Application Guide

IRDye® 800CW Maleimide Labeling

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Table of Contents

I. Introduction

IRDye 800CW Maleimide is a functional derivative of infrared dye IRDye 800CW that is reactive toward free–SH (thiol, sulfhydryl) groups. Most molecules that contain free–SH groups can be labeled with maleimide dyes, including IRDye 800CW 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 with IRDye 800CW using the maleimide functional group of IRDye 800CW Maleimide.

The following conditions provide good labeling efficiency:

Table 1. Labeling conditions for IRDye 800CW Maleimide.

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

*Ambient temperature is preferred, but 4 °C may be used if the protein 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 room 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 (MEM) to produce free sulfhydryl groups. After reduction, excess reducing agent should be removed prior to the labeling, since it will also react with the maleimide group.

Additional considerations:

- If it is not possible to remove the reducing agent after reduction, TCEP is the preferred choice.
- Contrary to some claims in the literature, we have observed that TCEP will react with the

maleimide group of the dye during labeling reaction. The ratio of TCEP and maleimide dye to the protein or peptide must be optimized for efficient reduction and labeling.

- The overall concentrations of the reactants affect the rate of the reaction.

III. Examples

Labeling of Affibody® Molecules with IRDye 800CW Maleimide

Affibody molecules are small proteins with unique binding sites capable of binding to different target proteins (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 S-S bridges formed by the C-terminal cysteine and must be reduced prior to labeling with IRDye 800CW 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 molecule in PBS (1 mg/ml) to get a final TCEP concentration of 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, piercenet.com).
5. Reconstitute IRDye 800CW Maleimide (0.5 mg, MW 1191) in 50 µl DMSO or water to get ~10 mM solution. Add 4 µl of maleimide solution (2.5-fold molar excess of dye over protein) to 100 µl reduced Affibody molecule solution. 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.
6. Mix and incubate at room temperature for 2-3 hours, protected from light.
7. Purify the dye labeled Affibody molecule by passing over two 0.5 ml Zeba Desalt Spin Columns consecutively.
8. Store the labeled Affibody molecule protected from light at -20 °C.

Labeling of Small Molecules with IRDye 800CW Maleimide

Glutathione is a small peptide which is available in the reduced form. LI-COR has used glutathione as a model compound to optimize the labeling of small molecules containing free thiols with IRDye 800CW 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 to 0.1 mM (1 µl stock to 100 µl) in PBS, pH 7.4.
3. Reconstitute IRDye 800CW Maleimide (0.5 mg, MW 1191) in 50 µl DMSO or water to get ~10 mM solution.
4. Add 2 µl dye-maleimide solution to the peptide (2-fold molar excess), mix well, and incubate at room temperature for one hour. The excess free dye can be removed from the dye-labeled peptide by using RP-HPLC as follows:

Table 2. RP-HPLC conditions for removing excess dye.

Column	Waters YMC ODS-AQ C18 Column (4.6 X 150 mm)
Gradient	0-100% Acetonitrile in aqueous 50 mM Triethylammonium Acetate (TEAA) buffer, pH 6.0 over 10 minutes
Flow Rate	1.5 mL/min
Monitor	Near 778 nm to track the free dye-labeled peptide and at 260 nm for free peptide

Labeling of Antibodies with IRDye 800CW Maleimide

Antibodies can be labeled with IRDye 800CW Maleimide by selective reduction of disulfide bonds in the hinge region using 2-Mercaptoethylamine (MEM). 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.

1. Reduction of antibodies to generate free thiols
    a. Dissolve the antibody to be reduced at a concentration of 10 mg/ml in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4 buffer containing 1 mM EDTA.
    b. To each milliliter of the antibody solution, add 6 mg of 2-Mercaptoethylamine (final concentration 50 mM). Mix to dissolve.
    c. Incubate the solution in a sealed tube for 90 minutes at 37 °C.
    d. Immediately purify the reduced IgG from excess 2-Mercaptoethylamine and reaction by-products by dialysis or desalting spin column such as Zeba Desalting Spin Columns. All buffers should contain 1-10 mM EDTA to preserve the free sulfhydryls from metal-catalyzed oxidation.
2. Labeling of reduced antibody with IRDye 800CW Maleimide dye

a. Reconstitute IRDye 800CW Maleimide (0.5 mg, MW 1191) in 50 µl DMSO or water to get ~10 mM solution. Protect from light.

b. Add 10-15 µl of maleimide dye solution (~10-fold molar excess of dye over protein) to 1-2 mg/ml reduced antibody solution in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4 buffer containing 1-10 mM EDTA. 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.

c. Mix and incubate at room temperature for 2-3 hours, protected from light. Purify the dye-labeled antibody by dialysis or desalting column such as a Zeba Desalting Spin Column. Store the labeled 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.

IV. References

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