IRDye®

Infrared Dye Reagents

Technical Note

IRDye 680LT Maleimide Labeling Application Guide

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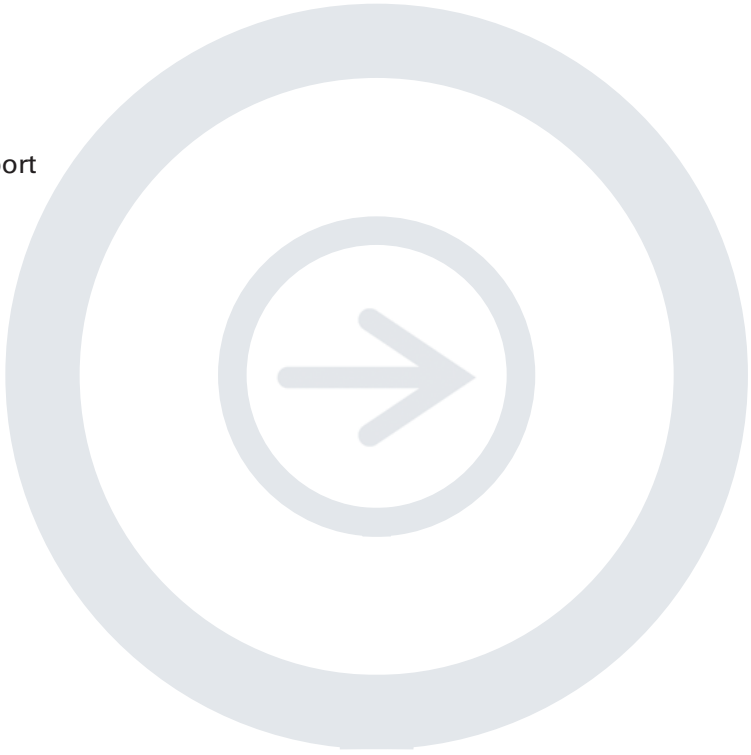

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I. INTRODUCTION

IRDye 680LT Maleimide is a functional derivative of infrared dye IRDye 680LT that is reactive with free –SH (thiol, sulfhydryl) groups. Most molecules that contain free –SH groups can be labeled with maleimide dyes, including IRDye 680LT Maleimide Infrared Dye.

II. LABELING REACTION CONDITIONS AND CONSIDERATIONS

Maleimide groups react with sulfhydryl groups at pH 6.5-7.5, forming a stable thioether bond. A protein, peptide, or biomolecule containing a reactive sulfhydryl group can be labeled using the functional group of IRDye 680LT Maleimide.

The following conditions provide good labeling efficiency:

Table 1. Labeling conditions for IRDye 680LT Maleimide.

Buffer:	Phosphate Buffered Saline (PBS), pH 7.2-7.4
Temperature:	Ambient*
Time:	2 hours
Dye equivalents per free-SH:	2-5

* Ambient temperature is preferred, but 4°C may be used if the target is not stable during incubation. If 4°C is used for the labeling reaction, an overnight incubation should be performed.

Generally, PBS works well for labeling, but other buffers with pH 6.5 to 7.5 can be used. Reactions above pH 8.0 should be avoided, since unprotonated amines can also react with maleimides. The labeling reaction is usually complete in 2 hours at ambient temperature, but the reaction can be carried out at 4°C for 16-18 hours. The labeled molecule should be purified by appropriate purification techniques. Dialysis, size exclusion chromatography, desalting spin columns, and HPLC all work well for purification.

Molecules containing disulfide bonds cannot be directly labeled with a maleimide. However, the disulfide bonds can be cleaved with reducing agents such as TCEP, DTT, or 2-Mercaptoethylamine (MEA) to produce free sulfhydryl groups. After reduction, excess reducing agent must be removed prior to the labeling, since it will also react with the maleimide group.
Additional considerations:

- Once excess reducing agent is removed, the dye should be added promptly to the labeling reaction as disulfide bonds will reform in the absence of reducing agent.
- If excess reducing agent cannot be removed from the labeling reaction, the molar excess of the reducing agent should be limited, as the dye will react with the maleimide as well in the labeling reaction.
- Contrary to some claims in the literature, we have observed that TCEP will react with the maleimide group of the dye during the labeling reaction. The ratio of TCEP and maleimide dye to the protein or peptide must be optimized for efficient reduction and labeling.
- Use the highest practical concentration of protein or peptide for the labeling reaction. Other things being equal, more concentrated solutions will react faster than less concentrated ones.

III. EXAMPLES

Labeling of Affibody® Molecules with IRDye 680LT Maleimide

Affibody molecules are small proteins with unique binding sites capable of binding to different target proteins (www.affibody.com). Commercial Affibody® molecules are engineered with a single C-terminal cysteine residue that can be coupled to any fluorescent dye. The Affibody molecules are partially dimerized due to disulfide (S-S) bridges formed by the C-terminal cysteine and must be reduced prior to labeling with IRDye 680LT Maleimide.

1. Prepare a fresh 500 mM solution of TCEP in water (47.5 mg/331 µl).

2. Add 1 µl of 500 mM TCEP to 99 µl Affibody molecules in PBS (1 mg/ml); final TCEP concentrtion is 5 mM (68-fold molar excess over Affibody dimer).

3. Incubate overnight at room temperature.

4. Remove excess TCEP by passing the reduced mixture through a 0.5 ml Zeba™ Desalt Spin Column (Pierce) (30-130 µl sample volume, www.piercenet.com).

5. Reconstitute IRDye 680LT Maleimide (0.5 mg, MW 1427) in 50 µl DMSO or water to a final concentration of ~10 mM.

6. Add 4 µl of maleimide solution (2.5-fold molar excess of dye over Affibody) to 100 µl reduced Affibody molecule solution.

