INSTRUCTIONS

Sulfhydryl Addition Kit

Pub. No. MAN0011437
Rev. B
Doc. Part No. 2161316

23460

Number	Description
23460	**Sulfhydryl Addition Kit,** contains sufficient components for one reaction containing 0.5-5.0mg of protein

Kit Contents:

SATA (*N*-succinimidyl-S-acetylthioacetate), 2mg

Hydroxylamine•HCl, 5mg

Conjugation Buffer Stock (10X), 20mL

BupH™ Phosphate Buffered Saline Pack, 1 pack, yields 500mL of 0.1M sodium phosphate, 0.15M sodium chloride; pH 7.2 when reconstituted

Dimethylformamide (DMF), 1mL

Ellman's Reagent (DTNB), 2mg

Cysteine•HCl•H_2O, 20mg

Dextran Desalting Column, 1 × 5mL

Column Extender, 1 each

Porous Discs, 2 each

Porous Disc Insertion Tool

Storage: Upon receipt store kit at 4°C. Kit is shipped at ambient temperature.

Introduction

Sulfhydryl groups on proteins, peptides and other molecules are important for many protein applications and reactions. The Thermo Scientific Sulfhydryl Addition Kit contains all the necessary components to introduce free sulfhydryl groups (–SH) into any molecule containing primary amino (NH_2) groups, purify the modified molecule and quantify the added sulfhydryl groups. The modified molecule can be used in a variety of sulfhydryl-specific methods such as conjugation to Maleimide-Activated KLH (Product No. 77611) for antibody production.

This kit uses SATA as a means of adding sulfhydryls to molecules. SATA contains an *N*-hydroxysuccinimide (NHS) ester that forms a stable amide bond with primary amines. Once the covalent linkage is formed, the modified molecule can be stored and the sulfhydryl group de-protected when needed. The reaction conditions for SATA are mild and non-denaturing, which preserves protein activity.

Material Preparation

Phosphate-buffered Saline (PBS)	Dissolve the dry-blend buffer with 500mL of ultrapure water. For long-term storage of excess buffer, sterile-filter the solution or add sodium azide to a final concentration of 0.02% and store at 4°C.
Protein	Dissolve 0.5-5.0mg of protein with up to 1mL of PBS. For proteins already in solution, make a 1:1 dilution of the protein with PBS or dialyze against PBS. **Note:** If the protein is in a buffer that contains primary amines (e.g., Tris or glycine), these compounds must be thoroughly removed by dialysis or desalting, as they will quench the SATA reaction.

Procedure for Adding Sulfhydryl Groups

Note: In the following reaction (Figure 1), 10-fold molar excess of SATA is added to the protein solution, which generally results in 1-5 sulfhydryl groups per protein (see Table 1 in the Appendix). For the volume of SATA in DMF to add to achieve 10-fold excess see Table 2 in the Appendix.

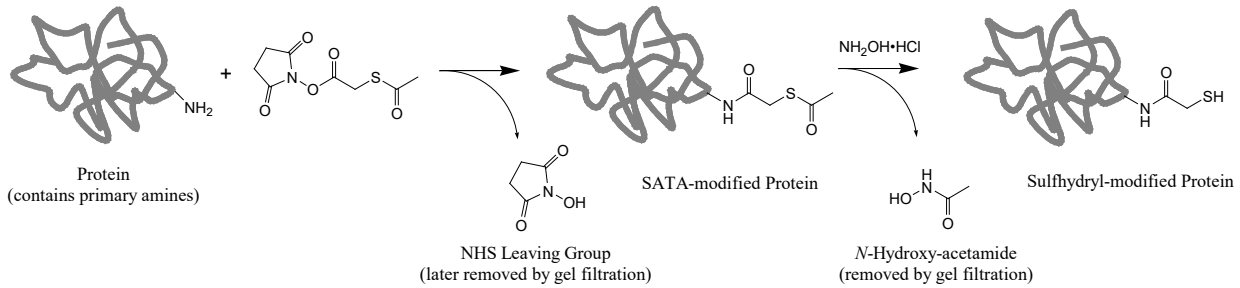

Figure 1. Reaction scheme for sulfhydryl modification of protein. The NHS ester of SATA reacts with primary amines on lysine residues. Hydroxylamine de-protects the latent sulfhydryl groups.

