

Thiol-Reactive Probes

Table 1. Contents and Storage Information.

Material	Amount	Concentration	Storage Upon Receipt	Stability
Reactive dye (lyophilized solids)	Various, see product label	NA	<ul style="list-style-type: none"> • $\leq -20^{\circ}\text{C}$ • Desiccate • Protect from light 	When stored as directed, dyes are stable for 6–12 months.

NA = Not applicable.

Introduction

Most of Molecular Probes' thiol-reactive reagents will react with thiol groups on proteins to give thioether-coupled products. These reagents react rapidly at near-neutral (physiological) pH and usually can be coupled with thiol groups selectively in the presence of amine groups.^{1,2}

Haloalkyl Derivatives

Haloalkyl reagents (primarily iodoacetamides) are among the most frequently used reagents for thiol modification. In most proteins, the site of reaction is at cysteine residues that either are intrinsically present or result from reduction of cystines. In addition, methionines can sometimes react with haloalkyl reagents.

Maleimides

Maleimides are similar to iodoacetamides in their application as reagents for thiol modification; however, they may be more thiol-selective than iodoacetamides because they apparently do not react with histidine or methionine. The optimum pH for the reaction of maleimides is near 7.0.

Hg-Link™ Phenylmercury Compounds

The potent Hg-Link™ phenylmercury compounds form stable thiolates with free sulfhydryls, but can also react with other thiol moieties, including nitrosylated thiols (SNO).³ Unlike the stable thioether bond formed by iodoacetamides and maleimides, the thiolate bond is reversible with HCl⁴ or reducing agents such as DTT.

TS-Link™ Reagents

TS-Link™ reagents or thiosulfates are extremely water soluble and react readily with free sulfhydryl groups to form disulfide bonds. In contrast to the thioether bonds formed by maleimides and iodoacetamides, the disulfide bonds formed by TS-Link™ reagents are reversible—the TS-Link™ hapten or fluorophore can easily be removed using a reducing agent such as DTT or TCEP, leaving the molecule of interest unchanged. Furthermore, like maleimides and iodoacetamides, TS-Link™ reagents are selective for thiols.

Before You Begin

Materials Required but not Provided

- 10–100 mM phosphate (such as phosphate-buffered saline (PBS)), Tris, or HEPES buffer with pH between 7.0–7.5.
- Dimethylformamide (DMF), dimethylsulfoxide (DMSO), or dH_2O for preparing dye stock solutions.
- Gel filtration column (Sephadex G-25 or equivalent), equilibrated in buffer of choice.
- (optional) Glutathione to stop the reaction.
- (optional) DTT (dithiothreitol, D1532) or TCEP (tris-(2-carboxyethyl)phosphine, T2556). These reagents can be used to reduce disulfide bonds in proteins to liberate free thiols; however, the reaction solutions must be purged of oxygen to maintain the thiols in their reduced form.

Experimental Protocol

Conjugation Reaction

A general procedure suitable for conjugation of most thiol-reactive probes—including Hg-Link™ phenylmercury compounds, iodoacetamides, maleimides, TS-Link™ reagents, and alkyl halides—to proteins is outlined below. Other thiolated biopolymers and oligonucleotide thiophosphates should behave similarly. Low molecular weight thiol conjugates may require alternative purification methods.

1.1 Dissolve the protein at 50–100 μM in a suitable buffer at pH 7.0–7.5 (10–100 mM phosphate, Tris, HEPES) at room temperature. In this pH range, the protein thiol groups are sufficiently nucleophilic so that they react almost exclusively with the reagent in the presence of the more numerous protein amines, which are protonated and relatively unreactive.

1.2 Reduction of disulfide bonds in the protein is best carried out at this stage. A 10-fold molar excess of a reducing agent such as DTT (D1532) or TCEP (T2556) is usually sufficient. If DTT is used, then dialysis is required to remove the excess DTT prior to introducing the reactive dye. TCEP is reactive towards haloalkyl derivatives such as bimanes⁵ and therefore must be removed by dialysis prior to conjugation. It is not necessary to remove excess TCEP during conjugation with iodoacetamides or maleimides.

1.3 It may be advisable to carry out thiol modifications in an oxygen-free environment because thiols can be oxidized to disulfides. This precaution is particularly important if the protein has been treated with a reagent such as dithiothreitol prior to thiol modification. In this case, all buffers should be deoxygenated and the reactions carried out under an inert atmosphere to prevent re-formation of disulfides.

