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A Nanogram Level Colloidal Gold Single Reagent Quantitative Protein Assay

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Abstract

We have developed a nanogram level quantitative protein assay based on the binding of colloidal gold to proteins adhered to nitrocellulose paper. The protein-gold complex produces a purple color proportional to the amount of protein present, and the intensity of the stain is quantified by densitometry. Typical assays require minimal starting material (10 – 20 μ l) containing 2 – 5 μ g protein. A small volume (2 μ l) of protein solution is applied to nitrocellulose paper in a grid array, and dried. The nitrocellulose is incubated in colloidal gold suspension with gentle agitation (4–16 h), rinsed with water and scanned. Densitometric analysis of the scanned images allows quantitation of the unknown sample protein concentration by comparison with protein standards placed on the same nitrocellulose grid. The assay requires significantly less sample than conventional protein assays. In this report, the Golddots assay is calibrated against weighed protein samples, and compared with the Pierce Micro BCA protein assay kit. In addition, the assay is evaluated with several known proteins with different physical properties.

Keywords

Protein Analysis; Colloidal Gold; Densitometry

Introduction

Analysis of protein concentration continues to be an important activity in most biomedical research laboratories. Procedures based on the ultraviolet absorbance of proteins, or their reactions with specific reagents are abundant in the literature [1–4]. In general these procedures require relatively large amounts of sample (μ g - mg), and are biased based on the particular protein property exploited in the assay. Unbiased protein assays based on amino acid analysis are considered a standard, but typically require large amounts of protein [5]. More recently, a fluorescent procedure has been developed, with somewhat higher sensitivity than earlier procedures [6]. In our laboratory, we found that the established assays consumed significant quantities of valuable sample preparations. As we were using colloidal gold to stain proteins on nitrocellulose Western blots, we investigated the possibility that the same reaction could be used to quantitatively estimate protein concentration [7]. We developed a protein assay in which a small volume (2 μ l) of protein solution is applied to nitrocellulose paper in a grid array,

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and dried. The nitrocellulose is incubated in colloidal gold suspension with gentle agitation (4–16 h), rinsed with water and digitized. Densitometric analysis of the scanned images allows quantitation of the unknown sample protein concentration by comparison with protein standards placed on the same nitrocellulose grid. In the present study we show that the Golddots assay has a working range from 1.5 – 1500 ng protein/dot, with a mean coefficient of variation of 5–7 %. Above 1500 ng/ 2ul spot, the assay begins to saturate. In addition, when tested against a panel of four different proteins, individual proteins exhibited differential staining intensity per ng protein analyzed [8]. The Golddots assay is therefore a sensitive and precise estimator of protein concentration, which is critical for proteomics and standard protein chemistry studies.

Materials and Methods

Colloidal Gold suspension was obtained from Bio-Rad Laboratories (Hercules, GA). The nitrocellulose Paper (NCP) was Protran BA 45 (0.45 μ m pore size), a product of Schleicher and Schuell (Keene, NH), and SDS was ultraPure 10% stock solution from Gibco (Grand Island, NY). Bovine serum albumin (BSA), bovine erythrocyte carbonic anhydrase, egg white lysozyme and soybean trypsin inhibitor (SBTI) were all products of Sigma-Aldrich (St. Louis, MO). The Micro BCA Protein Assay kit was obtained from Pierce (Rockford, IL). The IntenSE BL Silver Enhancement Kit was obtained from GE healthcare.

Golddots Assay

For analysis, protein samples (20 μ l) were adjusted to 0.1% SDS, and heated at 80 °C for 10 min. Serial dilutions were made with 0.1% SDS such that 2 μ l aliquots contained 1.5 –1500 ng of protein. In addition to unknown samples, a standard curve was constructed using BSA. All samples were spotted in duplicate on the same sheet of nitrocellulose. A template for guiding even rows and columns with approximately 1 cm spacing was obtained by using the flat plastic detachable 1000 μ l pipette tip holder tray support from the “Space Saver” rack series (Rainin). To deposit the samples, the nitrocellulose sheet is placed on absorbent tissue paper (e.g. Kimwipe), the template is positioned over the nitrocellulose, and is secured to the bench top with pieces of tape. Aliquots (2 μ l) were pipetted onto the nitrocellulose, as guided by the template, into a rectangular grid. This ordered array at 1 cm intervals provides for ample distance between dots for accurate background measurement and the use of a grid reading capability of densitometric software for rapid quantitation of the entire array for up to 96 dots per sheet (Figure 1).

Once all standards and samples were deposited on the nitrocellulose, the array was allowed to thoroughly dry at room temperature and was then placed into the colloidal gold suspension and incubated at room temperature with gentle rocking. The staining reached an end-point after about 2 hours but can be left over-night, since there is no additional staining of protein dots or background after the end-point of full color development has been achieved. (Figure 2) Upon reaching the end-point, the nitrocellulose was rinsed with water and was digitized. Digitization can be carried out wet or dry.

