INSTRUCTIONS

Ellman's Reagent

22582

0311.3

Number	Description
22582	**Ellman's Reagent**, 5,5'-Dithio-*bis*-(2-nitrobenzoic acid), 5g Molecular Weight: 396.35 CAS #: 69-78-3

Storage: Store product protected from moisture at 4°C. Product is shipped at ambient temperature.

Table of Contents

Introduction

In 1959 Ellman[1] introduced 5,5'-dithio-*bis*-(2-nitrobenzoic acid), also known as DTNB, as a versatile water-soluble compound for quantitating free sulfhydryl groups in solution (Figure 1, see Additional Information section). A solution of this compound produces a measurable yellow-colored product when it reacts with sulfhydryls. Consequently, Thermo Scientific Ellman's Reagent is very useful as a sulfhydryl assay reagent because of its specificity for -SH groups at neutral pH, high molar extinction coefficient and short reaction time.

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. Therefore, the rate of this reaction is dependent on several factors:[2] 1) the reaction pH, 2) the pKa' of the sulfhydryl and 3) steric and electrostatic effects. 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 was originally reported to be $13{,}600 M^{-1}cm^{-1}$ at 412nm and pH 8.0.[1] Consequently, this value has been cited frequently in the literature. Later studies have shown, however, that the molar extinction coefficient is more accurately reflected by a value of $14{,}150 M^{-1}cm^{-1}$ at 412nm.[2,3] The extinction of TNB is not affected by changes in pH between 7.6 and 8.6.[3] However, the extinction of TNB is different in other solvents (Table 1).

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. Both methods are presented in these instructions.

Ellman's Reagent may be used for applications other than the estimation of sulfhydryls in solution. It has been used for the determination of alkylthiols by HPLC using a pre-column derivatization procedure[4] and to study thiols in the active site of several enzymes including thiolase,[5] fatty acid synthase[6] and mevalonate 5-diphosphate decarboxylase.[7]

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Procedure for Quantitating Sulfhydryl Groups Using a Cysteine Standard

A. Material Preparation

- Reaction Buffer: 0.1M sodium phosphate, pH 8.0, containing 1mM EDTA
- Cysteine Hydrochloride Monohydrate: M.W. = 175.6, Product No. 44889 (see Related Thermo Scientific Products)
- Ellman's Reagent Solution: Dissolve 4mg Ellman's Reagent in 1mL of Reaction Buffer.

B. Procedure

1. Prepare a set of cysteine standards by dissolving Cysteine Hydrochloride Monohydrate at the following concentrations in Reaction Buffer:

Standard	Volume of Reaction Buffer	Amount of Cysteine (M.W. = 175.6)	Final Concentration
A	100mL	26.34mg	1.5mM
B	5mL	25mL of Standard A	1.25mM
C	10mL	20mL of Standard A	1.0mM
D	15mL	15mL of Standard A	0.75mM
E	20mL	10mL of Standard A	0.5mM
F	25mL	5mL of Standard A	0.25mM
G	30mL	0ml	0.0mM (Blank)

2. Prepare a set of test tubes, each containing 50µL of Ellman's Reagent Solution and 2.5mL of Reaction Buffer.
3. Add 250µL of each standard or unknown to the separate test tubes prepared in step 2.

   Note: For the unknown(s), make dilutions so that the 250µL sample applied to the assay reaction has a sulfhydryl concentration in the working range of the standard curve (0.1-1.0mM is ideal).
4. Mix and incubate at room temperature for 15 minutes.
5. Measure absorbance at 412nm.
6. Plot the values obtained for the standards to generate a standard curve. Determine the experimental sample concentrations from this curve.

   Note: The most accurate results are obtained from the linear portion of the standard curve; i.e, the portion yielding an r^2 value equal to 1.0. One or more of the high standards may exceed the linear range.

Procedure for Quantitating Sulfhydryl Groups Based on Molar Absorptivity

A. Material Preparation

- Reaction Buffer: 0.1M sodium phosphate, pH 8.0, containing 1mM EDTA
- Ellman's Reagent Solution: Dissolve 4mg Ellman's Reagent in 1mL of Reaction Buffer.

B. Measure Absorbance

1. For each unknown sample to be tested, prepare a tube containing 50µL of Ellman's Reagent Solution and 2.5mL of Reaction Buffer.
2. Add 250µL of each unknown to the separate test tubes prepared in step 1. As a blank, add 250µL of Reaction Buffer to a separate test tube prepared in Step 1.

   Note: For the unknown(s), make dilutions so that the 250µL sample applied to the assay reaction has a sulfhydryl concentration less than 1.0mM. Concentrations exceeding 1mM free sulfhydryl will result in high absorbance values and less accurate estimation of the concentration based on the extinction coefficient.
3. Mix and incubate at room temperature for 15 minutes.
4. With a spectrophotometer set to 412nm, zero the instrument on the blank and then measure absorbance of each sample.
5. Calculate the amount and concentration of sulfhydryls in the sample from the molar extinction coefficient of TNB ($14{,}150 M^{-1}cm^{-1}$), as exemplified in Section C.