1. Prepare Maleimide Conjugation Buffer (1X) by mixing 90mL of PBS, pH 7.2, with 10mL of Conjugation Buffer Stock (10X).
2. Invert the desalting column several times to suspend the resin. Position the column upright in a test tube or clamp and allow the resin to settle for several minutes.
3. Remove the top cap from the column and carefully pipette the storage solution (contains 0.02% azide) until 5-10mm of solution remains above the resin bed.
4. (Optional) Using the open end of the supplied porous disc insertion tool, insert and slide a porous disc to within 1mm of the resin bed. A top porous disc provides a "stop-flow" function, preventing disturbance and drying of the resin bed during use.
5. Twist off the column bottom end tab. Equilibrate the column by adding 15mL of Maleimide Conjugation Buffer (1X) and allowing it to flow through.
6. Add 500µL of DMF to the vial containing 2mg of SATA (17.3mM) and mix thoroughly to dissolve.
7. Add a 10-fold molar excess of SATA/DMF solution to 1mL of the protein (see Table 2). Incubate the reaction for 30 minutes at room temperature.

   Note: The SATA-modified protein may be stored at -20°C provided the protein is not adversely affected by freezing.
8. Add 100µL of Conjugation Buffer Stock (10X) to the vial containing 5mg of Hydroxylamine•HCl and mix thoroughly to dissolve.
9. To de-protect the latent sulfhydryl, add 100µL of hydroxylamine solution to the SATA-modified protein. Incubate mixture for 2 hours at room temperature.
10. To remove nonreacted reagents, apply ~1mL of de-protected sulfhydryl protein to the equilibrated desalting column. Add Maleimide Conjugation Buffer (1X) to the Desalting Column. Collect 1mL fractions and measure the absorbance at 280nm of each fraction to locate the protein. Pool fractions containing most of the protein.
11. Determine concentration of sulfhydryl-protein (i.e., the pooled protein sample) by comparing its absorbance at 280nm with the absorbance of the original protein solution. Alternatively, determine protein concentration using the Thermo Scientific Coomassie (Bradford) Assay Kit (Product No. 23236).
12. Sulfhydryl groups are unstable, therefore, initiate downstream procedures within 1 hour of sulfhydryl modification. Refrigerate the modified protein until ready to use.

Procedure for Determining Sulfhydryl Content

1. Prepare Maleimide Conjugation Buffer (1X) by mixing 90mL of PBS with 10mL of Conjugation Buffer Stock (10X).
2. Add 1mL of Maleimide Conjugation Buffer (1X) to the vial containing 2mg of Ellman's Reagent and mix for 5-10 minutes to dissolve completely. This 2mg/mL Ellman's Reagent stock solution is stable for up to 1 week stored frozen.
3. Dilute a portion of the stock Ellman's Reagent 1:2 with Maleimide Conjugation Buffer (1X) [i.e., add one part Ellman's Reagent to two parts Maleimide Conjugation Buffer (1X)] to make the 1.0mg/mL working Ellman's Reagent.
4. To prepare the cysteine (–SH group) standards, add 2mL of Maleimide Conjugation Buffer (1X) to the vial containing 20mg of cysteine and mix thoroughly to dissolve. This 10mg/mL stock cysteine solution is stable for up to 1 week stored frozen.
5. Dilute a portion of the stock cysteine 1:200 with Maleimide Conjugation Buffer (1X) to make a 0.05mg/mL working cysteine solution (285nmol/mL).
6. Prepare 10 serial dilutions of the working cysteine solution (i.e., dilute the 0.05mg/mL working cysteine 1:1 with Maleimide Conjugation Buffer (1X), then dilute that 0.025mg/mL solution 1:1, etc.). These 10 dilutions are used as cysteine standards.
7. In separate wells of a microplate, pipette duplicate samples containing 100µL of each of the following:
   - Maleimide Conjugation Buffer (1X) (reagent blank)
   - Each of the 10 cysteine standards
   - Sulfhydryl-modified protein

