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Thiol and Sulfide Quantitation Kit (T-6060)

Quick Facts

Storage upon receipt:

- –20°C
- Protect from light

Introduction

Molecular Probes' Thiol and Sulfide Quantitation Kit provides an ultrasensitive colorimetric assay for quantitating both protein and nonprotein thiols (also called sulfhydryls or mercaptans). In this assay, which is based upon a method reported by Singh, *et al.*,[1,2] thiols or inorganic sulfides reduce a disulfide-inhibited derivative of papain, stoichiometrically releasing the active enzyme (Figure 1A). The activity of the enzyme is then measured using the chromogenic papain substrate, *N*-benzoyl-L-arginine, *p*-nitroanilide (L-BAPNA) (Figure 1B). Although thiols and inorganic sulfides can also be quantitated using the traditional Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid); DTNB),[3] the enzymatic amplification step in our quantitation kit enables researchers to detect as little as 0.2 µM thiol (0.2 nanomole in a 1 mL reaction) — a sensitivity that is about 100-fold greater than that achieved using Ellman's reagent.

In addition to the disulfide-inhibited papain derivative and L-BAPNA substrate, the Thiol and Sulfide Quantitation Kit includes L-cysteine, Ellman's reagent and cystamine. The L-cysteine serves as a standard in the reaction, and Ellman's reagent is used for accurately determining the actual thiol concentration of L-cysteine standard solutions. Cystamine, when added to the reaction, permits the detection of poorly accessible thiols on proteins or thiols that have high pK_a values.[1] Cystamine, a disulfide, undergoes an exchange reaction with protein thiols, yielding 2-mercaptoethylamine (cysteamine), which then releases active papain (Figure 1C).

The Thiol and Sulfide Quantitation Kit also includes a procedure for the quantitation of maleimides — with a detection limit comparable to that for thiols.[4] Maleimide quantitation is useful, for example, to assess the extent of maleimide derivatization of a protein prior to its conjugation to a second protein. In addition, the kit can be used to detect phosphine, sulfite and cyanide with detection limits of about 0.5, 1 and 5 nanomoles, respectively.

