MANUAL

Total Thiol Quantification Assay (Ellman's test) Kit

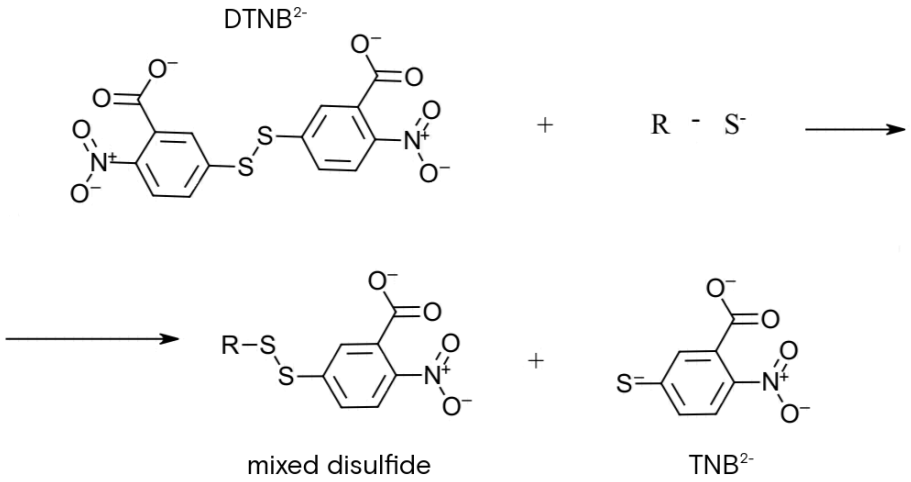
Catalog #: MA-THL

ISO 13485:2016

Introduction

Thiols are organosulfur compounds containing a thiol group or sulfhydryl group. A thiol group is the sulfur analogue of a hydroxyl group. Due to its high reactivity, thiol plays an important role in biomolecules. For example, cysteine residues contain thiol groups and can form cystine which has a disulfide bond to maintain the specific structure and stability of a protein. Many enzymes and cofactors such as coenzyme A rely on a thiol group as its key reactive group. In addition, thiols are easily oxidized, and glutathione is an important antioxidant in cells. These properties make changes in thiol levels a good indicator of diseases, oxidative stress, and enzyme activity.

Free thiols react with 5,5′-dithiobis-(2-nitrobenzoic acid), also known as DTNB or Ellman's reagent, to release 2-nitro-5-thiobenzoate (TNB^-) as the below figure shows. TNB^- ionizes to the TNB^{2-} dianion at neutral and alkaline pH in aqueous solvent, which shows yellow color. Because the formation of TNB^- is proportional to thiol, this reaction can be used for quantifying the amount of thiol.



RayBio® Total Thiol Quantification Assay (Ellman's test) Kit is designed based on the Ellman's reaction and optimized for the high-throughput microtiter form. It can be used to easily and quickly measure the amount of total free thiol in a given sample with a range of 0-40 nmole per well.

Storage

All components, except the DTNB reagent, can be stored at room temperature for up to 1 year from the date of shipment. The DTNB reagent should be stored at 4 ºC upon receipt. For prepared reagent storage, see table below.

Component	Size / Description			Storage	Storage After Preparation
	MA-THL-1	MA-THL-2	MA-THL-4		
DTNB reagent	1 vial	2 vials	4 vials	4 ºC	4 ºC
Assay Diluent F	50 ml	50 ml	100 ml	RT	RT
L-cysteine standard	2 vials	2 vials	4 vials	RT	Do not store and reuse. Keep on ice.
96 well microplate	1 plate	2 plates	4 plates	RT	RT

RT = room temperature

Note: Once reconstituted, the L-Cysteine standard should be used within the same day. Additional lyophilized L-cysteine standard is available for purchase. The DTNB reagent may be stored for longer after reconstitution. Protect from light, and discard if it turns yellow.

Additional Materials Required

1. Precision pipettes to deliver 2 µl to 1 ml.
2. Plate reader.
3. Serological Pipettes (25 ml).
4. Microcentrifuge tubes.
5. 50 ml conical tubes for preparing reagent working solution.

