INSTRUCTIONS

PIERCE
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Micro BCA™ Protein Assay Kit

23235

0412.2

Number	Description
23235	**Micro BCA™ Protein Assay Kit**, sufficient reagents for 480 tube assays or 3,200 microplate assays

Kit Contents:

Micro BCA™ Reagent A (MA), 240 ml, contains sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH

Micro BCA™ Reagent B (MB), 240 ml, contains 4% bicinchoninic acid in water

Micro BCA™ Reagent C (MC), 12 ml, contains 4% cupric sulfate, pentahydrate in water

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

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

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

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Introduction

The Micro BCA™ Protein Assay Kit is a detergent-compatible bicinchoninic acid formulation for the colorimetric detection and quantitation of total protein. An adaptation of the BCA Protein Assay Kit (Product No. 23225), the Micro BCA™ Kit has been optimized for use with dilute protein samples (0.5-20 µg/ml). The unique, patented method utilizes bicinchoninic acid (BCA) as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment.[1] A purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}). This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations.

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 caused by more than the mere sum of individual color-producing functional groups.[2]

The Micro BCA™ Protein Assay Kit uses concentrated reagents and a protocol that utilizes an extended incubation time at an elevated temperature (60°C, Test Tube Procedure only). The result is an extremely sensitive colorimetric protein assay in a test tube or microplate assay format.

Preparation of Standards and Working Reagent

A. Preparation of Diluted Albumin (BSA) Standards

Use Table 1 as a guide to prepare a fresh set of protein standards. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as your sample. Each 1 ml ampule of 2.0 mg/ml Albumin Standard is sufficient to prepare a set of diluted standards such that three replicates of each dilution may be included in the Test Tube Procedure.

Table 1. Preparation of Diluted Albumin (BSA) Standards

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	4.5 ml	0.5 ml of Stock	200 µg/ml
B	8.0 ml	2.0 ml of vial A dilution	40 µg/ml
C	4.0 ml	4.0 ml of vial B dilution	20 µg/ml
D	4.0 ml	4.0 ml of vial C dilution	10 µg/ml
E	4.0 ml	4.0 ml of vial D dilution	5 µg/ml
F	4.0 ml	4.0 ml of vial E dilution	2.5 µg/ml
G	4.8 ml	3.2 ml of vial F dilution	1 µg/ml
H	4.0 ml	4.0 ml of vial G dilution	0.5 µg/ml
I	8.0 ml	0	0 µg/ml = Blank

B. Preparation of the Micro 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:

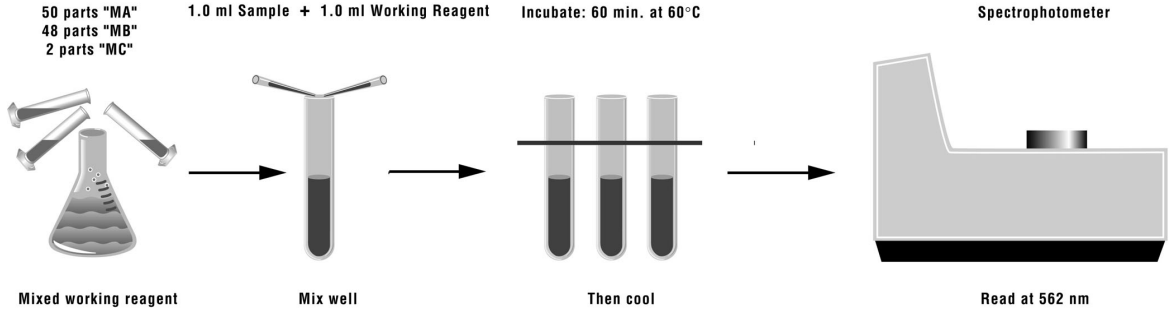
   (9 standards + 3 unknowns) (2 replicates) (1 ml) = 24 ml WR required (round up to 25 ml)

   Note: 1 ml of the WR is required for each sample in the Test Tube Procedure, while only 150 µl of WR is required for each sample in the Microplate Procedure.

2. Prepare fresh WR by mixing 25 parts of Micro BCA™ Reagent MA and 24 parts Reagent MB with 1 part of Reagent MC (25:24:1, Reagent MA:MB:MC). For the above example, combine 12.5 ml of Reagent MA and 12.0 ml Reagent MB with 0.5 ml of Reagent MC.

   Note: When Reagent MC is initially added to Reagents MA and MB, 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)



Test Tube Procedure (linear working range of 0.5-20 µg/ml)

1. Pipette 1.0 ml of each standard and unknown sample replicate into an appropriately labeled test tube.
2. Add 1.0 ml of the WR to each tube and mix well.
3. Cover tubes and incubate at 60°C in a water bath for 1 hour.
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 Micro 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 readings of all tubes are made within 10 minutes of each other.
6. Subtract the average 562 nm absorbance reading of the Blank standard replicates from the 562 nm reading of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 562 nm reading 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 (linear working range of 2-40 µg/ml)

1. Pipette 150 µl of each standard or unknown sample replicate into a microplate well.
2. Add 150 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 2 hours.

