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A modified protein assay from microgram to low nanogram levels in dilute samples

Ghanshyam D. Heda [a,b,*], Upasana Kunwar [a], Rajiv P. Heda [c]

[a] Department of Sciences and Mathematics, Mississippi University for Women, Columbus, MS 39701, USA
[b] Department of Medicine, University of Tennessee Health Sciences Center, Memphis, TN 38163, USA
[c] Mississippi School for Mathematics and Science, Columbus, MS 39701, USA

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ABSTRACT

In this article, we present a modified and improved protein assay that was previously described as "amidoschwarz assay" by Schaffner and Weissmann [13]. Our improved protein assay is user-friendly and 30–40 times more sensitive than the earlier method. The assay was developed into three formats (macro-, micro-, and nanoassay) with trichloroacetic acid (TCA) as protein precipitating agent, measuring up to 96 samples. The macro and micro formats of this assay require a single reagent staining with amido black of protein dots bound to nitrocellulose membrane with lowest protein measurements to 1 and 0.1 μg, respectively. On the other hand, the nanoassay, with combination staining of amido black followed by colloidal gold, can extend the detection limit to 2.5 ng of protein. Protein concentrations were determined by densitometry and/or spectrophotometry. This assay is compatible with many ionic and non-ionic detergents. This improved protein assay provides an additional choice to researchers in measuring total protein concentration accurately in dilute biological samples as low as 0.125 μg/ml prior to their biochemical analysis such as in comparative proteomics.

Protein assay is a primary requirement of many protein biochemistry research laboratories. With the recent surge in proteomics research, there is a need for a precise total protein measurement assay, especially when two samples containing small quantities of proteins need to be compared. In our laboratory, such a need arose when we were trying to measure the protein concentration in immuno-isolated protein-specific endocytic vesicles from mammalian cells that were transfected with either wild-type or mutant genes. Protein concentration in such vesicle populations is at low nanogram levels, and samples are often dilute. Our goal with such samples is to collectively measure membrane and cytosolic proteins of acidic and basic natures prior to subjecting them for proteomics analysis. We also wanted an assay that is capable of measuring protein concentrations when directly dissolved in two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] sample solubilization buffer (minus dye). The most common Lowry [1] and Bradford [2] assays with their later modifications can meet some of these requirements; however, they are not sensitive enough and cannot accommodate dilute samples. A later surge of colorimetric [3–9] and fluorescence-based protein assays [10], on the other hand, are sensitive. These methods, however, might not measure the total protein concentration in a sample because they do not include protein precipitating agents such as trichloroacetic acid (TCA) in their assay mixture. It is to be noted that the original version of commonly applicable Lowry assay [1] and its later modification [11] contained TCA that was later omitted from its commercial versions such as Bio-Rad *DC*™ protein assay [12]. The amidoschwarz 10B (amido black) dye-based protein assay described by Schaffner and Weissmann [13] was found to be most suitable for our purpose because this assay is based on the principle of precipitating all proteins, specifically membrane proteins with TCA. Moreover, the protein precipitation ability of TCA extends the usefulness of this assay to dilute biological samples containing low quantities of proteins. The drawbacks in the earlier method [13], however, were in sample loading, limitation on the number of samples that can be analyzed at a given time, and methods used to quantify proteins. Furthermore, the lowest protein concentration that could be measured was 5 μg/ml. We have overcome these problems by using modern equipment, resources, and an array of commercially available protein binding stains [14]. Multiple samples can be applied using a 96-well dot–blot apparatus providing more control over sample loading. Subsequently, protein concentration can be conveniently and accurately measured by densitometry and/or spectrophotometry. Various protein stains in

* Corresponding author at: Department of Sciences and Mathematics, Mississippi University for Women, Columbus, MS 39701, USA. Fax: +1 662 329 7238.
E-mail addresses: gheda@as.muw.edu, g.heda@juno.com (G.D. Heda).

[1] *Abbreviations used:* SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; CG, colloidal gold; AB, amido black; BSA, bovine serum albumin; IgG, immunoglobulin G; NC, nitrocellulose; PBS, phosphate buffer solution; SD, standard deviation; APN, amidoschwarz protein nanoassay; DTT, dithiothreitol.

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addition to amido black were tested individually as well as in combination. The combined effect of amido black (AB) followed by colloidal gold (CG) was found to be the most suitable combination staining of protein dots in terms of sensitivity and specificity. Like several other solid phase [3–7,13] and liquid phase [1,2,8–11] assays, our improved assay is also susceptible to protein-to-protein variations. The protein assay presented in this article, however, is unaffected by detergents that are normally used in solubilization of membrane proteins included in two-dimensional SDS–PAGE sample buffer [15].