Sample Tips and General Considerations

Samples should not contain insoluble particles or cells, which can interfere with the reading of absorbance. These particles may be removed by centrifugation or filtration. As thiols are easily oxidized, samples should be assayed as soon as possible or stored short-term at -80 °C. If the samples must be stored long-term, antioxidants or EDTA may be added to prevent the oxidation of thiol groups before freezing.

In addition, the assay cannot distinguish between thiols from different molecules. DNTB also contains a disulfide bond, which can be reduced by some reagents. To avoid interference, buffers containing thiols and strong reductants such as dithiothreitol (DTT) and 2-mercaptoethanol should be avoided.

As a colorimetric assay, the absorbance of sample itself can affect the result if it is not determined. To assess background signal from sample type, prepare a diluted sample to include in the assay by mixing 25 µl of sample and 0.25 ml of Assay Diluent F.

NOTE: Optimal methods of sample preparation will need to be determined by each researcher empirically based on researched literature and knowledge of the samples.

1.Cell lysate
For attached cells, remove supernatant from cell culture, wash cells twice with cold 1× PBS (for suspension cells, pellet the cells by spinning down the cells at 1,000× g for 10 minutes) making sure to remove any remaining PBS before adding lysis buffer. Solubilize the cells at 2×10^7 cells/ml in lysis buffer containing protease inhibitors. Pipette up and down to resuspend cells and rock the lysates gently at 2–8 °C for 30 minutes. The lysates can be centrifuged at 14,000× g for 10 minutes to remove the insoluble particles. Lysates can be assayed directly with or without dilution. The supernatant can also be assayed directly.

Lysates should be used immediately or aliquot and stored at -80 °C. Thawed lysates should be kept on ice prior to use. General tips for preparing lysate samples can be viewed on the online Resources page of the website: https://www.raybiotech.com/tips-on-sample-preparation/

2. Plasma samples
Collect blood with an anticoagulant such as citrate, EDTA or oxalate and mix by inversion. Centrifuge the blood at 1,000× g at 4 °C for 10 minutes. Collect plasma supernatant without disturbing the white buffy layer. Samples should be tested immediately or frozen at -80 °C for storage. Samples can be assayed directly.

3. Serum samples
Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 2,500× g for 20 minutes. Remove the yellow serum supernatant without disturbing the white buffy layer. Samples should be tested immediately or frozen at -80 °C for storage. Samples can be assayed directly.

4. Urine samples
To remove insoluble particles, spin at 10,000× g for 5 minutes. The supernatant can be assayed directly.

5. Enzyme assay products
DTNB can also react with the thiol groups of the enzyme and inactivate the enzyme with cysteine as a catalytic center. Thus, measuring the thiols after the enzymatic reaction is recommended. As the color development for this assay requires neutral or alkaline pH, pH adjustment is needed if the enzymatic reaction was terminated by acid. The assay mix can be assayed directly if it does not contain insoluble particles.

Reagent Preparation

1. DNTB working solution
   a. Briley spin the vial of DTNB reagent. Add 1 ml of Assay Diluent F into the vial and reconstitute the powder thoroughly by a gentle mix.
   b. In a 50 ml conical tube, make a 50-fold dilution of the DTNB reagent by mixing 0.5 ml of the DTNB reagent and 25 ml Assay Diluent F to prepare the DTNB working solution.

   Note: As the DTNB working solution is not stable, it should be discarded after each use. The volume of working solution prepared may be adjusted based on the number of standards and samples. Each well needs 0.25 ml of the DTNB working solution.

2. Preparation of L-cysteine standard samples
   a. Dissolve 1 vial of the L-cysteine standard in 0.5 ml of Assay Diluent F.
   b. In a microcentrifuge tube labeled as Standard 1, add 0.9 ml of Assay Diluent F and 100 µl of the reconstituted L-cysteine standard. Mix thoroughly.
   c. Label another 6 microcentrifuges as Standard 2 through 7. Add 200 µl of the Assay Diluent F to each tube.
   d. Perform a 2-fold serial dilution by transferring 200 µl liquid from Standard 1 to Standard 2. Repeat the transfer until Standard 7. Mix each tube thoroughly before the next transfer. The 7 standards prepared contain 1500, 750, 375, 187.5, 93.75, 46.88, 23.44 µM of L-cysteine, respectively.
   e. Label another microcentrifuge as Standard 8 and add 200 µl of the Assay Diluent F, which serves as the zero standard.