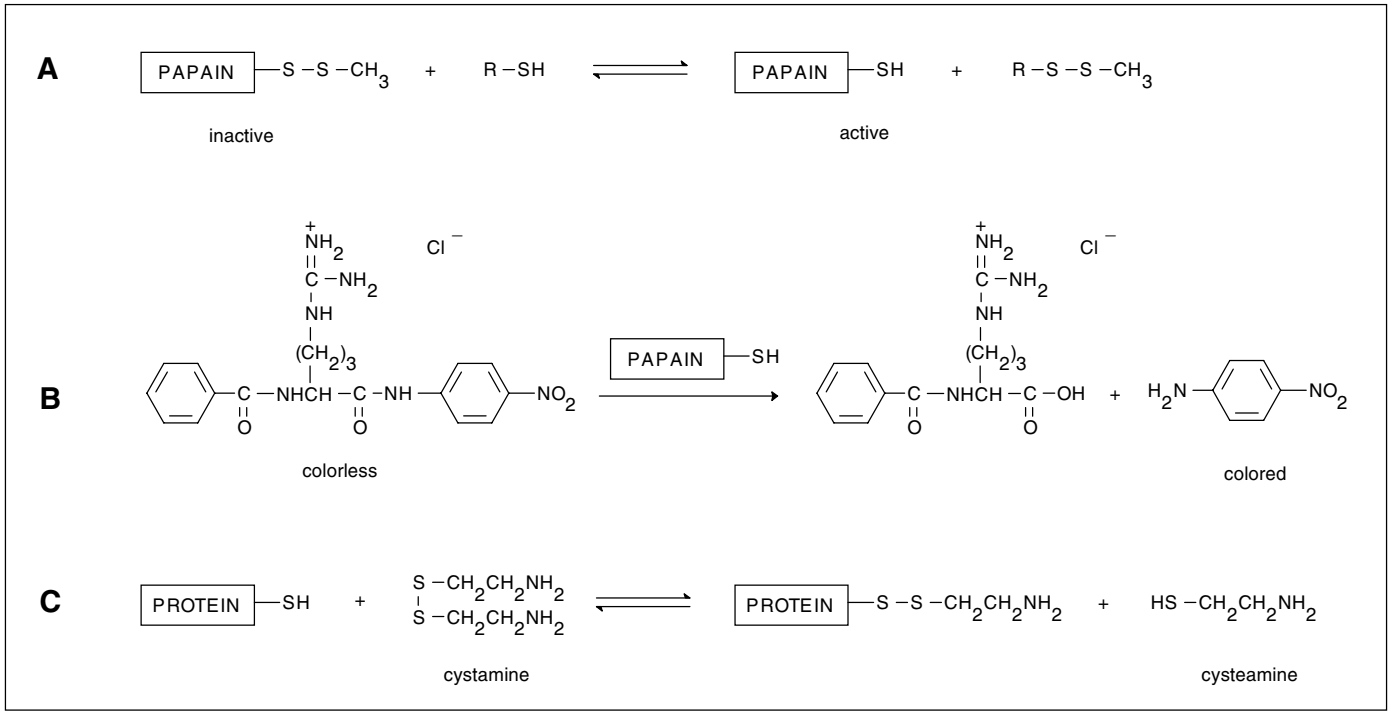

Figure 1. *Chemical basis for thiol detection by the Thiol and Sulfide Quantitation Kit: A) The inactive disulfide derivative of papain, papain–SSCH$_3$, is activated in the presence of thiols; B) Active papain cleaves the substrate L-BAPNA, releasing the p-nitroaniline chromophore; C) Protein thiols, often poorly accessible, exchange with cystamine to generate 2-mecaptoethylamine (cysteamine), which is easily detected.*

Materials

Reagents Supplied

- **Papain–$SSCH_3$** (MW = 23,000, Component A), 18 mg, lyophilized from buffer
- ***N*-benzoyl-L-arginine, *p*-nitroanilide, hydrochloride (L-BAPNA)** (MW = 434.9, Component B), 55 mg
- **Bis-Tris/EDTA buffer** (Component C), 40 mL of 50 mM bis-Tris, 1 mM EDTA, pH 6.3
- **Cystamine, dihydrochloride** (MW = 225.2, Component D), 10 mg
- **L-Cysteine** (MW = 121.2, Component E), 30 mg
- **Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid); DTNB),** (MW = 396.3, Component F), 10 mg

Each kit supplies sufficient material for about 50 assays using standard 1 mL cuvettes or 250 assays using a microplate format.

Storage and Handling

Upon receipt, the kit should be stored at -20°C, protected from light. Stored in this manner, the kit components should remain active for at least one year. After individual components have been made into solutions, storage at 4°C is recommended (see *Reagent Preparation*, below).

Reagents Required but Not Provided

- **Buffer A: 5 mM sodium acetate, 50 mM NaCl, 0.5 mM EDTA, pH 4.7.** Dissolve 41 mg sodium acetate and 292 mg NaCl in about 80 mL deionized water (dH_2O). Add 1 mL of 50 mM EDTA, pH 8.0, and adjust the pH to 4.7 with 1 M HCl. Add sufficient dH_2O to bring the volume to 100 mL. Degas as indicated in *Degassing*.

- **Buffer B: 40 mM sodium phosphate, 2 mM EDTA, pH 7.6.** Dissolve 0.55 g $NaH_2PO_4 \cdot H_20$ in about 80 mL dH_2O. Add 4 mL of 50 mM EDTA, pH 8.0, and adjust the pH to 7.6 with 1 M NaOH. Add sufficient dH_2O to bring the volume to 100 mL. Degas as indicated in *Degassing*.

- **Buffer C: 5 mM sodium acetate, pH 4.0.** Dissolve 41 mg sodium acetate in about 80 mL dH_2O. Adjust the pH to 4.0 with 1 M HCl. Add sufficient dH_2O to bring the volume to 100 mL. Degas as indicated in *Degassing*.