   Note: Limit incubations of microplate to less than or equal to 37°C, otherwise high background and aberrant color development may result. Most polystyrene assay plates deform, leach, and become cloudy at 60°C.
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, 562 nm readings are lower with the Microplate Procedure than with the Test Tube Procedure. Consequently, the lower limit of detection is greater (2.0 µg/ml) in the Microplate Procedure.
6. Subtract the average 562 nm absorbance reading of the Blank standard replicates from the 562 nm reading of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 562 nm reading 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 more Reagent MC)
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]
All tubes (including blank) are dark purple	Buffer contains a reducing agent	Dialyze or dilute sample
	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 Micro 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	Hydrogen Peroxide	Iron	Reducing Sugars
Catecholamines	Hydrazides	Lipids	Tryptophan
Cysteine	Impure Glycerol	Phenol Red	Tyrosine
EGTA	Impure Sucrose	Reducing Agents	Uric Acid

Other substances interfere to a lesser extent with protein estimation using the Micro BCA™ Protein Assay Kit. These have only minor (tolerable) effects below a certain concentration in the original sample. Maximum compatible concentrations for many substances in the Test Tube Procedure are listed in Table 2. Substances were compatible at the indicated concentration in the Test Tube Procedure 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. The Blank-corrected 562 nm absorbance measurements (for the 10 µg/ml BSA standard + substance) were compared to the net 562 nm readings of the same standard prepared in 0.9% saline.

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

The effects of interfering substances in the Micro BCA™ Protein Assay may be eliminated or overcome by 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 proteins 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 Micro BCA™ WR.[4] A protocol for performing this on samples to be assayed with BCA Protein Assay Reagent is available at the Pierce web site. Precipitation methods may not be suitable (i.e., accurate) for samples and standards at low concentrations, such as are used in the Micro BCA™ Assay.
- Increase the amount of copper in the WR (prepare WR using a greater proportion of Reagent MC; e.g., MA:MB:MC equal to 25:24:2 or 25:24:3), which may eliminate interference by copper chelating agents.

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

Related Pierce Products

23209	**Albumin Standard Ampules, 2 mg/ml**, 10 x 1 ml ampules, containing bovine serum albumin (BSA) at 2.0 mg/ml in a solution of 0.9% saline and 0.05% sodium azide
23212	**Bovine Gamma Globulin Standard, 2 mg/ml**, 10 x 1 ml ampules, containing bovine gamma globulin (BGG) at 2.0 mg/ml in a solution of 0.9% saline and 0.05% sodium azide
23231	**Micro BCA™ Reagent A (MA)**, 240 ml
23232	**Micro BCA™ Reagent B (MB)**, 240 ml
23234	**Micro BCA™ Reagent C (MC)**, 12 ml
23225	**BCA Protein Assay Kit***, working range of 20-2,000 µg/ml
23236	**Coomassie Plus™ Protein Assay Kit**, working range of 1-1,500 µg/ml

Additional Information

A. Please visit the Pierce web site for additional information on this product including:

- Frequently Asked Questions
- Tech Tip protocol: TCA or Acetone Elimination of Interfering Substances
- Application notes and more complete reference list

B. Response characteristics for different proteins

Each of the commonly used total protein assay methods exhibits some degree of varying response toward different proteins (Table 3). 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. Pierce Albumin Standard (BSA) (Product No. 23209) provides a consistent standard for protein estimations. Nevertheless, individual proteins, including BSA and IgG, differ slightly in their color responses in the Micro BCA™ Assay (Figure 1). Therefore, if great accuracy is required, the standard curve should be prepared from a pure sample of the target protein to be measured.

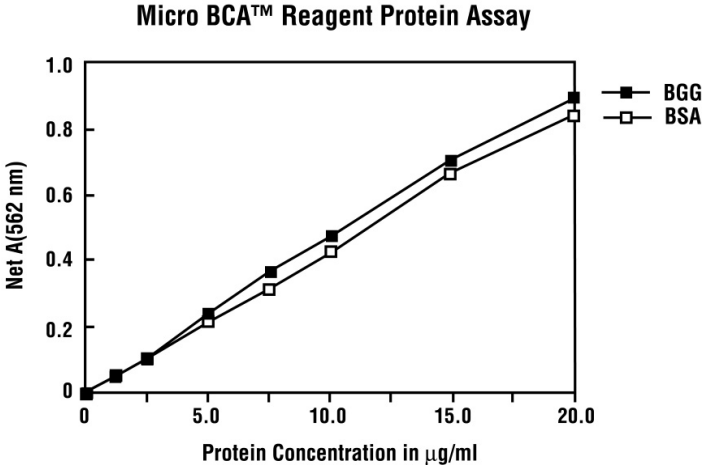


Figure 1: Typical color response curves for BSA and BGG using the Test Tube Procedure.

