

Technical Supplement:
Microparticle Reagent Optimization
BCA Assay for Microparticles
and Quick Elution Technique
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Introduction

The Microparticle-bound Protein Assay is a simple and quick way to directly measure the amount of surface-bound protein after coupling reactions. Use of this assay will allow one to:

- Quantitate microparticle-bound protein
- Optimize coupling conditions to achieve the most efficient coupling of precious proteins
- Determine the effect of protein loading on immunoreactivity
- Institute improved QC monitoring of manufactured products

Principle of Assay

The direct measurement of microparticle-bound protein is possible using the copper reduction/bicinchoninic acid (BCA) reaction. Copper (II) is reduced to Copper (I) by protein under alkaline conditions. The Copper (I) ion generated forms a soluble, intense colored complex with BCA^{1,2} that is detectable at 562 nm. Total microparticle-bound protein is measured by the reaction of a known amount of microparticle suspension with the BCA reagent. After formation of color, the microparticle is separated by centrifugation and the color is measured spectrophotometrically. The bound protein is reported as µg of protein per mg of particles.

Quick Elution Technique

This technique can be utilized as an analytical tool. Covalently bound protein can be measured by first eluting adsorbed protein with a combination of base and detergent. This technique, developed at Thermo Fisher Scientific, completely removes adsorbed protein in only 30 minutes. After elution, the remaining microparticle-bound protein is measured using the BCA assay. This permits the distinction between passively adsorbed and covalently bound protein. The non-elutable fraction is presumed to be covalently bound.

Microparticle (MP) Reagent Development

BCA protein measurement capability can assist in reagent development by answering the following questions:

1. Is there any protein on the microparticles?

Dye binding methods are commonly used to assay the decrease in supernatant protein after coupling. These methods are plagued with interferences from buffer components and generally do not have adequate sensitivity. The BCA assay gives a direct and sensitive measure of microparticle-bound protein.

2. Which conditions give the most efficient coupling?

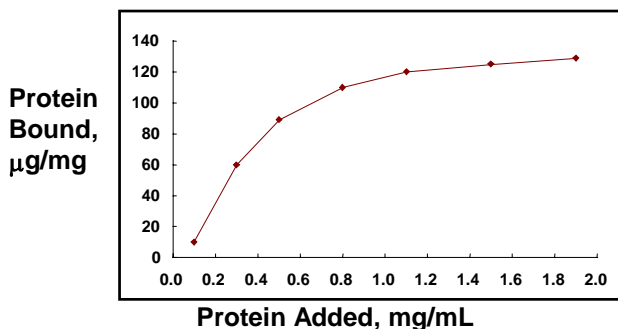
Use this assay to compare and optimize coupling conditions. Assess the effects of: pH, Concentration of reactants, Buffer, Covalent coupling vs. adsorption, Coupling reagents, Different microparticles

3. Has the coupling procedure worked satisfactorily?

The performance of sensitized microparticles is highly dependent on the quantity of bound protein. The BCA protein assay provides valuable data for optimization and quality control.

Shown below is a typical binding isotherm where the data (μg protein/mg microparticles) were obtained with the BCA microparticle-bound protein assay.

Binding Isotherm



Materials and Methods

1. Micro BCA™ Protein Assay Kit (Pierce Cat. No. 23235)
component description: Micro BCA reagent A (MA), Micro BCA reagent B (MB), Micro BCA reagent C (MC)
2. Prediluted Protein Assay Standards: Bovine Serum Albumin (BSA) Set (Pierce Cat. No. 23208)
Component Description:
 - BSA standard 1: 125 $\mu\text{g}/\text{mL}$
 - BSA standard 2: 250 $\mu\text{g}/\text{mL}$
 - BSA standard 3: 500 $\mu\text{g}/\text{mL}$
 - BSA standard 4: 750 $\mu\text{g}/\text{mL}$
 - BSA standard 5: 1000 $\mu\text{g}/\text{mL}$
 - BSA standard 6: 1500 $\mu\text{g}/\text{mL}$
 - BSA standard 7: 2000 $\mu\text{g}/\text{mL}$