Protocol

Reagent Preparation

Reagents for the Thiol and Sulfide Quantitation Kit, except for Component C, are provided as solids and must be made into stock solutions before use. For convenience, the stock solutions of Components A, B, D and F may be prepared directly in the component bottles, as described below. Once these solutions are prepared, they can be stored for up to six months at 4°C. A stock solution of L-cysteine (Component E) can be prepared by weighing out a portion of the solid and making the solution in a separate vial. L-Cysteine solutions, as thiol standards, are only stable for about two weeks at 4°C; however, the solid is more stable, and sufficient material is provided for preparing about six stock solutions.

1.1 Prepare a 1.2 mg/mL stock solution of papain–$SSCH_3$ by dissolving the 18 mg of papain–$SSCH_3$ (Component A) in 15 mL of Buffer C. The final buffer composition will be approximately 5 mM sodium acetate, 50 mM NaCl, pH 4.5, due to salt contributions from the lyophilyzed material. Sodium azide, at a final concentration of 2 mM, should be added as a preservative.

1.2 Prepare a 4.9 mM stock solution of L-BAPNA by first dissolving the 55 mg of L-BAPNA (Component B) in 1.5 mL of DMSO. Sonicate briefly to help solubilize the L-BAPNA and then add 24.5 mL of bis-Tris/EDTA buffer (Component C).

1.3 Prepare a 40 mM cystamine stock solution by dissolving the 10 mg of cystamine, dihydrochloride (Component D) in 1.11 mL of Buffer A (see *Reagents Required but Not Provided*). Prepare a working solution of 4 mM cystamine prior to running each set of assays by diluting 100 µL of the 40 mM cystamine stock solution into 0.9 mL Buffer A.

1.4 Prepare Ellman's reagent (100 mM DTNB) by dissolving the 10 mg of DTNB (Component F) in 250 µL of DMSO.

1.5 Prepare a 100 mM L-cysteine stock solution, when needed, by weighing out 5 mg from the bottle originally containing 30 mg L-cysteine (Component E) and dissolving the sample in 412 µL of Buffer A in a separate vial.

Degassing

Important: All solutions used for thiol and sulfide determination should be degassed thoroughly before use. We recommend that solutions be degassed on ice in a vacuum desiccator at about 25 Torr for 30 minutes and then <1 Torr for an additional 30 minutes prior to use. The desiccator should be filled with argon or nitrogen gas before opening to air. Degassed solutions should be sealed and used in the assay within a short period of time. When solutions are properly degassed, the standard curve of the corrected absorbance at 410 nm vs. thiol concentration should pass through the origin; insufficient degassing will result in a standard curve that has a positive intersection on the axis of thiol concentration.

Ellman's Assay for Calibration of L-Cysteine Thiol Standard

Ellman's reagent is provided with the Thiol and Sulfide Quantitation Kit in order to accurately determine the thiol content of L-cysteine thiol standard solutions. Because thiols are prone to oxidation in aqueous solutions, the actual thiol concentration of a L-cysteine thiol standard solution may differ significantly from the nominal molarity of the solution that was prepared. The thiol concentration should be determined prior to performing assays (see *Thiol and Sulfide Determination*) that will be used to create a standard curve. The protocol for this assay, which uses Ellman's reagent (100 mM DTNB in DMSO; step 1.4), is presented below. For greater confidence in this determination, we recommend performing the Ellman's reagent assay in duplicate.

2.1 Dilute 5 µL of degassed 100 mM L-cysteine stock solution into 5.0 mL of degassed Buffer A to make 5.0 mL of 0.1 mM L-cysteine working solution.

2.2 Add 0.69 mL of Buffer B (see *Reagents Required but Not Provided*) to each of two clean tubes.

2.3 Add 0.4 mL of the 0.1 mM L-cysteine working solution to one of the tubes and 0.4 mL of Buffer A to the other, to serve as a control.

2.4 Add 10 µL of Ellman's reagent (100 mM DTNB in DMSO) to each tube, mix well and incubate the solution at room temperature for one to two minutes.

2.5 Zero the absorbance of the spectrophotometer at 412 nm using dH_2O as a blank.

2.6 Measure the absorbance of the two solutions at 412 nm. Subtract the absorbance of the control at 412 nm from the absorbance of the L-cysteine-containing solution. This is the corrected absorbance value (ΔA_{412}).

