

Ellman's assay for in-solution quantification of sulfhydryl groups

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Introduction

Ellman introduced 5,5'-dithio-bis-(2-nitrobenzoic acid), also known as DTNB, as a compound used for quantitating free sulfhydryl groups in solution (Figure 1). A solution of this compound produces a yellow-colored product when it reacts with sulfhydryl groups. This yellow-colored product can be measured using a spectrophotometer at 412 nm.

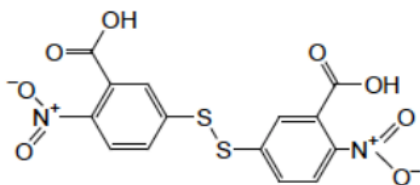


Fig. 1: Structure of Ellman's Reagent

Thiol chemistry is a rapidly expanding field in basic and applied bioscience. As a consequence, the quantitative measurement of -SH groups is a routine task in many applied disciplines where a quick and easy method is much preferred. Electrochemical and fluorimetric assays are very sensitive and accurate but they involve lengthy procedures (complete proteolysis, electrolysis, HPLC separation). Although spectrophotometric thiol assays such as Ellman's are less sensitive in comparison, they are rapid and simple and are therefore more popular for the quantification of sulfhydryls.

Assay Principle

DTNB reacts with a free sulfhydryl group to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB; see Figure 2). The target of DTNB in this reaction is the conjugate base (R-S-) of a free sulfhydryl group. TNB is the "colored" species produced in this reaction and has a high molar extinction coefficient in the visible range. The molar extinction coefficient

of TNB is reported to be $13,600 \text{ M}^{-1}\text{cm}^{-1}$ at 412 nm and pH 8.0.

Sulfhydryl groups may be estimated in a sample by comparison to a standard curve composed of known concentrations of a sulfhydryl-containing compound such as cysteine.

Alternatively, sulfhydryl groups may be quantitated by reference to the extinction coefficient of TNB.

Materials & Methods

- Flat-Bottom 96-well plate (NUNC)
- SPECTROstar^{Nano}
- Reaction Buffer (RB): 0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA
- Cysteine Hydrochloride Monohydrate (MW 175.6)
- Ellman's Reagent 5,5'-dithio-bis-(2-nitrobenzoic acid)

Experimental Procedure

Thaw Ellman's Reagent and Cysteine to room temperature prior to use. Prepare Ellman's Reagent Solution by dissolving 4 mg thawed Ellman's Reagent in 1 mL RB.

1. Dissolve Cysteine Hydrochloride Monohydrate in RB to make Standard A (1.6 mM). Prepare a set of cysteine standards by diluting

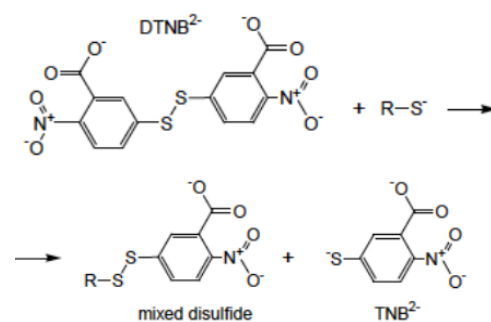


Fig 2. Reduction of Ellman's Reagent

Standard A in 2-fold dilution series to 0.1 mM.

2. Prepare a set of test tubes, each containing 50 μL of Ellman's Reagent Solution and 2.5 mL of RB
3. Add 250 μL of each standard or unknown to the separate test tubes prepared in step 2.
4. Mix and incubate at room temperature for 15 minutes. Add 200 μL of each solution to a 96-well plate.
5. Use the SpectroStarNano to take a full absorbance spectrum of the compound (see table 1)
6. Plot the OD values obtained 412 nm for the standards to generate a standard curve. Determine the experimental sample concentrations from this curve using the formula (1) below.

Tab. 1 Instrumental settings

Optic settings	Absorbance, endpoint	
Wavelength settings	Spectra	250-500 nm
	Resolution	2
Pathlength correction	On, volume 250 μL	
Speed and precision	precise	

$$C_{\text{Sulfhydryls}} = \frac{\text{Absorbance of Unknown} - Y_{\text{int of Std. Curve}}}{\text{Slope of Std. Curve}} \quad (1)$$

Results & Discussion

Cysteine Standard Curve

The standard curve itself can be a useful indicator of the strength of the assay. By taking the full spectrum of absorbance values for each solution, the isosbestic point can be determined either tabularly or graphically to confirm that the molar ratio between the Ellman's Reagent and test sample are equivalent across each test solution. The isosbestic point for Ellman's assay is approximately 356 nm (Figure 3). A smooth peak at 412 nm also indicates that your solution falls within the working range of the assay.