Densitometry

The stained nitrocellulose sheet can be scanned by a standard desktop scanner, or by a laser densitometer. Some of the data presented here was obtained using a Molecular Dynamics Personal Densitometer, and the stained spots quantitated using Molecular Dynamics ImageQuant software (available from GE Healthcare). We have also collected data using an Epson Perfection 1660 PHOTO scanner, and analyzed the images using UN-SCAN-IT-gel software (Silk Scientific, Orem Utah). While the more expensive laser densitometer has some advantages for digitizing dots at the higher end of stain density, it is too powerful for some of

the lighter staining dots. Indeed, the use of the Epson scanner revealed a more sensitive range for the assay, extending it down to the range of 1.5–100 ng. The measured spot intensities were transferred to Quattro Pro (Corel) or Excel (Microsoft) for statistical analysis. Graphs were prepared using SigmaPlot (Systat Software Inc.)

Results and Discussion

The appearance of a typical Golddots assay is shown in Figure 1. The figure demonstrates a regular grid of Golddots 1 cm apart representing dilution series of BSA, bovine erythrocyte carbonic anhydrase and casein (Blotto) in duplicate ranging from 125–1000 ng protein, as determined by dry weight. The data in the figure show the reproducibility of the assay, and the differences in staining sensitivity amongst these proteins. To optimize the assay, we determined the spot density for three concentrations of BSA as a function of time (Figure 2). As shown in the figure, the spot density increases with time up to about 2 hours, after which it remains stable.

Quantitative dose-response Golddot analyses of BSA are presented in Figure 3. Using the Epson scanner, two linear ranges of spot density were revealed: a low range (1.5–100 ng) and a higher range (100–400 ng). The upper range can be further expanded through the use of the Molecular Dynamics Personal Densitometer, where spots up to 1500 ng can be measured (not shown). One advantage of the Golddots method is that the same blot can be scanned by different instruments to maximize the data range. As is evident in the figure, the two linear ranges have different slopes. In Figure 3, the regression lines are calculated as a 2 segment linear, piecewise fit using SigmaPlot. Alternatively, a non-linear, least squares fit to polynomial expression can be used to cover the whole range with a single expression (not shown). The differences in densitometric measurement reflect the two different methods used. The Molecular Dynamics Densitometer measured transmitted light, while the Epson scanner measures reflected light. Thus, the lighter spots are more easily measured by the surface scanner, while the more dense, higher protein levels are better quantitated by the transmitted light system. We attempted to further expand the range of the assay using the IntenSE silver enhancement kit. Silver enhancement had the effect of increasing the slope of the dose response curve in the lowest range, but did not extend the useful range of the assay to smaller protein samples, as the enhancement process also increased the background staining (data not shown).

To ascertain the generality of the assay for different proteins, we compared the staining density of BSA with lysozyme. The data in Figure 4 clearly show that the spot density is a linear function of the amount of protein applied over the range of 100–1000 ng/spot for each protein, but the slope of the regression line is different for the two proteins. Statistical analysis of the reproducibility of the duplicate dots showed that the mean coefficient of variation (i.e. Standard Deviation/Mean) for BSA was 5 %, and for lysozyme 7.6%.

We examined the dose response of 4 pure proteins, BSA, bovine erythrocyte carbonic anhydrase, lysozyme and soybean trypsin inhibitor, and in addition, we tested Blotto, (non-fat dry milk) which is ~80% casein. We observed linear relationships between Golddot intensity and the amount of protein spotted for all five samples. Linear regression analysis was performed on each of the dose-response curves, and the slopes of each are shown in Table 1 as the sensitivity [9]. Based on the work of De Roe, *et al.*, we expected the slopes of the dose response curves to be proportional to protein molecular weight, but, while four of these are roughly correlated with molecular mass, the response of Lysozyme is anomalously high [8]. While we saw little effect of pI on the staining intensity, it is conceivable that the high staining observed with lysozyme results from its high pI. We conclude that the sensitivity of the Golddots assay for a particular protein is not easily predicted. Thus, while differences in sensitivity amongst

different proteins were observed, it is easy to calibrate the assay for particular proteins or mixtures of proteins relative to one well behaved standard, such as BSA.

Table 2 shows a comparison of the sample size requirements for the Golddots assay relative to the Pierce Micro BCA kit. While the Micro BCA assay is more rapid, it consumes considerably more sample. Recently, Noble and co-workers compared a number of dye-based spectrophotometric and fluorometric protein assays, including the BCA assay[10]. Based on their analysis, the Golddots assay compares favorably with the best assays in their survey in useful range, and consumes less sample. In addition, the stained nitrocellulose and digitized images provide a permanent record of the protein concentration determination.