   NOTE: The remaining maleimide dye solution can be stored at -20°C protected from light, and used for dye labeling reactions for up to 2 weeks.

7. Mix and incubate at room temperature for 2-3 hours, protected from light.

8. Purify the dye labeled Affibody solution by consecutively passing over two 0.5 ml Zeba Desalt Spin Columns.

9. Store the 680LT- Affibody conjugate protected from light at -20°C.

Labeling of Small Molecules with IRDye 680LT Maleimide

Glutathione, a small peptide, is available in the reduced form which can be used as a model compound to illustrate the labeling of small molecules with IRDye 680LT Maleimide. The following procedure should serve as a general guideline for labeling small molecules containing a free–SH group.

1. Prepare a 10 mM stock solution of glutathione in water by dissolving 3 mg of glutathione in 1 mL of water.

2. Dilute glutathione stock to 0.1 mM (1µl stock to 100 µl) in PBS, pH 7.4.

3. Reconstitute IRDye 680LT Maleimide (0.5 mg, MW 1427) in 50 µl DMSO or water to a final concentration of ~10 mM.

4. Add 2 µl IRDye 680LT Maleimide dye solution to the peptide (2-fold molar excess), mix well, and incubate at room temperature for one hour.

5. Remove excess free dye from the reaction mix using RP-HPLC.

Labeling Antibodies with IRDye 680LT Maleimide

Antibodies can be labeled with IRDye 680LT Maleimide by reduction of disulfide bonds using 2-Mercaptoethylamine (MEA). 2-Mercaptoethylamine is a mild reducing agent which selectively reduces the two disulfide bonds in the hinge region of IgG, thereby producing two heavy chain-light chain molecules, each containing one antigen binding site.

Reduction of antibodies to generate free thiols

1. Prepare the antibody at a concentration of 10 mg/ml in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4 buffer containing 1 mM EDTA.

2. Prepare 500 mM MEA stock solution by mixing 57 mg of MEA with 1mL of water.

3. Add 500 mM MEA to a final concentration of ~50 mM (e.g. 10 µL stock into 100 µL of antibody).

4. Incubate the solution in a sealed tube for 90 minutes at 37°C.

5. Immediately purify the reduced IgG from excess 2-Mercaptoethylamine and reaction by-products by desalting spin column such as Zeba™ Desalt Spin Columns (Pierce).

   NOTE: All buffers should contain 1-10 mM EDTA to preserve the free sulfhydryls from metal-catalyzed oxidation.

Labeling of reduced antibody with IRDye 680LT Maleimide dye

1. Reconstitute IRDye 680LT Maleimide (0.5 mg, MW 1427) in 50 µl DMSO or water to a final concentration of ~10 mM.

2. Add 10-15 µl of maleimide dye solution (~5-10 fold molar excess of dye over protein) to 1-2mg of reduced antibody solution.

   NOTE: The remaining maleimide dye solution can be stored at -20°C protected from light, and used for dye labeling reactions for up to 2 weeks.

3. Mix and incubate at room temperature for 2-3 hours, protected from light.

4. Purify the dye labeled antibody by dialysis or desalting column such as a Zeba Desalting Spin Column.

5. Store the IRDye 680LT conjugated antibody, protected from light, at 4°C for up to two weeks. If storing longer than two weeks, the dye-labeled antibody can be aliquoted, lyophilized, and stored at -20°C.

6. To improve shelf life and stability of labeled conjugates, add sodium azide (0.01%) and bovine serum albumin (1-10 mg/mL).

For further questions, please send a detailed inquiry to biohelp@licor.com.

REFERENCES

1. E. B. Getz, M. Xiao, T. Chakrabarty, R. Cooke, P. R. Selvin. 1999. A comparison between the sulfhydryl reductants tris (2-carboxyethyl)phosphine and dithiothreitol for use in protein bio chemistry. Anal. Biochem. 273: 73-80

2. J. C. Han et al. 1994. A procedure for quantitative determination of tris (2-carboxyethyl) phosphine, an odorless reducing agent more stable and effective than dithiothreitol. Anal. Biochem. 220: 5-10

3. T. L. Kirley. 1989. Reduction and fluorescent labeling of cysteine containing proteins for subsequent structural analysis. Anal. Biochem. 180: 231-236

4. M.E. Levison et al. 1969. Reduction of biological samples by water-soluble phosphines: Gammaglobulin. Experiantia. 25: 126-127

5. J. C. Mery et al. 1993. Disulfide linkage to polyacrylic resin for automated Fmoc peptide synthesis. Immunochemical application of peptide,resins and mercaptoamide peptides. Int. J. Peptide Protein Res. 42: 44-52

6. D. E. Shafer, J. K. Inman, A. Lees. 2000. Reaction of Tris (2-carboxyethyl) phosphine (TCEP) with Maleimide and alphahaloacylgroups: anomalous elution of TCEP by gel filtration. Anal. Biochem. 282: 161-164

7. R. Scherer. 2008. LI-COR IRDye® 680LT Maleimide conjugation report. Int. Materials Science & Engineering, Vanderbilt University.

8. K. Tyagarajan, E. Pretzer, J. E. Wiktorowicz. 2003. Thiol-reactive dyes for fluorescence labeling of proteomic samples. Electrophoresis. 24: 2348-2358

9. P. Blauenstein, et al. 1995. Experience with the iodine-123 and technitium-99 m labeled anti-granulocyte antibody Mab47: a comparison of labeling methods. Eur. J. nuclear Med. 22:690-698.

10. G. Hermanson. 1996. Bioconjugate Techniques.

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