg/ml) is sufficient to prepare a set of diluted standards for either working range. There will be sufficient volume for three replications of each diluted BSA standard, if desired.

2. Preparation of the BCA Working Reagent (WR)

To prepare WR, mix 50 parts of BCA Reagent A with 1 part of BCA Reagent B. When BCA Reagent B is initially added to BCA Reagent A, a turbidity is observed that quickly disappears upon mixing to yield a clear-green WR. Prepare sufficient volume of WR based upon the number of tests to be done. Each test tube sample to be done requires 2.0 ml of the WR, while the microwell plate samples require only 200 µl. The WR is stable for at least 1 day when stored in a closed container at room temperature (RT).

The Test Tube Protocol (Sample to WR ratio = 1:21)

1. Pipet 0.1 ml of each standard or unknown sample into appropriately labeled test tubes. Use 0.1 ml of the diluent for the blank tubes.
2. Add 2.0 ml of the WR to each tube, mix well.
3. Incubate all the tubes at the selected temperature and time:
   a. Standard Protocol: 37°C for 30 minutes (working range = 20-2,000 µg/ml)
   b. Room Temp. Protocol: RT for 2 hours (working range = 20-2,000 µg/ml)
   c. Enhanced Protocol: 60°C for 30 minutes (working range = 5-250 µg/ml)

   Note: Increasing the incubation time or temperature increases the net A(562) for each test and decreases both the minimum detection level of the reagent and the working range of the protocol.
4. After incubation, cool all tubes to RT.
5. Measure the absorbance at 562 nm of each tube vs. a water reference.

   Note: Because the BCA Reagent 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, if the A(562) readings of all the tubes can be done in 10 minutes or less, no significant error is introduced.
6. Subtract the average A(562) reading for the blanks from the A(562) reading for each standard or unknown sample.
7. Prepare a standard curve by plotting the average blank corrected A(562) reading for each BSA standard vs. its concentration in µg/ml. Using the standard curve, determine the protein concentration for each unknown sample.

The Microwell Plate Protocol (Sample to WR Ratio = 1:9)*

1. Pipet 25 µl of each standard or unknown sample into the appropriate microwell plate wells. Use 25 µl of the diluent for the blank wells. (working range = 20-2,000 µg/ml)

   * **Note:** If sample size is limited, 10 µl of each unknown sample and standard can be used. However, the working range of the assay will be limited to 125-2,000 µg/ml.
2. Add 200 µl of the WR to each well, mix the plate well on a plate shaker for 30 seconds.
3. Cover the plate and incubate the plate at 37°C for 30 minutes.
4. After incubation, cool the plate to RT.

5. Measure the absorbance at or near 562 nm on a plate reader.

   * **Notes:**

   A. Wavelengths from 540-590 nm have been used successfully with this method.

   B. The Microwell Plate Protocol uses a higher volume ratio of protein sample to WR to obtain the same sensitivity as the Standard Test Tube Protocol. This is due to the shorter light path employed with a plate reader. If higher A(562) readings are required: 1) increase the incubation time to 2 hours or 2) increase the incubation temperature to 60°C.

   C. Increasing the incubation time, incubation temperature or ratio of sample volume to WR increases the net A(562) for each well and lowers both the minimum detection level of the reagent and the working range of the protocol.

6. Subtract the average A(562) reading for the blanks from the A(562) reading for each standard or unknown sample.

7. Prepare a standard curve by plotting the average blank-corrected A(562) reading for each BSA standard vs. its concentration in µg/ml. Using the standard curve, determine the protein concentration for each unknown sample.

