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A MODIFIED PROTEIN ASSAY FROM MICROGRAM TO LOW NANOGRAM LEVELS IN DILUTE SAMPLES

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Abstract

In this paper we present a modified and improved protein assay that was previously described as ‘amidoschwarz assay’ by Schaffner and Weissmann (*Anal. Biochem.* 56, 1973, 502–514). Our improved protein assay is user-friendly and 30 to 40 times more sensitive than the earlier method. The assay was developed into 3 formats (macro, micro, and nanoassay) with 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 µg and 0.1 µg respectively. The nanoassay on the other hand 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.

Keywords

Protein assay; amido black; colloidal gold; TCA; densitometry; spectrophotometry; nitrocellulose membrane

Introduction

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 need arose when we were trying to measure the protein concentration in immunisolated protein-specific endocytic vesicles from mammalian cells that were transfected with either wild-type or mutant gene. 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

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proteins of acidic and basic nature, prior to subjecting them for proteomic analysis. We also wanted an assay that is capable of measuring protein concentrations when directly dissolved in 2-dimensional SDS-PAGE 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 neither sensitive enough nor can 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, may not measure the total protein concentration in a sample, as they do not include protein precipitating agents such as 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 like 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 the most suitable for our purpose as this assay is based on the principle of precipitating all proteins, specifically membrane proteins with TCA. Moreover, protein precipitation ability of TCA extends the usefulness of this assay to dilute biological samples containing low quantities of proteins. The drawbacks in 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 the 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 addition to amido black were tested individually as well as in combination. The combined effect of amido black followed by colloidal gold 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 paper, however, is unaffected by detergents that are normally used in solubilization of membrane proteins, included in 2-dimensional SDS-PAGE sample buffer [15].

Materials and Methods

Materials

Nitrocellulose membranes with supported base (pore size: 0.2 µm), Bio-Rad DC™ protein assay kit with BSA stock solution, and colloidal gold total protein stain were purchased from Bio-Rad Laboratories (Hercules, CA). Amido black 10B stain was purchased from MP Biomedicals (Solon, OH), Ponceau-S from IBI Scientific (Peosta, IA), BLOT-FastStain™ from G-Biosciences (St. Louis, Missouri), and GelCode® blue stain was purchased from Thermo Scientific, (Rockford, IL). LLC-PK₁ cell line was obtained from ATCC (Manassas, VA). Goat anti-rabbit IgG was obtained from Zymed Laboratories (San Francisco, CA). 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 either from Sigma Chemical Co (St. Louis, MO) or Thermo Scientific.

Methods

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

Macroassay

Protein samples (1 to 10 μg) were transferred to different microfuge tubes, and water was added to adjust volume to 270 μl . To each sample a 30 μl of macroassay buffer (1M Tris-HCl, pH 7.5 with 1% SDS) and 60 μl of 60% TCA were added, giving the final concentration of 10%. The entire mixture (360 μl total volume) was briefly vortexed and incubated at room temperature for 5 min. A supported NC membrane was cut to the required size for a 96-well Bio-Dot[®] microfiltration apparatus (Bio-Rad Laboratories, Hercules, CA), and pre-soaked in 6% TCA solution for about 15 min. Supported base to NC membrane prevented its shrinking and expansion during staining and destaining for accurate densitometric analysis. To prevent the damage to the apparatus from acidic conditions of TCA, a thin layer of ≈ 5 grams 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) amido black (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 to 1 μg) were transferred to different microfuge tubes, and volume 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 the final concentration of 10%. Sample loading (30 μl total volume), membrane staining and destaining procedures were similar to that of the macroassay.

Nanoassay

Protein samples in the lower nanogram range (2.5 to 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 was added, giving the final concentration of 10%. Sample loading (30 μl total volume), and staining of membrane with AB was performed as described above for macro and micro assays. 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), membrane was rinsed with destain-I (70% methanol, 2% acetic acid) for 15 min followed by destain-II (90% methanol, 2% acetic acid) for 5 min. Membrane was then transferred back to destain-I for 5 min, and rinsed with ample quantities of water (200 ml) 3 times, 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, 0.1% Tween 20, pH 7.6) 3 times, 15 min each while shaking. Membrane is finally stained with colloidal gold total protein stain for 3–5 min while shaking and 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 the 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 Inc., La Jolla, CA). 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 is still damp, and transferred to microfuge tubes. To each tube a 600 μ l (macroassay) or 20 μ l (microassay) of elution buffer consisting of 25 mM NaOH, 0.05 mM 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 μ l eluate (macro and microassays) on NanodropTM 1000 spectrophotometer or a 300 μ l eluate (macroassay only) using 96-well plates in LT-4000 microplate reader (Labtech International, UK). Samples can be read immediately or within 24 hrs when kept in dark at 4°C. Protein concentrations were determined by plotting standard curves using GraphPad Prism 5.0 software program.