2.7 The thiol concentration of the L-cysteine working solution is calculated using the formula below:

$$\text{mM of thiol} = \frac{\Delta A_{412} \times 1.1 \text{ mL}}{13{,}600 \times 0.4 \text{ mL}} \times 1000$$

where ΔA_{412} is the corrected absorbance value (using 1 cm-pathlength cuvettes), and 13,600 is the molar extinction coefficient ($cm^{-1}M^{-1}$) of the 5-thio-2-nitrobenzoate generated from Ellman's reagent in reacting with the free thiol of the L-cysteine. The concentration of the thiol determined for the 0.1 mM L-cysteine working solution will be used for generating a standard curve in the papain–$SSCH_3$-based assay below.

Thiol and Sulfide Determination

The Thiol and Sulfide Quantitation Kit is designed to detect thiols in the range of ~0.2 µM to 1.5 µM (~0.2 nanomole to 1.5 nanomoles in a 1 mL assay volume). The following step-by-step protocol describes the method for thiol determination; cysteine is used as a thiol standard. The same procedure can be used, with appropriate standards, for quantitation of inorganic sulfide, phosphines, sulfite or cyanide — for these, the determination limits are about 0.2, 0.5, 1 and 5 nanomoles, respectively.

3.1 Determine the number of tubes that will be needed for the standard-curve and experimental reactions. Add 15 µL of the 4 mM cystamine working solution (step 1.3) to each tube. As discussed in the introduction, cystamine is required as an intermediate disulfide for the determination of protein thiols (sulfhydryls) and for the determination of certain thiols that have high pK_a values.

3.2 To the standard-curve reaction tubes, add 0, 2, 5, 8, 12 and 15 µL of 0.1 mM L-cysteine working solution (step 2.1). Add sufficient Buffer A to make the volume added in this step equal 15 µL; the total volume in each tube will now be 30 µL.

3.3 To the tubes for the experimental samples, add 15 µL sample volumes, prepared in Buffer A. The experimental samples added should contain 0.2 nanomole to 1.5 nanomoles of the thiol or inorganic sulfide; a dilution series may be required to attain this target range.

3.4 Prepare a 0.6 mg/mL papain–$SSCH_3$ working solution by mixing equal volumes of 1.2 mg/mL papain–$SSCH_3$ stock solution (step 1.1) and Buffer B (see *Reagents Needed but Not Provided*). A 0.5 mL volume will be used in each reaction; prepare a slight excess. This working solution of papain–$SSCH_3$ should be freshly prepared before each set of assays; between experiments, the papain–$SSCH_3$ is best stored as the 1.2 mg/mL stock solution.

3.5 Add 0.5 mL of the 0.6 mg/mL papain–$SSCH_3$ working solution to each assay tube and mix well.

3.6 Incubate the reactions at room temperature for about one hour.

3.7 Add 0.5 mL of the 4.9 mM L-BAPNA solution (step 1.2) to each assay tube and mix well. For consistent incubation periods (see below), the L-BAPNA substrate can be added to the individual tubes, with starting times offset by one minute, for example.

3.8 Incubate the reactions at room temperature for one hour. The exact time interval is not critical; however, it is essential that all reactions be incubated for the same length of time.

3.9 Zero the absorbance of the spectrophotometer at 410 nm using dH_2O as a blank, and measure the absorbance at 410 nm of each reaction. For accuracy, the absorbance value must be between 0.1 and 1. Dilution of the sample may be necessary.

3.10 Calculate the corrected absorbance by subtracting the absorbance value for the control reaction lacking L-cysteine from absorbance values for both the standard reactions and experimental reactions. For the samples that required dilution, multiply the corrected value by the dilution factor.

3.11 Plot a curve of the corrected absorbance at 410 nm of the L-cysteine standards vs. the calibrated thiol content of these samples as determined from results of the Ellman's assay.