Performance Characteristics of the BCA Protein Assay Reagent

Response Curves for BSA and BGG

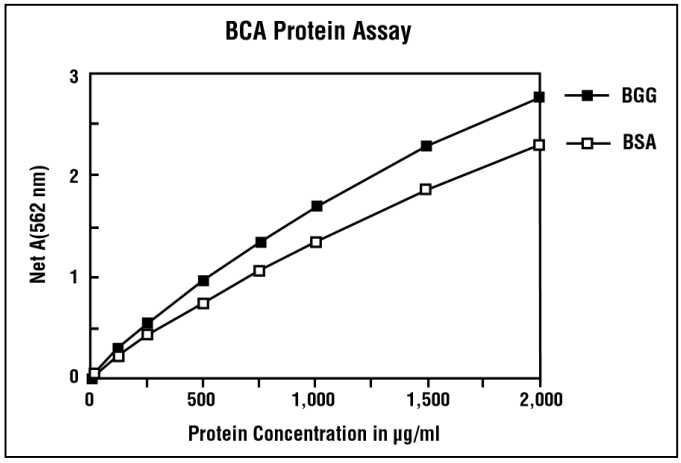

Figure shows the typical linear color response curves for BSA and BGG using the Standard (37°C/30-minute) Test Tube Protocol.

2. Protein-to-protein Variation

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 utilize BSA or immunoglobulin (IgG) as the standard against which the concentration of protein in the sample is determined. Using either of these proteins as the standard works well in most assay methods. However, if great accuracy is required, the standard curve should be prepared from a pure sample of the target protein to be measured.

Table 2 shows typical BCA Protein Assay Reagent protein-to-protein variations in color response. 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.

3. Compatible Substances

The substances listed in Table 3 were found to be compatible with the Standard Tube BCA Protein Assay Protocol if the error in the estimate of the protein concentration (BSA at 1,000 µg/ml) caused by the presence of the substance in the sample was less than or equal to 10%.

The substances in Table 3 were tested over a period of three weeks using freshly prepared WR with each run. The blank corrected A(562) readings (for the 1,000 µg/ml BSA standard + substance) were compared to the net A(562) of the 1,000 µg/ml BSA standard prepared in 0.9% saline.

4. Substances Known to Interfere

The following substances have been reported to interfere with the accurate estimation of protein concentration with the BCA Protein Assay Reagent. They should be avoided as components of the sample buffer.

Ascorbic Acid	Catecholamines	Creatinine	Cysteine	EGTA	Impure Glycerol
Hydrogen peroxide	Hydrazides (Na_2BH_4 and $NaCNBH_3$)		Iron	Lipids	Melibiose
Phenol Red	Impure Sucrose	Tryptophan	Tyrosine	Uric Acid	

Troubleshooting

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

1. The Presence of Incompatible Substances in the Sample

Interference in the BCA Protein Assay may be eliminated or overcome by:

a Removing the interfering substance by dialysis or gel filtration.

b. Diluting the sample to the point that the substance no longer interferes. (This works if the starting protein concentration of the sample is high.)

c. Precipitating 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 directly in the alkaline BCA WR.[15]

d. Increasing the amount of copper in the WR (use 4 ml or even 6 ml of Reagent B/100 ml of Reagent A instead of the 2 ml of Reagent B/100 ml of Reagent A that is called for in the instructions for preparing the BCA WR). This may eliminate interference by copper chelating agents.

Note: For greatest accuracy of the estimate of protein concentration in the sample, the protein standards must be treated identically to the sample.

2. Alternative Total Protein Reagents

Occasionally, interference by a reducing substance or metal chelating substance contained in the sample cannot be overcome easily by any of the above methods. If that occurs, try the Pierce Coomassie® Plus Protein Assay Reagent Kit, Product No. 23236. The Coomassie® Plus Protein Assay Reagent is a modified Bradford formulation. This reagent contains the Coomassie® dye that binds to protein in an acidic medium and produces a blue color that is measured at 595 nm.

3. Reading at Wavelengths other than 562 nm

If a photometer or plate reader with a 562 nm filter is not available, the purple color may be measured at any wavelength between 540 nm and 590 nm.[4,5] The maximum absorbance of the BCA-Cu^{+1} complex occurs at 562 nm. Taking the absorbance measurements at any wavelength other than 562 nm will result in a lower slope for the standard curve and may increase the minimum detection level for the protocol.