Cell lysates

LLC-PK₁, a pig kidney epithelial cell line was grown at 37°C in the presence of 5% CO₂ in a nutrient rich growth media [16]. Cells were lysed with 1% SDS containing HaltTM protease and phosphatase inhibitor cocktail (Thermo Scientific). Cell lysates were used to compare improved amidoschwarz assay with Bio-Rad DCTM protein assay.

Results

TCA precipitation of proteins

First, the effects of TCA on protein precipitation were confirmed. For this purpose, we used BSA at sub-microgram (0.1 to 1 μ g) and microgram (1 to 10 μ g) concentrations. Phosphate buffer (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) was used as a control and compared with the 10% TCA in assay mixture. Although linearity was obtained in both cases, the amount of BSA that could be measured was significantly less in the control assay (data not shown). For example, control assay with PBS could measure only about 20% of 1 μ g BSA when compared to TCA containing assay. TCA therefore appears to be a necessary reagent to recover all of the proteins in a sample, and was used in all formats of amidoschwarz assays.

Macro and Microassays

Serially diluted BSA and other protein samples produced precise spherical circles after AB staining. Staining of membranes beyond 15 min had no additional effect on the staining of protein dots. In fact, longer staining produced darker background and required longer destaining. Stained membranes could be scanned wet or dry without compromising with the results, and stored for at least one year in the dark without losing intensity. Densitometric data plotted from Quantity One® analysis software produced sharp linear curves for both macro and microassays with small standard deviation (SD) and strong correlation coefficient (r^2) values (Figs. 1b, 2b). Quantity One® data represent the six independently conducted assays, where samples were run in triplicate for each concentration of BSA. Portion of the membrane where TCA was passed, stained slightly darker than the surrounding region as previously reported [13]. The darker background in the blank samples (without protein), that is sometime difficult to see with the naked eyes, was detected by sensitive scanners producing higher baseline. As a result blank sample in standard curves produced a higher y-intercept, without affecting the linearity of curves (Figs. 1b, 2b, 3b).

Protein concentrations were also confirmed by spectrophotometry. Stained protein dots were eluted by the procedure described in methods. Samples eluted from macroassay can be analyzed individually using Nanodrop™ spectrophotometer or simultaneously (up to 96) using LT-4000 multi-well plate reader. Samples eluted from the microassay on the other hand, due to its small volume size can be analyzed only by Nanodrop™ spectrophotometer.

The absorbency of eluted protein samples from macroassay on Nanodrop™ spectrophotometer at 630 nm produced linear curves with small SD and high r^2 values (Fig. 1c). However, spectrophotometry was found to be less favorable for microassay as indicated by relatively lower r^2 values, suggesting less than consistent protein elution from the NC membrane (Fig. 2c). Additionally, the absorbance of sample eluates from macroassay was also measured at 630 nm in a LT-4000 spectrophotometer (a 96-well plate reader). Like Nanodrop™; 96-well plate reader also produced linear curve with strong r^2 values (data not shown).

Nanoassay

To enhance the sensitivity of microassay (see above), a set of commonly used protein binding stains besides AB were tested individually as well as in combination. These stains were Ponceau S, GelCode® (a commercial version of colloidal coomassie blue), Colloidal Gold (CG), BLOT-FastStain™ (a commercial protein stain for membranes). Most of the stains when used individually were as good as or weaker () than AB, in terms of specificity, linearity, and sensitivity based on visual appearance and densitometric data (not shown).

Next, we tested the effects of these stains when used in combination. Staining of the protein dots by AB followed by CG (AB+CG) appeared to be superior. This combination (AB+CG) therefore was pursued further with a serial dilution of BSA in the lower nanogram range. A standard curve using BSA in the range of 2.5 to 60 ng produced linear curve with low SD and high r^2 value (Fig. 3). With AB+CG combination staining we can now measure the protein concentration as small as 2.5 ng, about 40-fold increase in sensitivity than the method of Schaeffner & Weissman [13]. A reverse order of our chosen staining (i.e., CG +AB), however, produced a non-specific and non-linear response especially in sub-nanogram range, i.e., 50 ng (data not shown).