3. Particle Control
 - SeraMag® particle control 5 mL (Seradyn 30152103010150)
 - Streptavidin / CML particle control 5 mL (Seradyn 29000701010150)
4. Particle Blank
 - SeraMag® particle blank (Seradyn 24152105)
 - CML or PS particle blank (Part numbers vary)
5. DI water
6. Hexadimethrine Bromide (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) Sigma H9268 1% (W/V) Polybrene
Note: This reagent is used for the flocculation step of the procedure for nonmagnetic beads ONLY
7. **For Quick Elution Technique:** Alkaline SDS (0.20 M tris base/1.0% SDS).
Mix as follows for 20 mL:
 - 4 mL 1.0 M tris base
 - 2 mL 10% SDS
 - 14 mL deionized water

Note: We recommend an alkaline-SDS with 0.20 M tris base. This formulation, which has a pH in the range of 10-11, is gentler while still effective in eluting the non-covalent protein fractions in the 30 - minute incubation time. We have confirmed by electrophoresis that IgG molecules stay intact during this treatment.

Appropriate Labware including:

- Microcentrifuge tubes
- Pipettes and Tips (25 – 500 µL)
- Vortex
- Water bath
- MicroCentrifuge
- 96-well microtiter plate
- Plate reader (reading 562 nm)

Before You Begin:

- The procedure given is intended to describe the application of the BCA reagent for assay of microparticle bound protein. The package insert should be consulted for a full description of the method.³
- The BCA assay gives a nonlinear standard curve; therefore, all unknown samples should be calculated from a standard curve in which the unknown is bracketed by standards.
- In-house studies have shown that the color yield obtained from protein bound to microparticles is slightly lower than that obtained from the same amount of protein in solution. Despite this limitation, the BCA assay provides valuable information for analysis and quality control of particle coupling reactions.

- Every protein gives a unique reaction with the BCA reagent.² Therefore, if more accurate measurements are desired, a standard curve with the protein of interest should be performed.
- Microparticle suspensions are frequently blocked with inert proteins such as BSA. In this case, an aliquot may be taken for assay prior to adding the blocking protein. It is useful to measure the total bound protein (sensitizing protein + blocking protein) by performing total bound protein assay on an aliquot of particles washed with plain buffer.
- Most commonly used biological buffers and detergents do not interfere in this assay. However, bis-tris, bis-tris propane, and tricine interfere with the assay. The following substances give high interference: NHS (N-hydroxysuccinimide), sodium salicylate, phenol, phenol red.
Note: Washing in plain buffer to remove these compounds is necessary before performing the assay.

Procedure:

1. Set heating bath to 55°C.
Note: Thermo Fisher Scientific recommended reaction parameters vary slightly from those given in the reagent package insert.
2. Centrifuge tube labeling:
 - Label eight micro centrifuge tubes as follows: BSA 0 (blank), BSA 1 to BSA 7 for the seven BSA standards.
 - Label an additional micro centrifuge tube for the particle control.
 - Label micro centrifuge tubes for each sample and also include the particle blank.
Note: Run test samples in duplicate.
3. Prepare the standards by mixing 25 µl of each prediluted standard (Cat. No. 23208) and 475 µl of DI Water. Include a standard 0 consisting of 500 µl of DI Water.
4. **Note: Before taking particle samples, re-suspend the particle sample thoroughly by vortex mixing until no visible clumping is seen.**
 - Dilute the Particle Control: Add 50 µL of the particle control to the control tube and add water to bring the total volume to 500 µL.
 - Dilute the Test Samples: Add 50 µL of your protein coupled particle sample (If measuring streptavidin SeraMag® particle level 4 or level 5, add only 25 µL sample) to each tube and add water to bring the total volume to 500 µL.
 - Preparation of the particle blank: Prepare a particle blank using the same volume of uncoated microparticle and the same % solids as the sample.
5. Prepare fresh BCA reagent (Cat. No. 23235).
 Total number of the tubes X 0.6 mL = minimum volume of BCA reagent.
 - Mix the three BCA reagent components together using the following proportion:

