Open access • Book Chapter • DOI:10.1007/978-1-4939-8793-1_6

The Cydex Blue Assay: A One-Step Protein Assay for Samples Prior to SDS Electrophoresis. — Source link

Thierry Rabilloud

Institutions: University of Grenoble

Published on: 01 Jan 2019 - Methods of Molecular Biology (Humana Press, New York, NY)

Topics: Bradford protein assay, Sodium dodecyl sulfate and Protein purification

The Cydex Blue Assay: A One-Step Protein Assay for Samples Prior to SDS Electrophoresis

Thierry Rabilloud

The cydex blue assay: a one step protein assay for samples prior to SDS electrophoresis

Thierry Rabilloud [1,2,3]

1: CNRS UMR 5249, Laboratory of Chemistry and Biology of Metals, Grenoble, France
2: Univ. Grenoble Alpes, Laboratory of Chemistry and Biology of Metals, Grenoble, France
3: CEA Grenoble, BIG/CBM, Laboratory of Chemistry and Biology of Metals, Grenoble, France

to whom correspondence should be addressed:
Laboratoire de Chimie et Biologie des Métaux, UMR CNRS-CEA-UJF 5249, BIG/CBM, CEA Grenoble, 17 rue des martyrs, F-38054 Grenoble Cedex 9, France
thierry.rabilloud@cea.fr

running title: protein assay in the presence of detergents

keywords: cyclodextrin, detergents, dye-binding, protein assay, RIPA buffer, SDS electrophoresis, SDS buffer, Bradford assay

Abstract

Determination of protein concentrations prior to SDS electrophoresis is made difficult by the simultaneous presence of SDS and reducers in the buffers used for protein extraction. Reducers interfere with the copper-based assays while SDS interferes with the dye-binding assays. The combined use of cyclodextrins with a commercial Bradford reagent concentrate, described in this chapter, allows to determine protein concentrations in a Laemmli type buffer, containing both SDS and reducers, in a single step (without any precipitation) with a simple spectrophotometric assay. The use of various cyclodextrins brings compatibility not only with SDS, but also with other nonionic and ionic detergents such as sodium deoxycholate or detergents of the Triton type.

1.Introduction

In gel detection methods are generally used for two purposes. the first one is just protein visualization prior to band excision, often prior to a proteomic analysis of the excised band(s) [1]. The second and more important use is a comparative quantitative analysis for sample comparisons. A typical case is the one of 2D gel-based proteomics, in which the comparisons between samples is based on the intensity of the signals detected on the gels for the separated proteins [2] . In the frame of SDS electrophoresis, comparative quantification between different gel lanes is used either for not too complex samples, or as a normalization method prior to specific detection, e.g. by western blotting (e.g. [3, 4]).

However, gel detection has its own limits, and optimal performances require similar amounts of proteins to be loaded on the different 2D gels making a gel series, or on the different lanes making a SDS gel or a series of gels. This is due to threshold effects, e.g. lower limits of detection on the one side and saturation on the other side, and these limitations occur for any detection method.

This situation means in turn that the protein concentration must be reasonably determined in the samples that will be used for the electrophoretic separations. For various reasons, e.g. avoidance of protein degradation during sample preparation and storage, samples are generally directly solubilized in strongly denaturing buffers, i.e. containing high concentrations of urea for 2D electrophoresis or high concentrations of SDS and reducers for SDS electrophoresis. Alternatively, lysis buffers that do not destroy the nuclei and release most of the cytoplasmic proteins can be used prior to dilution in the final electrophoresis buffer. A classical example is the so called RIPA (Radio Immuno Precipitation Assay), which contains a cocktail of nonionic and anionic detergents.

Sensitive protein assays are based on two major principles. The first and most ancient one is the reducing effect of the peptide bond on the copper ion at high pH (the so called biuret reaction). The Cu(I) ion thus generated is the used to produce a color signal, either using its reducing properties (in the Lowry assay [5] or using a specific chelator (in the BCA assay [6].