Assay Validation and Comparative Analysis

Total cellular lysates from LLC-PK₁ cells and 5 other commercially obtained protein samples were used to compare the improved amidoschwarz protein nanoassay (APN) with Bio-Rad DC™ protein assay [12] that is based on the principles of Lowry et al [1]. APN showed 18.5% (n=3) higher protein concentration in cell lysates when compared with Bio-Rad DC™ protein assay. This increased protein concentration value in complex mixture of proteins such as in cellular lysates by APN is may be due to increased sedimentation of overall proteins by TCA.

Commercially obtained protein samples of different molecular weights included in this comparative analysis were rabbit IgG, ribonuclease-A, γ -globulin, recombinant human insulin, and lysozyme. Percentage difference in protein concentration measurements by each one of these assays were compared with BSA and presented in Table 1. Protein concentration values for IgG, γ -globulin remained indifferent from BSA when determined by either types of assay. However, APN measured relatively less protein for low molecular weight proteins like insulin, ribonuclease-A, and to some extent, lysozyme. Regardless of their binding efficiency all of the proteins examined produced linear curves with $r^2 \geq 0.9$ when assayed with increasing protein concentrations (data not shown).

Detergent Compatibility

Since all formats of amidoschwarz protein assays contained SDS, the compatibility of various detergents (ionic and non-ionic) was tested against 1% SDS. Common membrane solubilizing detergents used in 2-dimensional SDS-PAGE, in addition to 10% Tween-20, and 5% digitonin were included in this test. These detergents at the concentration present in most membrane solubilizing sample buffers [15] produced background that was lower than the background produced by 1% SDS alone (Table 2). Among the detergents tested, only 5% digitonin produced background that was roughly twice than that of 1% SDS. Additionally, BSA standard curves prepared in the presence of 2-dimensional SDS-PAGE sample buffer minus bromophenol blue dye (i.e., 7M Urea, 2M thiourea, 4% CHAPS, 60 mM DTT, 40 mM Tris, 0.5% pH 3–10 ampholytes) by APN produced linear curves with r^2 value 0.9 (data not shown).

Discussion

We present a convenient and improved version of amidoschwarz assay [13] as an alternative to several liquid and solid phase proteins assays. Inclusion of TCA facilitates precipitation of total proteins regardless of their biochemical properties, eliminating the need for concentrating dilute samples prior to protein assay. The three different formats (nano, micro, macro) of this assay measuring the total protein concentrations in low nanogram to high microgram range (2.5 ng to 10 μ g) also eliminates the need for using different protein assay methods in a given experimental procedure. One such example can be checking the progress in protein purification procedures, from high protein containing samples at the initial steps to low protein containing purified fractions in the later steps. In our case, we found the usefulness of this assay in measuring protein concentration in endocytic vesicles that we routinely isolate from cell homogenates.

All formats of this assay can utilize densitometry; however, spectrophotometry was limited to macro and microassays as an alternative measurement method. NanodropTM spectrophotometer suitable for handling small size volumes (2 μ l) can be used for macro as well as microassays. Only macroassays have an additional advantage of being measured by 96-well plate readers that require larger sample volume. Additionally, densitometric measurements with the flexibility of using any desktop scanner make this assay convenient and affordable.

Several sensitive protein assays are reported in literature where single staining reagents are used [3–9]. Among them, solid phase assays are either less sensitive [3, 7] or require additional steps and reagents [4–6]. Liquid phase assays on the other hand, require spectrophotometry, disallowing the ease of densitometry [8, 9]. In our experiments, whenever CG was used first as a single staining reagent or in subsequent combination with other stains, it produced non-specific background and non-linear curves, especially in the sub-nanogram range (i.e., <100 ng/dot; data not shown). This, however, was not the case with AB, a mostly hydrophobic stain. Non-specifically bound AB stain from hydrophilic NC membrane can be easily removed with methanol containing destains. CG, a hydrophilic stain, on the other hand binds relatively stronger to NC membrane leaving higher background, as this cannot be washed off completely with methanol. Since negatively charged stains can bind to peptides lacking charged amino acids, CG by virtue of its negative charge [17] binds to AB stained protein dots producing the synergistic effects.

Molecular mass, isoelectric point, hydrophobicity of proteins, pH tolerance, and incompatibility with various reagents are some of the major factors that interfere with precise protein measurements [1–13]. Protein-to-protein variations in amidoschwarz assay are minimum and are reported previously by Schaffner and Weissmann [13]. In this study,

we examined the performance of our chosen combination staining (AB+CG) on five proteins of different molecular weight by APN. The poor and inconsistent staining of low molecular weight proteins like insulin (MW 5.8 kDa), ribonuclease-A (MW 13.7 kDa), and lysozyme (MW 14.3 kDa) (Table 1), is not uncommon [3, 7]. This could be due to poor binding of small size proteins to NC membranes [18], dyes [4] or its passage through the membrane and into the filtrate [5, 13]. These collective findings suggest that molecular mass and the inherent structure of the protein molecule may determine their binding with NC membrane and/or dyes. Since all solid phase protein assays reported in literature suggest variable binding of proteins with membranes and dyes, it is best to use either the same protein or protein of similar molecular weight and properties as standard.