1.4 Prepare a 1–10 mM stock solution of the reactive dye in a suitable solvent immediately prior to use. **Protect all stock solutions from light as much as possible by wrapping containers in aluminum foil.**

Note that the haloalkyl derivatives are not stable in solution of any kind. These reagents are also extremely sensitive to light, and their solutions must be protected from illumination. Immediately prior to use, prepare only as much dye solution as is needed. Maleimides are somewhat more stable, but we do not recommend storing them in solution for more than 24 hours.

Information about suitable solvents is given in the data tables of *Molecular Probes' The Handbook: A Guide to Fluorescent Probes and Labeling Technologies* or is available at our website (probes.invitrogen.com). In most cases, DMSO is a suitable solvent for the stock

solutions. The Alexa Fluor® Hg-Link™ phenylmercury compounds are soluble in H₂O. If the reagent is known to be water soluble, then an aqueous stock solution can be used, but stock solutions in water should not be stored for more than a few hours.

1.5 Add sufficient protein-modification reagent from a stock solution to give approximately 10–20 moles of reagent for each mole of protein. Add the reagent dropwise to the protein solution as it is stirring.

Note that maleimide reaction conditions are essentially the same as those for iodoacetamides; however, the selectivity of maleimides toward thiol groups may be greater, allowing somewhat more latitude in the buffer's pH. Decomposition to maleamic acids above pH 8.0 is a competing reaction.

1.6 Allow the reaction to proceed for 2 hours at room temperature or overnight at 4°C. **For haloalkyl reactive dyes and to a lesser extent maleimides, TS-Link™ reagents, and Hg-Link™ phenylmercury compounds, it is essential to protect the reaction mixture from light as much as possible.**

1.7 Upon completion of the reaction with the protein, an excess of glutathione, mercaptoethanol, or other soluble low molecular weight thiol can be added to consume excess thiol-reactive reagent, thus ensuring that no reactive species are present during the purification step.

1.8 Separate the conjugate on a gel filtration column, such as a Sephadex G-25 column or equivalent matrix, or by extensive dialysis at 4°C in an appropriate buffer.

Determination of the Degree of Labeling

Lyophilized Protein Conjugates

If sufficient buffer-free material is available, weigh a few mg of the lyophilized protein and dissolve it at 1 mg/mL in a suitable buffer that is free of other proteins or dyes.

Protein Conjugate Solutions

It is necessary to determine the conjugated protein concentration using: (1) a fluorometric assay such as that provided in our NanoOrange® Protein Quantitation Kit (N6666) or our CBQCA Protein Quantitation Kit (C6667), providing that the fluorescence from a fluorescent protein conjugate does not interfere with the assay; (2) a colorimetric assay such as the Bradford or Lowry method; or (3) an approximate value estimated from the initial amount of protein used (e.g., 10 mg in step 1) minus a 10–15% expected loss during the conjugation and purification procedure.

2.1 The degree of labeling can be calculated using the following formula:

$$\frac{A_x}{\epsilon} \times \frac{\text{MW of protein}}{\text{mg protein/mL}} = \frac{\text{moles of dye}}{\text{moles of protein}}$$

where A_x = the absorbance value of the dye at the absorption maximum wavelength. ϵ = molar extinction coefficient of the dye or reagent at the absorption maximum wavelength (see Chapter 2 of Molecular Probes' *The Handbook: A Guide to Fluorescent Probes and Labeling Technologies*).

2.2 In the case of a biotinylated conjugate, the degree of labeling can be estimated with HABA reagent as described in the protocol of our FluoReporter® Biotin-XX Protein Labeling Kit (F2610) and elsewhere.⁶

General Reference

- *Bioconjugate Techniques* by Greg Hermanson (Academic Press, 1996), available directly from Molecular Probes (B7884), is an excellent source of additional information on conjugation techniques of all kinds.

References

1. Hermanson, G., *Bioconjugate Techniques*, Academic Press (1996); 2. *Bioconjug Chem* 3, 2 (1992); 3. *Analyst* 83, 670 (1958); 4. *J Chromatogr* 160, 239 (1978); 5. *Anal Biochem* 318, 325 (2003); 6. *Methods Enzymol* 18A, 418 (1970).

Contact Information

Molecular Probes, Inc.

29851 Willow Creek Road
Eugene, OR 97402
Phone: (541) 465-8300
Fax: (541) 335-0504

Customer Service:

6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338
Fax: (541) 335-0305
probesorder@invitrogen.com

Toll-Free Ordering for USA:

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Technical Service:

8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353
Toll-Free (800) 438-2209
Fax: (541) 335-0238
probetech@invitrogen.com

Invitrogen European Headquarters

Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100
Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

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