The second principle used in protein assay is metachromasia, i.e. the ability of a dye to change its color (i.e. its absorption wavelength) depending on the environment, here the binding to proteins. The most popular method is the Bradford assay using Coomassie Blue G250 [7], but other dyes or metal-dye complexes have been used to build protein assays (e.g. [8])

These principles also determine in turn the interfering chemicals that will be able to bias the protein assays. In the copper-based assays, reducers present in the samples will induce copper reduction without the presence of any protein. In the dye-based assays, chemicals that will be able to bind to the dye and induce the metachromatic shift will be interfering, and this is the case of almost all detergents used for biochemical purposes.

This means in turn that buffers used for SDS electrophoresis, which contain both high concentrations of reducers and of SDS, are not easily amenable to protein assays as the buffer strongly interferes with both types of assay. The classical solution is then to precipitate the sample first, in order to remove the interfering substances, then to measure the protein concentration on the redissolved protein precipitate (e.g. in [9, 10]). Although efficient, such protocols are more cumbersome than direct assays and more prone to errors because of an increased number of steps.

A protocol has been however recently described for a single step assay of protein concentrations in SDS-reducers containing samples. This protocol is based on the Bradford assay and uses cyclodextrins to complex the detergent present in the sample and thus to remove the detergent interference [11]. A simplified version of this assay is presented here.

2.Materials

All solutions are prepared with ultrapure, deionized water and stored at +4°C without the addition of any preservative.

1. Semi-diluted Bradford reagent: This reagent is prepared from a commercial

concentrate (Bio Rad, catalog number #500-0006). 20 ml of this reagent are added to 30 ml of ultrapure water to prepare the working semi-diluted reagent (*see* **Note 1**)

2. BSA standard: Bovine serum albumin (e.g. Sigma, catalog number) is dissolved at 10mg/ml in ultrapure water.

3. Cyclodextrin reagent: alpha-cyclodextrin (e.g. Sigma, catalog number #28705) or beta-cyclodextrin (e.g. Alfa, catalog number # A14529) are dissolved at 5 mg/ml in ultrapure water. The dissolution is facilitated if the water is warmed prior to its addition to the cyclodextrin powder.

4. SDS sample buffer: A classical composition is used. The sample buffer contains 2% (w/v) SDS, 5% (v/v) beta mercaptoethanol, 10% (v/v) glycerol, 62.5 mM Tris-HCl buffer pH 6.8. Low concentrations of bromophenol blue (up to 0,004%) can be tolerated (*see* **Note 2**). For practical reasons we use a 2x concentrated buffer (4% SDS, 10% mercaptoethanol, 20% glycerol, 125 mM Tris buffer pH 6.8)

5. RIPA Buffer: There are several variants of this buffer described in the literature. The RIPA buffer used here contains 1% (v/v) IGEPAL 630, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM sodium chloride and 50 mM Tris-HCl buffer, pH 8.0. For practical reasons we use a 2x concentrated buffer (2% (v/v) IGEPAL 630, 1% sodium deoxycholate, 0.2% SDS, 300 mM sodium chloride and 100 mM Tris-HCl buffer, pH 8.0).

Semi-micro spectrophotometric cuvettes (maximum volume 3 ml) and a spectrophotometer able to operate at 595 nm are also needed. As the assay proceeds in the visible region of the spectrum, UV transparency is not needed, offering more flexibility regarding the material of the cuvettes.

3.Methods

1. BSA standard preparation: Using the 2x concentrated buffers (either RIPA or SDS sample buffer), water and the 10mg/ml BSA solution, prepare serial dilutions of BSA in the working buffer (RIPA 1x or SDS sample buffer 1x). The principle is that

different amounts of BSA will be added in the same sample volume to build the standard range. We use a 0-2 mg/ml concentration range by steps of 0.2 mg/ml, i.e. 0; 0.2; 0.4; 0.6 etc… mg/ml up to 2 mg/ml. Wider steps (e.g. 0.25 mg/ml) can be used but will result in a slightly lower precision.
The BSA standards tubes at their final dilution can be stored frozen at -20°C for several weeks

2. Samples preparation: Samples are considered prepared either in the RIPA buffer or in the SDS sample buffer. Depending on the expected concentration, serial dilutions (e.g. 2 fold, 5 fold and 10 fold) may be required (*see* **Note 3**). These dilutions must be prepared in the same buffer as the one used for the sample preparation and for the standard curve.