C. Example Calculation of the Free Sulfhydryl Concentration

A 250µL aliquot of the unknown mixed with 2.5mL of Reaction Buffer and 50µL of Ellman's Reagent Solution gave an absorbance of 0.879 (after subtracting the blank) using a 1cm spectrophotometric cuvette. Calculate the sulfhydryl concentration in µmoles per mL of unknown. The reported molar absorptivity (molar extinction coefficient, which is expressed in units of $M^{-1}cm^{-1}$) of TNB in this buffer system at 412nm is 14,150.[2] Molar absorptivity, E, is defined as follows:

$$E = \frac{A}{bc}$$ where A = absorbance, b = path length in centimeters, c = concentration in moles/liter (=M)

Solving for concentration gives the following formula: $c = \frac{A}{b\mathrm{E}}$

In the present example, A = 0.879, b = 1cm and E = 14,150$M^{-1}cm^{-1}$. Therefore, $c = \frac{0.879}{1(14{,}150)} = 6.21\times10^{-5}\,\mathrm{M}$

This value represents the concentration of the solution in the spectrophotometric cuvette. To calculate the concentration of the unknown sample, it is necessary to account for dilution factors as follows:

The total volume of the solution being measured is

2.50mL of Reaction Buffer
+ 0.25mL of Unknown Sample
+ 0.05mL of Ellman's Reagent Solution
2.80mL of solution

If the concentration of the assay solution is 6.21 x 10^{-5} M, then 2.80mL of that solution contains

$$2.80\mathrm{mL}\times\frac{1\mathrm{L}}{1000\mathrm{mL}}\times(6.21\times10^{-5}\,\mathrm{moles/L}) = 1.74\times10^{-7}\,\mathrm{moles}$$

These 1.74 x 10^{-7} moles of sulfhydryl in the assay solution were contributed by the original 0.25mL sample. Therefore, the concentration of free sulfhydryl in the original unknown sample is

$$\frac{1.74\times10^{-7}\,\mathrm{moles}}{0.25\mathrm{mL}}\times\frac{1000\mathrm{mL}}{\mathrm{L}} = 6.96\times10^{-4}\,\mathrm{M}$$

Troubleshooting

Problem	Possible Cause	Solution
Sulfhydryl content is lower than expected	Sulfhydryls in sample are not in reduced (free) state; i.e., have become oxidized	Minimize time delay between assay of sample and its use in applications that depend on its free sulfhydryl content
		Maintain 1-5mM EDTA in sample to chelate divalent metal ions, which can oxidize sulfhydryls
		Ensure reduction of sulfhydryls using a reducing agent (see Related Thermo Scientific Products)

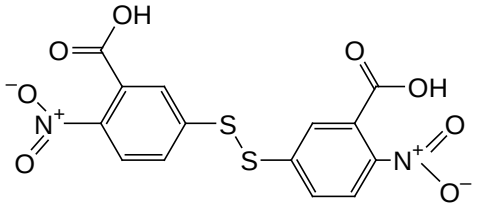
Related Thermo Scientific Products

44889 **Cysteine•HCl•H_2O**, 5g, M.W. 175.6

77712 **Immobilized TCEP Disulfide Reducing Gel**, 5mL

44999 **SulfoLink® Immobilization Kit for Peptides**, 2mL

Additional Information



Ellman's Reagent;
5,5'-Dithio-*bis*-(2-nitrobenzoic acid)
$C_{14}H_8N_2O_8S2$
Mol. Wt.: 396.35

Figure 1. Structure of Ellman's Reagent

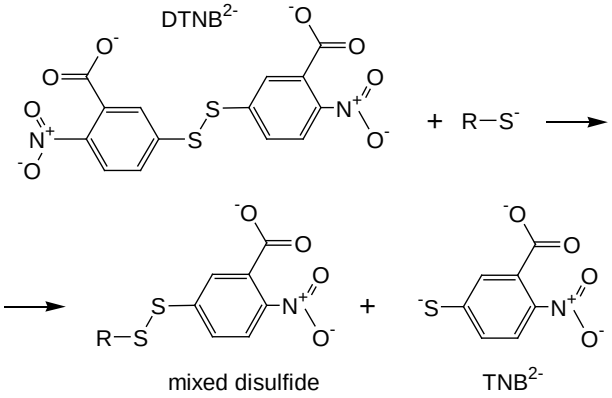


Figure 2. Reduction of Ellman's Reagent

Table 1. Molar extinction coefficients of Ellman's Reagent in various solvents

Solvent	E at 412nm
2% SDS	12,500
0.1M phosphate, pH 8.0, 1mM EDTA	14,150
Buffered 6M guanidine hydrochloride	13,700
6M guanidine hydrochloride	13,880
8M urea	14,290

Cited References

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2. Riddles, P.W., *et al.* (1983) Reassessment of Ellman's reagent. *Meth Enzymol* **91:**49-60.
3. Riddles, P.W., *et al.* (1979) Ellman's reagent: 5, 5´-dithiobis(2-nitrobenzoic acid) - a reexamination. *Anal Biochem* **94:**75-81.
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5. Masamune, S, *et al.* (1989) Bio-Claisen condensation catalyzed by thiolase from *Zoogloea ramigera.* Active site cysteine residues. *Chemtracts-Organic Chem* **2:**247-51.
6. Tsukamoto, Y. and Wakil, S.J. (1988) Isolation and mapping of the ß-hydroxyacyl dehydratase activity of chicken liver fatty acid synthase. *J Biol Chem* **263:**16225-9.
7. Alvear, M., *et al.* (1989) Fractionation of chicken liver mevalonate 5-diphosphate decarboxylase by sulfhydryl-directed reagents: evidence of a functional dithiol. *Biochem Biophys Acta* **994:**7-11.