Amidoschwarz protein assay's compatibility with a wide range of ionic and non-ionic detergents is known [13]. In this paper, we mainly focused on detergents that are normally included in sample solubilization buffer for 2-dimensional SDS-PAGE. Since none of these detergents produced background darker than the blank, this assay can be used even after the protein samples are dissolved in sample solubilization buffer (minus dye). This added convenience makes this assay useful for precious samples.

In conclusion, the modified amidoschwarz protein assay method presented in this paper has several advantages. They include: (a) measurement of total proteins by virtue of having TCA in assay mixture; (b) the ability to use dilute samples; (c) a common method for measuring protein concentrations in higher microgram (1–10 µg), lower microgram (0.1 to 1 µg), and nanogram (2.5 ng to 100 ng) ranges; (d) the ability to measure up to 96 samples simultaneously; (e) compatibility with a wide range of membrane solubilizing detergents, and (f) more than one convenient method for quantifying protein (densitometry and/or spectrophotometry).

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Abbreviations used

TCA	trichloroacetic acid
BSA	bovine serum albumin
NC	nitrocellulose membrane
AB	amido black
CG	colloidal gold
APN	amidoschwarz protein nanoassay
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Fig. 1.

Macroassay. (a) BSA was applied from 1 to 10 µg per well on a supported NC membrane using a Bio-Dot® microfiltration apparatus. Protein samples were precipitated with 10% TCA, and stained with amido black. After destaining NC membrane was photo-scanned by Epson Perfection V33 desktop scanner. (b) Densitometric analysis. Density volume of each protein dot was measured by Quantity One® 1-D analysis software. Percentage of total density of protein standards from six independent experiments ($n=6$) was plotted against the protein concentrations. (c) Spectrophotometric analysis. Protein samples from each dot were salt-eluted from NC membrane as described in methods. A 2 µl of sample eluate from each concentration was applied on Nanodrop™ 1000 spectrophotometer for 5x, absorbance at 630 nm were recorded and averaged. Data from six such independent assays ($n=6$) were plotted. Both types of analysis produced linear curves with strong correlation coefficient (r^2) values.

Fig. 2.

Microassay. (a) BSA samples were applied from 0.1 to 1 μg per well on supported NC membrane as described in methods. Densitometric (b) and spectrophotometric (c) analysis were performed as described in legend to Fig. 1. Densitometric analysis produced slightly stronger r^2 value unlike the spectrophotometric analysis.

Fig. 3.

Nanoassay. BSA samples were applied from 2.5 to 60 ng per dot on a supported NC membrane as described in methods. After staining with amido black (AB) for 15 min, NC membrane was restained with colloidal gold (CG) for 3–5 min. Destained membrane was photo-scanned by Epson Perfection V33 desktop scanner, and quantified using Quantity One® 1-D analysis software. Densitometric analysis produced strong r^2 value.

Table 1

Comparative Analysis of Different Proteins by Bio-Rad *DC*TM and Amidoschwarz Protein Nanoassay (APN).

Protein samples	Bio-Rad <i>DC</i> TM Assay (%)	APN (%)	Mr (~kDa)
BSA*	100	100	66
Goat anti-rabbit IgG*	108	106	160
Ribonuclease-A	80	56	13.7
Insulin (hu recombinant)**	79	48	5.8
γ -Globulin*	98	102	120
Lysozyme*	88	66	14.3

Stock protein samples were prepared as 1 mg/ml (w/v) either in water (*) or in 0.1M HCl (**). Samples were further diluted 100-fold for APN. BSA was used as standard. Percentage difference in protein concentration for each of the test proteins was compared with BSA.

Table 2

Interference of Detergents in Amidoschwarz Protein Nanoassay

Detergents	Intensity (mm ²)	Change in background (%) ^a
1% SDS	94	0
7M Urea	none detected	not applicable
2M Thiourea	none detected	not applicable
60 mM DTT	20	(79)
4% CHAPS	72	(24)
10% Tween-20	79	(16)
5% Digitonin	178	89

^a Change in background was compared with 1% SDS.