3. Working reagent for SDS samples: Mix one volume of 5 mg/ml alpha cyclodextrin solution and one volume of Semi-diluted Bradford reagent. Mix by inversion of the tube. Each point (blank, standard curve and samples) requires 1ml of this reagent. The working reagent is stable at least one week at room temperature

4. Working reagent for RIPA samples: Mix one volume of 5 mg/ml beta cyclodextrin solution and one volume of Semi-diluted Bradford reagent. Mix by inversion of the tube. Each point (blank, standard curve and samples) requires 1ml of this reagent. The working reagent is stable at least one week at room temperature

5. Assay procedure : Prepare the required numbers of 1.5 ml of 2 ml microcentrifuge tubes (*see* **Note 4**). In each tube, pipet 1ml of working reagent (*see* **Note 5**). Add 10 µl of sample per tube, either buffer alone for the blank, or BSA standards for building the standard curve or samples to determine their concentration. Mix by several inversions of the tube. The color takes 5 minutes to develop and is stable for 1 hour. Read the absorbance at 595 nm. The simplest procedure is to use the blank tube (buffer + color reagent) to zero the spectrophotometer. Alternatively, use water to zero the spectrophotometer and read the absorbance of all tubes including the blank(s).
Use the BSA dilution series to determine the standard curve (*see* **Note 6**), then determine the protein concentration of your samples. Typical standard curves are

shown in Figure 1.

6. Use your samples for SDS separations (*see* **Note 7**), as shown on Figure 2.

4. Notes

1: The commercial concentrated Bradford reagent is a rather viscous liquid, and is uneasy to pipet reproducibly in series, as need for a protein assay (standard curve + samples). The use of an intermediate dilution makes the pipetting much easier.

2: Bromophenol blue turns yellow in the very acidic environment of the Bradford assay, and this yellow form does not interfere with the reading at 595 nm

3 : The assay operates between 1-20 µg of protein in a 1 ml assay, and the best linearity is achieved between 2-10 µg of protein '(see also Figure 1). As the sample volume needs to be kept constant, this implies to use serial dilutions of the sample to get at least one measurement in the linear part of the standard curve. As the color is obtained after 5 minutes, additional sample dilutions can be performed and measured during the one hour stability period if needed.

4: It is advised not to rely on a single measurement, neither for the standard curve nor for the samples. Triplicate measurements result in a very high confidence determination of the protein concentration, but duplicate measurements are often sufficient.

5: Different working reagents are used for samples in RIPA buffer and for samples in SDS buffer. This means in turn that samples of each type must be read against a standard curve built in the same buffer type and with the adequate working reagent.

6: Different batches of the commercial concentrate and/or aging of the reagent result in slightly different response curves. Maximum precision requires the standard curve to be determined for each series of assays, but an often sufficient precision is obtained when the standard curve is run every week and at every change of a reagent, whichever comes first.

7: If RIPA samples are to be used in SDS electrophoresis, they will be diluted in SDS buffer. This means in turn that the protein concentration can be determined directly in the RIPA buffer or after dilution in the SDS buffer. It is preferable to determine the protein concentration directly in the RIPA buffer, using the beta-cyclodextrin reagent. The various concentrations of Igepal and sodium deoxycholate present in the sample after dilution in SDS induce variable interferences with the alpha-cyclodextrin reagent.