Calculating Sulphydryl Concentrations of Unknown Solutions

Similarly, the absorbance spectra of the unknown solutions can be compared to those of the standard curve to ensure that the concentrations of the solutions fall within the range of the standard curve (Figure 4). The absorbance values for Unknown Solutions A and B were plotted along the standard curve, corresponding to a sulphydryl concentrations of 1.234 mM and 0.810 mM, respectively (Figure 5).

Conclusion

Ellman's assay is a useful tool that can be used to determine the sulphydryl concentration of unknown solutions. The assay can also be adapted to accommodate larger volumes of test sample for

readings in a cuvette by using Beer's Law and the extinction coefficient of TNB.

Ellman's Reagent may be used for applications other than the estimation of sulphydryls in solution. It has been used for the determination of alkylthiols by HPLC using a pre-column derivatization procedure and to study thiols in the active site of several enzymes including thiolase fatty acid synthase and mevalonate 5-diphosphate decarboxylase as reduction in compound and sample/cell usage.

References

1. Thermo Scientific. (2011). Ellman's Reagent
2. IUPAC. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled A.D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997).
3. Riener, C. K., Kada, G., & Gruber, H. J. (2002). Quick measurement of protein sulphydryls with Ellman's reagent and with 4,4'-dithiopyridine. *Analytical and Bioanalytical Chemistry*, 373 (4-5), 266-276.

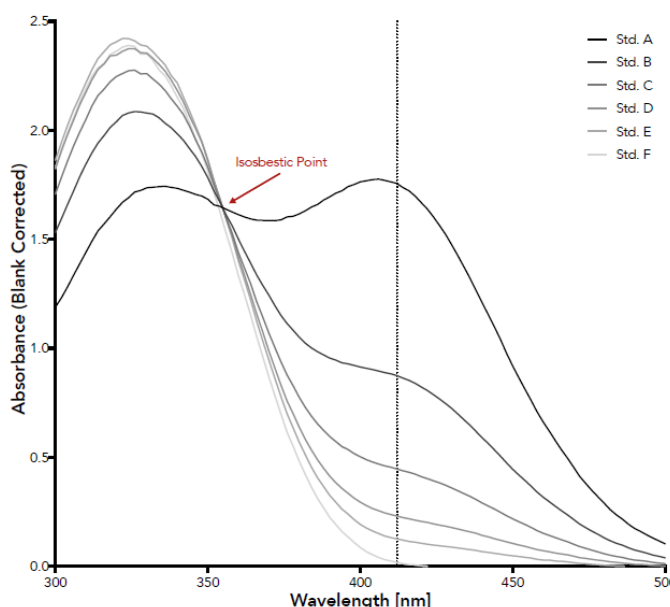


Fig. 3: Absorbance spectra of cysteine standards

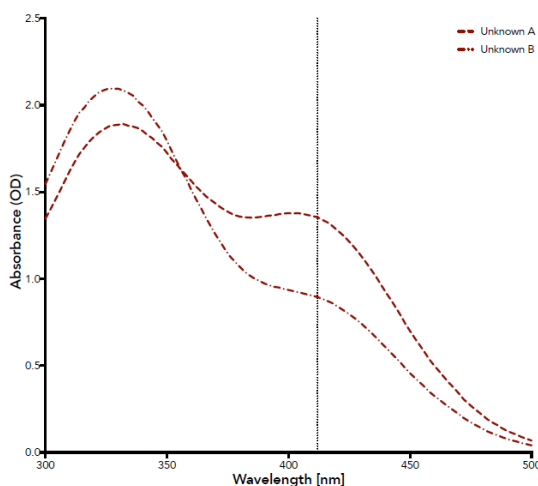


Fig. 4. Absorbance Spectra of Sulphydryl-Containing Unknown Solutions

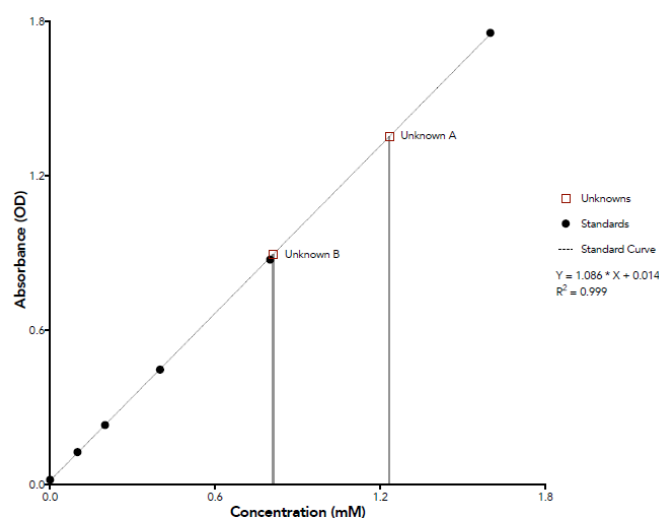


Fig. 5. Quantification of Sulphydryl Groups in Three Unknown Test Solutions