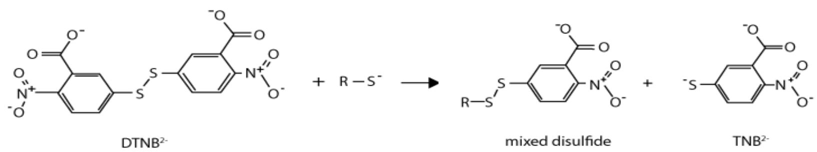
Ellman's Assay

Introduction

Ellman's reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid), also known as DTNB, is a water-soluble compound reacts with free sulfhydryl groups in solutions at slightly alkaline condition to yield a mixed disulfide and highly chromogenic 5-nitro-2-thiobenzoic acid (TNB) with molar extinction coefficient (molar absorptivity) of 14.15 $mM^{-1}cm^{-1}$ at 412 nm.



This is a standard protocol prepared by BroadPharm for determination of free thiols in small peptides and proteins, which are usually in their reduced from and are stable to oxidation in acidic solutions. Ellman's assay is necessary before conjugation of Fab' fragments and thiolated antibodies to liposomes containing maleimide.

Quantification of sulfhydryls using cysteine standards

1. Prepare 0.1 M sodium phosphate buffer solution, pH 8.0 (Reaction Buffer).
2. Dissolve 4 mg/mL of Ellman's reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) in the Reaction Buffer (Ellman's Reagent Solution).
3. Dissolve 5.268 mg of cysteine hydrochloride monohydrate (MW = 175.6) in Reaction Buffer at an initial concentration 1.5 mM (Standard A in the table below). Prepare a set of cysteine standards by serially diluting Standard A based on the table below.
4. Add 50 µL of Ellman's reagent to each standard and mix well.
5. Incubate at room temperature for 15 min.
6. Measure the optical absorbance at 412 nm.
7. Plot the absorbance values obtained for each standard versus cysteine concentrations (calibration curve), and determine the concentration of free sulfhydryl in the sample from the curve.

Final Concentration (mM)	Cysteine	Reaction Buffer (mL)	Standard
1.50	5.268 mg	20	A
1.25	5 mL of Standard A	1	B
1.00	4 mL of Standard A	2	C
0.75	3 mL of Standard A	3	D
0.50	2 mL of Standard A	4	E
0.25	1 mL of Standard A	5	F
0.00	0	5	Blank

Quantification of sulfhydryls using extinction coefficient calculation

1. Prepare 0.1 M sodium phosphate buffer solution, pH 8.0 (Reaction Buffer).

2. Dissolve 4 mg Ellman's Reagent in 1 mL of Reaction Buffer (Ellman's Reagent Solution).
3. For each sample, prepare two test tubes containing 25 µL of Ellman's Reagent Solution and 1.250 mL of Reaction Buffer.
4. Add 0.125 µL of unknown sample to a test tube prepared in Step 3.
5. Prepare a blank by adding 125 µL of Reaction Buffer to the other test tube prepared in Step 3.
6. Mix well and incubate at room temperature for 15 minutes.
7. Zero the spectrophotometer on the blank at 412 nm, and measure the optical absorbance of the sample.

Note: High concentrations (>1 mM) of free sulfhydryl may decrease the accuracy of the measured concentration of the sulfhydryl due to its higher absorbance values. In this case, it is recommended to dilute the sample solution so that 0.1786 mL aliquot contains less than 1 mM free sulfhydryl.

Calculate the free sulfhydryl concentration

A= ƐbC

Where

A: absorbance at 412nm;

b is the path length (cm),

C is concentration of the solution in the spectrophotometric cuvette (M).

Ɛ: Molar absorptivity,

Example:

A 125 µL aliquot of unknown sample is mixed with 1.250 mL of Reaction Buffer and 25 µL of Ellman's Reagent Solution gave an absorbance of 0.750 (after subtracting the blank) using a 1 cm spectrophotometric cuvette. Calculate the sulfhydryl concentration in µmoles per mL of unknown sample.

$0.75= 14150*1*C$

$C=5.300x\ 10^{-5}$ M (mol/L)

To calculate the concentration of sulfhydryl in the sample, the total volume of the assay and total moles of free sulfhydryl are needed.

$V_{total\ =}\ V\ \text{reaction-buffer} + V_{\text{Ellman\_ Reagent\_Buffer}} + V_{sample}$

$V_{total} ==1.25+ 0.025+0.125=1.4ml$

Therefore, the number of moles of sulfhydryl in the aliquot:

$1.4\times10^{-3}\times 5.3\times10^{-5} = 7.42\times 10^{-8}$ mol

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Since the assay solution contains only 0.1786 mL of the original sample. The concentration of free sulfhydryl in the original sample is obtained as follow:

$C\ sample = 7.42\times 10^{-8}/(0.125\times 10^{-3})$

$=5.92\times 10^{-4}\ M$

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