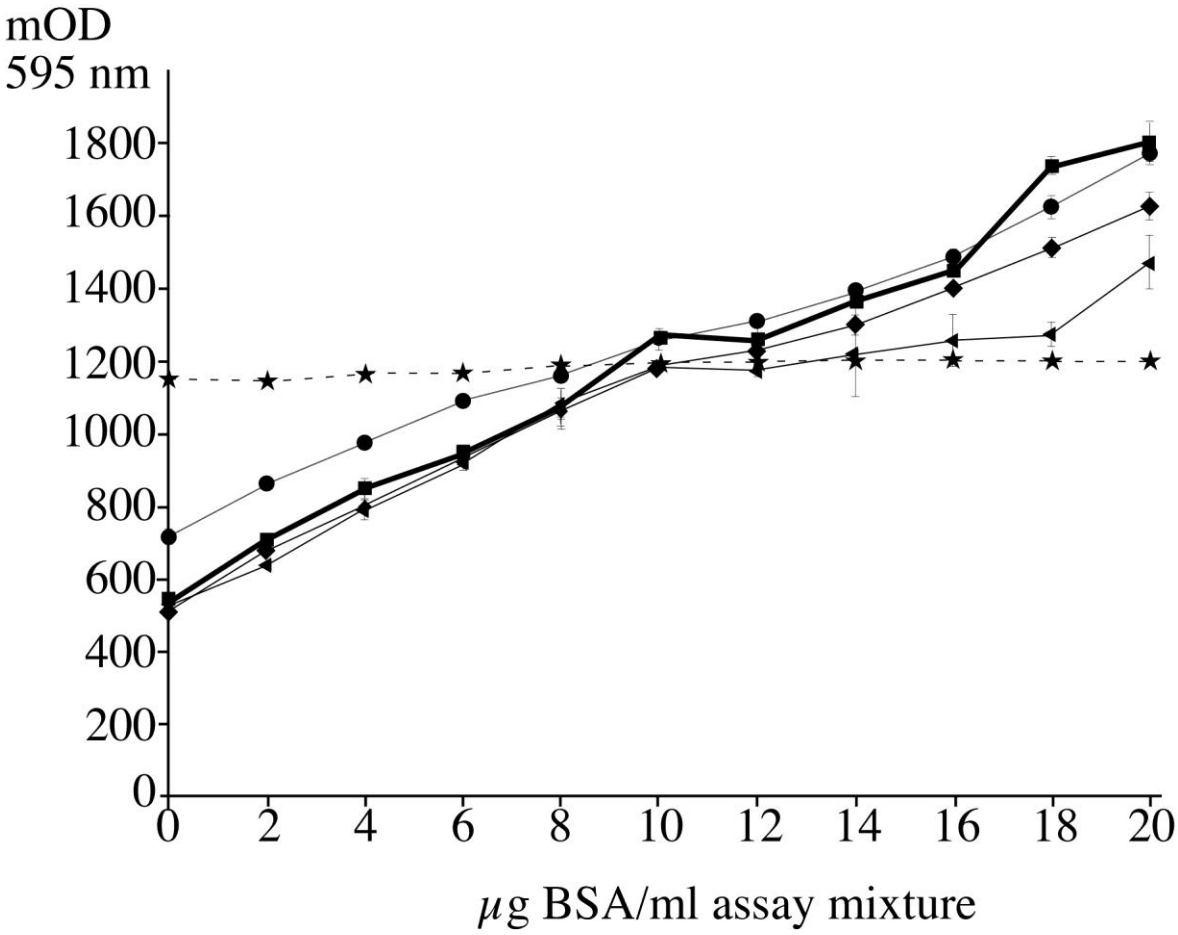

Figure 1: standard curves for BSA in various conditions

Standard curves were constructed using BSA in various conditions.

Squares and thick line: sample in water, standard Bradford reagent
Stars and dotted line: sample in 10µl SDS buffer, standard Bradford reagent
Circles and grey line: sample in 10µl RIPA buffer, Bradford reagent with beta cyclodextrin
Triangles: sample in water, Bradford reagent with alpha cyclodextrin
Diamonds: sample in SDS buffer, Bradford reagent with alpha cyclodextrin

Determinations made in duplicate using water as a blank. Non visible error bars mean that they are smaller than the symbols.