Labeled Tubes	L-cysteine Standard (µl)	Assay Diluent F (µL)	Standard Conc (µM)

1	100 µl of Reconstituted Stock	900 µl	1500
2	200 µl of Tube #1	200 µl	750
3	200 µl of Tube #2	200 µl	375
4	200 µl of Tube #3	200 µl	187.5
5	200 µl of Tube #4	200 µl	93.75
6	200 µl of Tube #5	200 µl	46.88
7	200 µl of Tube #6	200 µl	23.44
8	0µL	200 µl	0

Note: The L-cysteine standard should be kept on ice during assay.

Assay Procedure

1. In the microplate, add 25 µl of each standard or sample to wells in duplicate or triplicate.
   Note: include diluted sample prepared in Sample Tips and General Considerations
2. Add 0.25 ml of the DNTB working solution into each well.
3. Read the absorbance at 412 nm (A412) by plate reader within 10 minutes. No incubation is needed.

Data Analysis

1. Subtract the blanks
Average the absorbance value of the blank wells (Standard 0 µM) and subtract this from the absorbance values of all the other wells. These are the corrected absorbance.

2. Plotting the standard curves
Make a plot of corrected absorbance at 412 nm as a function of L-cysteine concentration.

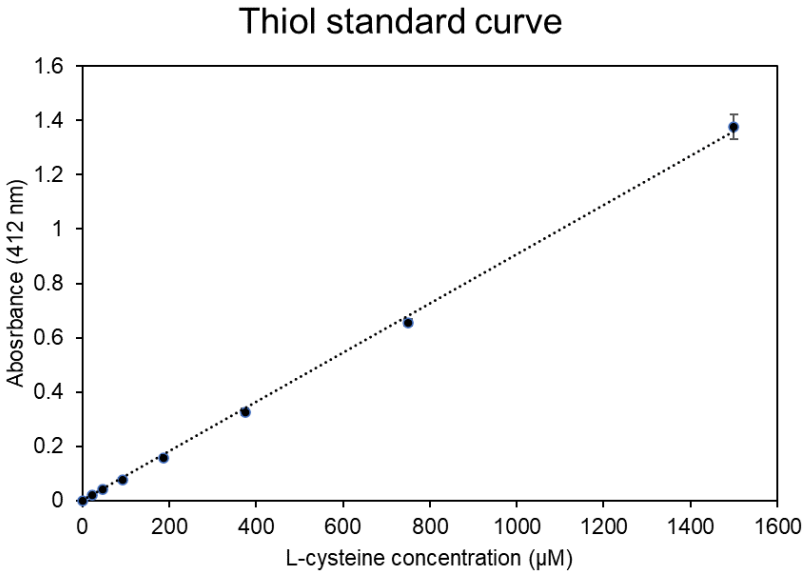
3. Determination of sample thiol concentration

$$\text{Thiol } (\mu M) = \frac{A_{412Sample} - A_{412Background}}{\text{Slope}} \times DF$$

$A_{412Sample}$ = Optical density (OD) reading of the Sample
$A_{412Background}$ = Optical density (OD) reading of the diluted sample, see Sample Tips and General Considerations. If there is no background, the value is 0.
Slope is from the plot of thiol concentration vs. A_{412}.
DF = Sample Dilution factor (DF = 1 for undiluted Samples)

Note: If the calculated thiol concentration of the sample is higher than 1500 µM, the sample should be diluted in Assay Diluent F to make the expected concentration in the range of standard samples and repeat the assay.

Typical Data



The standard curve above is for demonstration only. A standard curve must be run with each assay.

The tested intra-assay CV from three independent assays is less than 4%. The inter-assay CV from three independent assays is 8.5%.

This product is for research use only.