Table 3. Protein-to-Protein Variation. Absorbance ratios (562 nm) for proteins relative to BSA using the Test Tube Procedure.

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

Protein Tested	Ratio
Albumin, bovine serum	1.00
Aldolase, rabbit muscle	0.80
α-Chymotrypsinogen, bovine	0.99
Cytochrome C, horse heart	1.11
Gamma globulin, bovine	0.95
IgG, bovine	1.12
IgG, human	1.03
IgG, mouse	1.23
IgG, rabbit	1.12
IgG, sheep	1.14
Insulin, bovine pancreas	1.22
Myoglobin, horse heart	0.92
Ovalbumin	1.08
Transferrin, human	0.98
Average ratio	**1.05**
Standard Deviation	**0.12**
Coefficient of Variation	**11.4%**

C. Alternative Total Protein Assay Reagents

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

D. Cleaning and Re-using Glassware

Care must be exercised when re-using glassware. The Micro BCA™ WR is sensitive to metal ions, especially copper ions. All glassware must be cleaned and then given a thorough final rinse with ultrapure water.

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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., Braun, R. and Fitzpatrick, J. (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., Jarvis, K. and Hyland, K. (1989). Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal. Biochem*. **180**:136-9.

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Nguyen, V.T., Ndoye, A., Grando, S.A. (2000). Pemphigus vulgaris antibody identifies pemphaxin. *J. Biol. Chem.* **275:**29466-76.

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Table 2. Compatible Substance Concentrations in the Micro BCA™ Protein Assay (see text for details).

Substance	Compatible Concentration
Salts/Buffers	
ACES, pH 7.8	10 mM
Ammonium sulfate	--------
Bicine, pH 8.4	2 mM
Bis-Tris, pH 6.5	0.2 mM
Borate (50 mM), pH 8.5 (#28384)	1:4 dilution*
B-PER® Reagent (#78248)	1:10 dilution*
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)	1:600 dilution*
Na-Citrate (0.6 M), MOPS (0.1 M), pH 7.5 (#28386)	1:600 dilution*
Cobalt chloride in TBS, pH 7.2	--------
EPPS, pH 8.0	100 mM
Ferric chloride in TBS, pH 7.2	0.5 mM
Glycine	n/a
Guanidine•HCl	4 M
HEPES, pH 7.5	100 mM
Imidazole, pH 7.0	12.5 mM
MES, pH 6.1	100 mM
MES (0.1 M), NaCl (0.9%), pH 4.7 (#28390)	1:4 dilution*
MOPS, pH 7.2	100 mM
Modified Dulbecco's PBS, pH 7.4 (#28374)	undiluted
Nickel chloride in TBS, pH 7.2	0.2 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	1:10 dilution*
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)	5 mM (16.7 mM)
Sodium phosphate	100 mM
Tricine, pH 8.0	2.5 mM
Triethanolamine, pH 7.8	0.5 mM
Tris	50 mM
TBS; Tris (25 mM), NaCl (0.15 M), pH 7.6 (#28376)	1:10 dilution*
Tris (25 mM), Glycine (192 mM), pH 8.0 (#28380)	1:10 dilution*
Tris (25 mM), Glycine (192 mM), SDS (0.1%), pH 8.3 (#28378)	undiluted
Zinc chloride in TBS, pH 7.2	0.5 mM

Substance	Compatible Concentration
Detergents**	
Brij®-35	5.0%
Brij®-56, Brij®-58	1.0%
CHAPS (CHAPSO)	1.0% (5.0%)
Deoxycholic acid	5.0%
Nonidet P-40 (NP-40)	5.0%
Octyl β-glucoside	0.1%
Octyl β-thioglucopyranoside	5.0%
SDS	5.0%
Span® 20	1.0%
Triton® X-100	5.0%
Triton® X-114	0.05%
Triton® X-305, X-405	1.0%
Tween®-20, Tween®-80	5.0%
Tween®-60	0.5%
Zwittergent® 3-14	--------
Chelating agents	
EDTA	0.5 mM
EGTA	--------
Sodium citrate, pH 4.8 (or pH 6.4)	5 mM (16.7 mM)
Reducing & Thiol-Containing Agents	
N-acetylglucosamine in PBS, pH 7.2	--------
Ascorbic acid	--------
Cysteine	--------
Dithioerythritol (DTE)	--------
Dithiothreitol (DTT)	--------
Glucose	1 mM
2-Mercaptoethanol	1 mM
Thimerosal	--------
Misc. Reagents & Solvents	
Acetone	1.0%
Acetonitrile	1.0%
Aprotinin	1 mg/L
DMF, DMSO	1.0%
Ethanol	1.0%
Glycerol (Fresh)	1.0%
Hydrazide	--------
Hydrides (Na_2BH_4 or $NaCNBH_3$)	--------
Hydrochloric Acid	10 mM
Leupeptin	10 mg/L
Methanol	1.0%
Phenol Red	--------
PMSF	1 mM
Sodium Hydroxide	50 mM
Sucrose	4%
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