Note that the SDS buffer results in such an interference that no assay is feasible with the classical Bradford reagent. The linearity of the assay is optimal below 10µg of protein per ml of assay.
average slopes for the 0-10µg concentration range
standard bradford: 73 mOD/µg protein in 1ml assay
bradford with alpha cyclodextrin, sample in water: 66 mOD/µg protein in 1ml assay
bradford with alpha cyclodextrin, sample in SDS buffer: 67 mOD/µg protein in 1ml assay
bradford with beta cyclodextrin, sample in RIPA buffer: 54 mOD/µg protein in 1ml assay.
Note also the higher blank value with beta cyclodextrin.

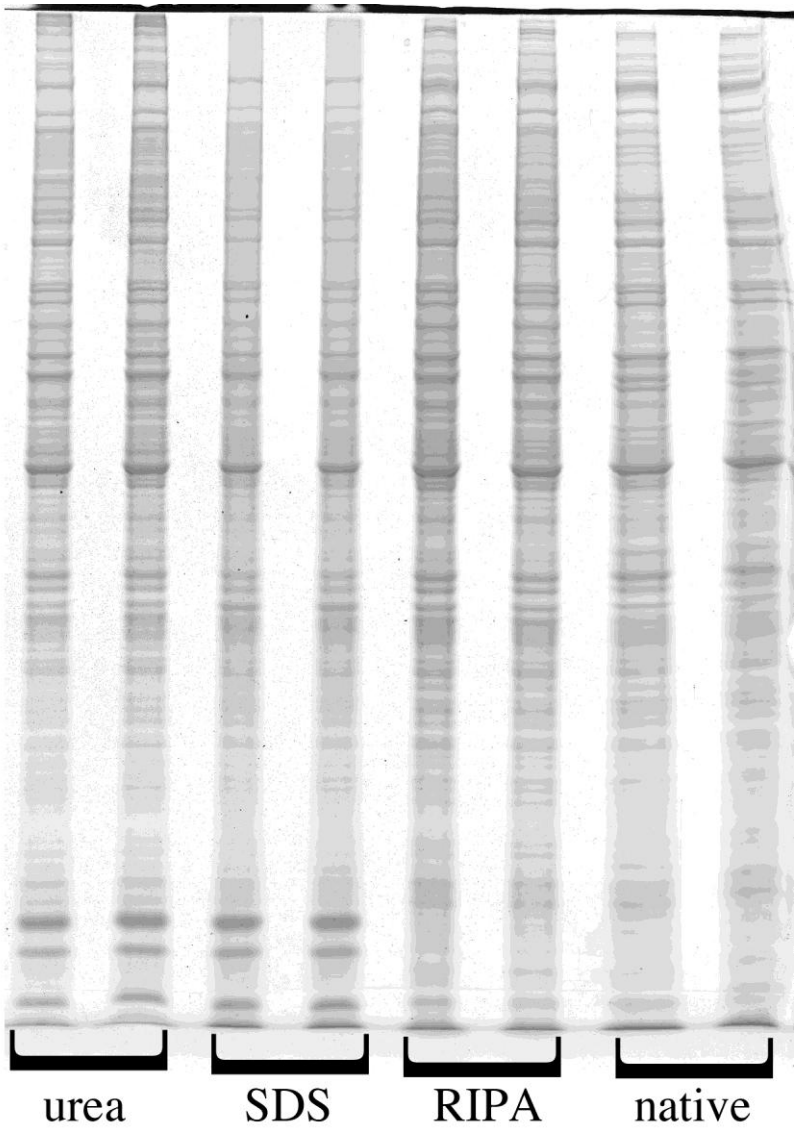

Figure 2: Example of SDS separation after protein determination

RAW264.7 cells were harvested and distributed in several aliquots, on which different protein extractions were performed

Native: cell pellets were treated with 6 volumes of native lysis buffer (Hepes NaOH 10 mM pH 7.5, KCl 50 mM, MgCl2 2 mM, 3-[tetradecyldimethylammonio]-propane-1 sulfonate (SB 3-14) 0.1% for 30 minutes at 0°C. The lysate was cleared by centrifugation (2000g, 10 minutes, 4°C). The supernatant was recovered and the protein concentration measured by a classical Bradford assay.