We also tested the Golddots assay for interference by common reagents likely to be found in protein samples. The pH (2.8 – 10.3) of the sample had no effect on spot density. Glycerol (5%) and sucrose (up to 20%) did not change the spot density. At sucrose concentrations > 10%, the viscosity of the sample decreased the spot diameter, but was compensated for by darker staining of the smaller dot. Thiol reagents (dithiothreitol and mercaptoethanol) result in hyper darkening of the gold spots and must be avoided. While it might be possible to diminish the effects of thiol reagents on gold staining, it is better to assay protein content in the sample before adding the reducing agent. The assay is probably unsuitable for those proteins which require the constant presence of reducing agents. We used SDS in preparing protein samples for the analyses reported here, so detergents do not appear to be a problem.

Many protein assays have been developed in the last century of biochemistry, and some have played a key role in the development of the science. The method of Lowry and coworkers, for example, is said to be the most often cited reference in the biochemical literature. [1;11]. The present work describes yet another protein assay, which offers ease of use, high precision and nanogram sensitivity. Its chief advantages over other procedures are that it consumes little of the sample, thus facilitating application of other analyses with the sample, and it utilizes stable materials (SDS, nitrocellulose paper and colloidal gold) commonly available in the laboratory. Like almost all such assays, it displays differential sensitivity with different proteins. A previous study of protein-colloidal gold interactions in solution postulated a monomolecular shell model with the number of binding sites on a gold particle for different proteins inversely proportional to protein molecular weight [8]. When we analyzed the dose response curves for different proteins in the Golddots assay, we did not observe such a simple relationship. While the slope of the dose response curves (a measure of assay sensitivity which depends on both affinity and binding capacity) were generally proportional to protein molecular weight, two proteins, SBTI and casein, displayed an anomalously high sensitivity for their size [9]. A better correlation was obtained between Golddots sensitivity and pI, although in this analysis, lysozyme was anomalous. These differences in assay sensitivity are similar to biases built into other protein assays. Despite this bias, the sensitivity, precision and ease of use of this assay make Golddots a useful procedure for the modern biomedical research laboratory.

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Figure 1. Golddots Assay. Duplicate samples (2 μ l) of three proteins were spotted on nitrocellulose paper, dried at room temperature, and stained with colloidal gold suspension (6 h). The nitrocellulose was then dried and digitized. Each row in the grid is a dilution series composed of 1000 ng (A), 500 ng (B), 250 ng (C) and 125 ng (D). Rows 1 & 2 are BSA, 3 & 4 carbonic anhydrase and 5 & 6 non-fat dry milk (Blotto).

Figure 2.

Time course of Golddots Assay. Triplicate samples of three concentrations of BSA were spotted on 7 identical pieces of nitrocellulose paper, and incubated with colloidal gold as described in the methods section. At the indicated times, one piece of paper was removed, dried and analyzed by densitometry. The samples contained 5.35 ng (◆), 22.5 ng (■) and 169 ng (●) of BSA per dot. Each point is the mean of three determinations; the error bars indicate the standard deviation.

Figure 3.

Dose response of the Golddots Assay. Triplicate spots of BSA (1.5 – 400 ng) were analyzed by the Golddots procedure. Each point is the mean of three values; the error bars are the standard deviations. Densitometry was performed using the Epson scanner (see Materials and Methods).

Figure 4.

Golddots dose-response curves for BSA and lysozyme. Golddots assays were carried out with serial dilutions of BSA (●) and lysozyme (○) 62.5–1000 ng/spot. The amount of protein spotted was determined by dry weight. The integrated spot intensity is plotted as a function of protein amount. The points represent the mean of duplicate dots, and the error bars the standard deviation. The lines are derived from a linear regression analysis of the data.

Table 1

Golddots sensitivity vs. protein molecular weight

Protein	Sensitivity (Spot Density/ng) ^d	Molecular weight	pI
Lysozyme	689.7	14,400	11.0
SBTI ^a	429.9	21,500	4.5
Blotto (caseins)	620.9	24,000	4.6
CA ^b	1096	29,000	6.0
BSA ^c	1239	66,000	5.82

^a Soybean Trypsin Inhibitor^b Carbonic Anhydrase (Bovine Erythrocyte)^c Bovine Serum Albumin^d Sensitivity is the slope of the dose-response regression line (see Figure 2).

Table 2

Comparison of the sample requirements and sensitivity of the Golddots and Micro BCA protein assays

Property	Golddots	Micro BCA
Range	1.5 –1500 ng	0.5 – 25 µg
Sample required	1.0 µg – 10 µl	20 µg – 200 µl
Assay volume	2 µl	100 µl