MA(Micro BCA reagent A) : MB(Micro BCA reagent B) : MC(Micro BCA reagent C) = 5 : 4.8 : 0.2

For example : 20 mL BCA reagent =
10 mL MA + 9.6 mL MB + 0.4 mL MC

- Add 0.5 mL of the mixed Micro BCA reagent to each tube (all controls, samples and blanks), cap, and vortex.
6. Incubate samples 50±5 minutes in the water bath set at 55°C.
- Place all samples in and remove all samples from the water bath at the same time.
 - For Magnetic Microparticles, for optimal results re-suspend MP by inversion of the rack of tubes every 15 minutes.
Note: The temperature is critical for optimal performance of the assay.
7. Remove the samples from water bath, and cool to room temperature for 1 hour.
8. Centrifuge samples.
- Magnetic Microparticles: Centrifuge samples for 1 minute at 14,000 RPM using a microcentrifuge.
 - Nonmagnetic Microparticles: To flocculate the microparticles for easy centrifugation, add 50 µL of a 1% solution of Hexadimethrine Bromide. Vortex each sample, then centrifuge the samples for 5 minutes at 14,000 RPM using a microcentrifuge.
Note: For small nonmagnetic particles i.e. 0.2 µm, the time of centrifugation may require extension to 15 minutes.
9. Pipette 200 µL of each supernatant into a 96-well microtiter plate.
10. Read the microplate on a **plate reader** at 562 nm wavelength.
11. Review assay results against the following criteria:
1. If the correlation coefficient (R^2 factor) is lower than 0.975, repeat the BCA assay procedure.
 2. If the absorption value of the sample falls outside of the standard range, repeat the assay using a different particle sample dilution and particle blank with the same percent solids as the new sample dilution.
Note: The new dilution of the particle sample is calculated in reference to the absorption (the absorption reading target is 0.5 ~ 1.0).
 3. If the strepavidin-coated SeraMag® particle control is not in the range of 41- 62 µg/mg, repeat the BCA assay procedure.

Quick Elution Technique

1. Add 500 μL of alkaline SDS to an appropriate amount of MP sample (same amount as for BCA protein assay).
2. Mix gently and allow to stand at room temperature for 30 minutes.
3. Centrifuge in a high-speed microcentrifuge to pellet MPs.
4. Remove the supernatant carefully. It is better to leave a little supernatant than to remove any particles.
5. Wash the pellet once with 500 μL of alkaline-SDS. This will dilute any protein-containing supernatant left on the pellet. Discard supernatant.
6. Add DI H_2O to pellet to 500 μL these samples are now ready to go to BCA procedure step 5.

Calculations

1. Plot absorbance vs. μg of BSA as standard curve.
2. Calculate μg of protein per mg of microparticles. To calculate μg of protein per mg of microparticles, divide the value for μg of protein obtained from the standard curve by the mg of microparticles used in the assay.

For example, if 50 μL of 1% solids Microparticle (MP) suspension were used, this corresponds to 0.5 MP in the assay

The calculation tool: Microparticle-Bound Protein Assay – MS Excel Calculations Sheet can be utilized to calculate the protein concentration at μg (protein) / mg (particle).

References

1. Smith P, Krohn R, Hermanson G, et al. Measurement of Protein Using Bicinchoninic Acid. *Anal. Biochem.* 150, 76-85 (1985).
2. Wiechelmann K, Braun T, and Fitzpatrick J. Investigation of the Bicinchoninic Acid Protein Assay: Identification of the groups responsible for color formation. *Anal. Biochem.* 175, 231-237 (1988).
3. Pierce. Instructions MicroBCA Protein Assay Kit, Pierce, Rockford IL.