Materials and methods

Materials

Nitrocellulose membranes with supported base (pore size = 0.2 μm), a Bio-Rad *DC*™ protein assay kit with bovine serum albumin (BSA) stock solution, and CG total protein stain were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Amido black 10B stain was purchased from MP Biomedicals (Solon, OH, USA), Ponceau-S was purchased from IBI Scientific (Peosta, IA, USA), BLOT-FastStain™ was purchased from G-Biosciences (St. Louis, MO, USA), and GelCode® blue stain was purchased from Thermo Scientific (Rockford, IL, USA). The LLC-PK_1 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Goat anti-rabbit immunoglobulin G (IgG) was obtained from Zymed Laboratories (San Francisco, CA, USA). TCA, ribonuclease A from bovine pancreas, recombinant human insulin, lysozyme from chicken egg white, γ-globulin from bovine blood, and all other routine laboratory chemicals were of analytical grade and purchased from either Sigma Chemical (St. Louis, MO, USA) or Thermo Scientific.

Methods

All assays were run in triplicate using BSA as standard. Deionized water of less than 1 μohm conductivity obtained from a Direct-Q3 (UV) water filtration system (EMD, Millipore, Billerica, MA, USA) was used.

Macroassay

Protein samples (1–10 μg) were transferred to different microfuge tubes, and water was added to adjust the volume to 270 μl. To each sample, 30 μl of macroassay buffer (1 M Tris–HCl [pH 7.5] with 1% SDS) and 60 μl of 60% TCA were added, giving a final concentration of 10%. The entire mixture (360 μl total volume) was briefly vortexed and incubated at room temperature for 5 min. A supported nitrocellulose (NC) membrane was cut to the required size for a 96-well Bio-Dot® microfiltration apparatus (Bio-Rad Laboratories) and pre-soaked in 6% TCA solution for approximately 15 min. The supported base to NC membrane prevented its shrinking and expansion during staining and destaining for accurate densitometric analysis. To prevent damage to the apparatus from acidic conditions of TCA, a thin layer of approximately 5 g of dry sodium bicarbonate powder was layered in its vacuum manifold plate. Protein samples were applied to the membrane that was assembled onto a Bio-Dot® microfiltration apparatus as per the manufacturer's directions. Samples were completely drained under the slow vacuum, and each well was washed with 600 μl of 6% TCA. The membrane was carefully removed with forceps and stained for 15 min with 0.1% (w/v) AB prepared in a solution consisting of 45% methanol and 10% acetic acid. Stained membrane was quickly rinsed with water and destained with a solution consisting of 90% methanol and 2% acetic acid until a clear background was obtained.

Microassay

Protein samples (0.1–1 μg) were transferred to different microfuge tubes, and volume was adjusted to 20 μl with water. To each tube, 5 μl of microassay buffer (0.5 M Tris [pH 7.5] with 0.5% SDS) and 5 μl of 60% TCA were added, giving a final concentration of 10%. Sample loading (30 μl total volume), membrane staining, and destaining procedures were similar to those of the macroassay.

Nanoassay

Protein samples in the lower nanogram range (2.5–60 ng) were transferred to microfuge tubes and adjusted to 20 μl with water. To each tube, 5 μl of microassay buffer (see above) and 5 μl of 60% TCA were added, giving a final concentration of 10%. Sample loading (30 μl total volume) and staining of membrane with AB were performed as described above for macro- and microassays. A dedicated set of clean glass dishes and wearing of clean gloves while handling the NC membrane was necessary due to the sensitive nature of nanoassay. After AB staining (see macroassay method above), membrane was rinsed with destain-I (70% methanol and 2% acetic acid) for 15 min, followed by destain-II (90% methanol and 2% acetic acid) for 5 min. Membrane was then transferred back to destain-I for 5 min and rinsed with ample quantities of water ($\geqslant$200 ml) three times for 15 min each. The sequential destaining allowed slow dehydration and rehydration of supported NC membrane and prevented it from curling. The membrane was then washed with TBS-T buffer (20 mM Tris, 0.137 M NaCl, and 0.1% Tween 20, pH 7.6) three times for 15 min each while shaking. The membrane was finally stained with CG total protein stain for 3–5 min while shaking and was destained by quick rinsing with water.

Densitometry

Stained protein dots on NC membrane were photo-scanned either wet or dry using a standard desktop scanner (Epson Perfection V33) or under epi-white light in a ChemiDoc™ image analyzer (Bio-Rad Laboratories). Scanned images were either saved as or converted into TIFF format and subjected to Quantity One® 1-D analysis software (Bio-Rad Laboratories). Precise circles of same size were drawn around the stained protein dots. Density volume within each circle was measured as intensity/mm^2 and plotted using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Standard curves can also be plotted using percentage of density volume within a given range of serially diluted samples of protein standard.

Spectrophotometry

Spectrophotometry was used as an alternative and/or confirmatory method for protein concentration measurements by macro- and microassays. Individual protein dots were sliced while the membrane was still damp and transferred to microfuge tubes. To each tube, 600 μl (macroassay) or 20 μl (microassay) of elution buffer consisting of 25 mM NaOH, and 0.05 mM ethylenediaminetetraacetic acid (EDTA) in 50% ethanol was added. The tubes were periodically vortexed over a period of 15 min, and the eluates were transferred to another tube. The absorbance at 630 nm was measured by applying 2 μl of eluate (macro- and microassays) on a Nanodrop 1000™ spectrophotometer or 300 μl of eluate (macroassay only) using 96-well plates in an LT-4000 microplate reader (Labtech International, East Sussex, UK). Samples can be read