4. Cleaning and Re-use of Glassware

Care must be exercised when re-using glassware. The BCA Reagent is sensitive to metal ions, especially copper ions. All glassware must be cleaned and then given a thorough final rinse with high-quality deionized water.

References

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Table 1: Preparation of the Diluted BSA Standards

Standard Test Tube or Microwell Plate Protocol
Working Range = 20 - 2000 µg/ml

Volume of the BSA to Add	Volume of Diluent to Add	Final BSA Concentration
300 µl of (Stock)	0 µl	2000 µg/ml
375 µl of (Stock)	125 µl	1500 µg/ml (A)
325 µl of (Stock)	325 µl	1000 µg/ml (B)
175 µl of (A)	175 µl	750 µg/ml (C)
325 µl of (B)	325 µl	500 µg/ml (D)
325 µl of (D)	325 µl	250 µg/ml (E)
325 µl of (E)	325 µl	125 µg/ml (F)
100 µl of (F)	400 µl	25 µg/ml (G)

Enhanced Test Tube Protocol
Working Range = 5 - 250 µg/ml

Volume of the BSA to Add	Volume of Diluent to Add	Final BSA Concentration
100 µl (Stock)	700 µl	250 µg/ml (a)
400 µl (a)	400 µl	125 µg/ml (b)
300 µl (b)	450 µl	50 µg/ml (c)
400 µl (c)	400µl	25 µg/ml (d)
100 µl (d)	400 µl	5 µg/ml (e)

Table 2: Protein-to-Protein Variation

Protein Tested avg. "test" net Abs. Ratio = ---------- avg. BSA net Abs.	Product No. 23225 BCA @ 562 nm
Albumin, bovine serum	1.00
Aldolase, rabbit muscle	0.85
a-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
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Average Ratio =	1.02
Standard Deviation =	0.15
Coefficient of Variation =	14.7%

Coefficient of Variation =
(standard deviation / average ratio) x 100

Table 3: Substances found to be compatible with the BCA Protein Assay Reagent using the Standard Test Tube Protocol: (Amount listed refers to the actual concentration in the sample.)

Substance	Compatible Concentration
Detergents	
Brij®-35	5.0%
Brij®-56	1.0%
Brij®-58	1.0%
CHAPS	5.0%
CHAPSO	5.0%
Deoxycholic acid	5.0%
Lubrol® PX	1.0%
Octyl b-glucoside	5.0%
Nonidet P-40 (NP-40)	5.0%
Octyl b-thioglucopyranoside	5.0%
SDS	5.0%
Span® 20	1.0%
Triton® X-100	5.0%
Triton® X-114	1.0%
Triton® X-305	1.0%
Triton® X-405	1.0%
Tween® 20	5.0%
Tween® 60	5.0%
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
Melbiose	--------
2-Mercaptoethanol	0.01%
Potassium thiocyanate	3.0 M
Thimerosal	0.01%
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 (Prod.	undiluted
B-PER™ Reagent	undiluted
Calcium chloride in TBS, pH 7.2	10 mM
Na-Carbonate/Na-Bicarbonate (0.2 M), pH 9.4 (Prod. #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 (Prod. #28388)	1:8 dilution*
Na-Citrate (0.6 M), MOPS (0.1 M), pH 7.5 (Prod. #28386)	1:8 dilution*

Substance	Compatible Concentration
Salts/Buffers continued	
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	1 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 (Prod. #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 (Prod. #28376)	undiluted
Tris (25 mM), Glycine (192 mM), pH 8.0 Prod. #28380)	1:3 dilution*
Tris (25 mM), Glycine (192 mM), SDS (0.1%), pH 8.3 (Prod. #28378)	undiluted
Zinc chloride in TBS, pH 7.2	10 mM
Misc. Reagents & Solvents	
Acetone	10%
Acetonitrile	10%
Aprotinin	10 mg/L
DMF	10%
DMSO	10%
Ethanol	10%
Glycerol (Fresh)	10%
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 dH_2O. A blank indicates that the material is incompatible with the assay.