3.12 Read the thiol (or sulfide) concentration of experimental samples from the standard curve.

Maleimide Determination

The Thiol and Sulfide Quantitation Kit can also be utilized to quantitate maleimides. In this protocol, the maleimides of a sample are first reacted with a known amount of a thiol standard (L-cysteine), present in slight excess, and then the remaining unreacted thiol is determined using the papain–$SSCH_3$-based assay.

4.1 To the tubes for the experimental samples, add 15 µL sample volumes, prepared in Buffer B. The experimental samples added should contain 0.1–1.0 nmol maleimide; a dilution series may be required to attain this target range.

4.2 Add 15 µL of the 0.1 mM L-cysteine working solution (from step 2.1); the precise thiol equivalent (~1.5 nanomoles) of this L-cysteine addition can be determined from the results of the Ellman's reagent assay.

4.3 Add 220 µL of Buffer B to each experimental reaction tube. Mix and incubate the solution at room temperature for 40 minutes.

4.4 For standards, add 0, 4, 8, 12 and 15 µL of the 0.1 mM L-cysteine working solution to separate tubes. Add sufficient Buffer A to bring the volume of each tube to 15 µL.

4.5 Add 235 µL of Buffer B to each standard-reaction tube, mix and incubate the solution at room temperature for 40 minutes.

4.6 Prepare a 0.6 mg/mL papain–$SSCH_3$ working solution by mixing equal volumes of the 1.2 mg/mL papain–$SSCH_3$ stock solution (step 1.1) and Buffer B (see *Reagents Required but Not Provided*). A 0.5 mL volume will be used in each reaction; prepare a slight excess. This working solution of papain–$SSCH_3$ should be freshly prepared before each set of assays; between experiments, the papain–$SSCH_3$ reagent is best stored as the 1.2 mg/mL stock solution.

4.7 Add 0.5 mL of the 0.6 mg/mL papain–$SSCH_3$ working solution to each tube (experimental reactions as well as the standard reactions) and mix well.

4.8 Incubate the reactions at room temperature for about one hour.

4.9 Add 0.5 mL of 4.9 mM L-BAPNA solution (step 1.2) to each assay tube and mix well. For consistent incubation periods (see below), the L-BAPNA substrate can be added to the individual tubes, with starting times offset by one minute, for example.

4.10 Incubate the reactions at room temperature for one hour. The exact time interval is not critical; however, it is essential that all reactions be incubated for the same length of time.

4.11 Zero the absorbance of the spectrophotometer at 410 nm with dH_2O as a blank, and measure the absorbance at 410 nm of each reaction. For accuracy, the absorbance value must be between 0.1 and 1. Dilution of the sample may be necessary.

4.12 Calculate the corrected absorbance by subtracting the absorbance value for the control reaction lacking L-cysteine from absorbance values for both the standard reactions and experimental reactions. For the samples that required dilution, multiply the corrected value by the dilution factor.

4.13 Plot a standard curve of the corrected absorbance at 410 nm of the L-cysteine standards vs. calibrated thiol content of these samples as determined from results of the Ellman's reagent assay.

4.14 Read the thiol concentration of experimental samples from the standard curve.

4.15 Calculate the nanomoles of maleimide by subtracting the measured nanomoles of thiol (from step 4.14) from the initial nanomoles of thiol added to each experimental reaction (~1.5 nanomoles of thiol; from step 4.2).

Microplate Assays

The Thiol and Sulfide Quantitation Kit protocol is readily adaptable to a microplate format. In the protocols above, simply reduce all volumes fivefold for final reaction volumes of about 200 µL. For accurate measurement of the L-cysteine standard, it may be necessary to dilute the solution before pipetting.

References

1. Anal Biochem 213, 49 (1993); **2.** Methods Enzymol 251, 229 (1995); **3.** Arch Biochem Biophys 82, 70 (1959); **4.** Bioconjug Chem 5, 348 (1994); **5.** Methods Enzymol 19, 226 (1970); **6.** Biochemistry 3, 180 (1964); **7.** Arch Biochem Biophys 95, 271 (1961); **8.** Chem Pharm Bull 40, 3000 (1992).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
T-6060	Thiol and Sulfide Quantitation Kit *50-250 assays*	1 kit

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