Urea: cell pellets were resuspended in a equal volume of PBS and the suspension was extracted by 4 times its volume of urea lysis buffer (9M urea, 1.25M thiourea, 5% CHAPS, 12.5 mM TCEP, 25mM spermine base, 25mM spermine tetrahydrochloride) and the proteins were extracted for 1 hour at room temperature. The lysate was cleared by centrifugation (15000g, 15 minutes 20°C) The supernatant was recovered and the protein concentration measured by a classical Bradford assay.

RIPA: cell pellets were treated with 8 volumes of 1x RIPA buffer for 30 minutes at

0°C. The lysate was cleared by centrifugation (2000g, 10 minutes, 4°C). The supernatant was recovered and the protein concentration measured by the modified Bradford assay using the beta cyclodextrin working reagent.

SDS: cell pellets were treated with 8 volumes of 1x SDS sample buffer for 30 minutes 70°C. The lysate was cooled down at room temperature and the DNA sheared by passing through a syringe fitted with a 0.8mm needle . The supernatant was recovered and the protein concentration measured by the modified Bradford assay using the alpha cyclodextrin working reagent.

Protein determinations for RIPA and SDS samples were made on 10x dilutions in the corresponding buffer (10µl of the final dilution in 1ml of final assay mixture)

Forty micrograms of proteins, as determined from the various assays, were diluted in SDS sample buffer with bromophenol blue (40µl final volume). Native and RIPA samples, after dilution in SDS, were heated at 95°C for 5 minutes. The samples were loaded on the gel, migrated and stained with colloidal Coomassie Blue [12]

Each lane corresponds to a different sample, starting from a different culture aliquot. This means that the sample preparations and protein concentration determination processes were carried out independently for each lane.
The comparable staining intensities in the various lanes shows that the protein determination was accurate

References

[1] Schirle, M., Heurtier, M. A., Kuster, B., Profiling core proteomes of human cell lines by one-dimensional PAGE and liquid chromatography-tandem mass spectrometry. *Mol Cell Proteomics* 2003, *2*, 1297-1305.

[2] Rabilloud, T., The whereabouts of 2D gels in quantitative proteomics. *Methods Mol Biol* 2012, *893*, 25-35.

[3] Welinder, C., Ekblad, L., Coomassie staining as loading control in Western blot analysis. *J Proteome Res* 2011, *10*, 1416-1419.

[4] Eaton, S. L., Roche, S. L., Llavero Hurtado, M., Oldknow, K. J.*, et al.*, Total protein analysis as a reliable loading control for quantitative fluorescent Western blotting. *PLoS One* 2013, *8*, e72457.

[5] Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951, *193*, 265-275.

[6] Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K.*, et al.*, Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985, *150*, 76-85.

[7] Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, *72*, 248-254.

[8] Fujita, Y., Mori, I., Kitano, S., Determination of proteins by using the color reaction with pyrocatechol violet-molybdenum(VI) complex. *Chem Pharm Bull (Tokyo)* 1984, *32*, 4161-4164.

[9] Bensadoun, A., Weinstein, D., Assay of proteins in the presence of interfering materials. *Anal Biochem* 1976, *70*, 241-250.

[10] Peterson, G. L., A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem* 1977, *83*, 346-356.

[11] Rabilloud, T., A single step protein assay that is both detergent and reducer compatible: The cydex blue assay. *Electrophoresis* 2016, *37*, 2595-2601.

[12] Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W., Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 1988, *9*, 255-262.