   Note: If the protein concentration is low (i.e., 0.5-1.0mg/mL), use 200µL samples rather than 100µL. Also use 200µL of Maleimide Conjugation Buffer (1X) and 200µL of each standard.
8. To each 100µL sample in the microplate, add 10µL of working Ellman's Reagent (0.5mg/mL) and mix well. If using 200µL samples, add 20µL working Ellman's Reagent to each sample.
9. Use a plate reader to measure the absorbance of each well at 405nm. Absorbance measurements at 412nm or 420nm are also acceptable.
10. Calculate sulfhydryl content by either of the following methods:
    - Plot the absorbance values of the cysteine standards. Determine slope of the curve and the Y-intercept and determine sulfhydryl content as follows:

$$\text{sulfhydryl content (moles)} = \frac{A_{405} \text{ of the sample} - \text{Y-intercept}}{\text{slope}}$$

    - Alternatively, divide the absorbance value of each cysteine standard by its cysteine content (mole of cysteine) then calculate the average absorbance units (average AU per mole of cysteine) for all the standards. Determine sulfhydryl content as follows:

$$\text{sulfhydryl content (moles)} = \frac{A_{405} \text{ of the sample}}{\text{average AU per mole cysteine}}$$

11. Using the results from Step 10 and the molecular weight of the protein, calculate the number of sulfhydryls per protein molecule as follows:

$$\text{sulfhydryl/protein} = \frac{\text{moles of cysteine in sample}}{\text{moles of protein}}$$

Appendix

A. Varying the Molar Ratio

The number of free sulfhydryls added to a particular protein is unique to each protein and depends largely on the number of available lysine residues within the protein. It is also related to the amount of protein and SATA added to the reaction mixture. To decrease or increase sulfhydryl incorporation, simply decrease or increase the amount of SATA that is added to the reaction. The results of using different molar ratios of SATA-to-BSA are reported in Table 1.

Table 1. Effect of varying molar ratio of SATA-to-protein on sulfhydryl incorporation using bovine serum albumin (BSA).

SATA Molar Excess	Moles –SH Incorporated per Mole BSA
10	4.9
25	21.2
50	23.6
100	29.4

B. Determine Volume of SATA to Add

Adding the amount of SATA (10-fold molar excess) indicated in Table 2 generally results in 1-5 sulfhydryl groups per protein (mole/mole). Actual sulfhydryl incorporation depends on the number of accessible amines on the protein.

Table 2. Volume (µL) of SATA in DMF to add per milliliter of protein of known concentration and molecular weight (MW). For example, for a 50kDa protein at a concentration of 2mg/mL, add 22µL of SATA/DMF to 1mL of protein.

Protein MW × 10^3	Protein Concentration (mg/mL) 0.5	1	2	3	4	5
5	56	110	220	280	440	–
10	28	55	110	170	220	280
25	11	22	44	68	88	112
50	6	11	22	34	44	56
100	3	6	11	17	22	28
200	1.4	2.8	6	9	11	14

Related Thermo Scientific Products

22322	**Sulfo-SMCC** (sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate), 50mg
26102	**SATA** (*N*-succinimidyl S-acetylthioacetate), 50mg
22582	**Ellman's Reagent** (5,5'-dithio-*bis*-[2-nitrobenzoic acid]), 5g
17904	**Glycerol,** 1L
29810	**Ethylene Glycol (50% aqueous),** 200mL
89890	**Zeba™ Spin Desalting Columns, 2mL,** 25 columns
89892	**Zeba Spin Desalting Columns, 5mL,** 25 columns
44999	**SulfoLink™ Immobilization Kit for Peptides,** contains all reagents generally required for preparing five affinity columns
77666	**Imject™ Maleimide Activated mcKLH Spin Kit**, contains sufficient materials for five conjugation reactions
77112	**Imject Maleimide Activated BSA Kit**, contains sufficient materials for five conjugation reactions
77126	**Imject Maleimide Activated Ovalbumin**, contains sufficient materials for five conjugation reactions

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition. The information in this guide is subject to change without notice.

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Revision	Date	Description
B	31 July 2024	Correcting spin column usage.
A	17 October 2015	New document for Pierce™ Streptavidin Agarose Resins.