INSTRUCTIONS

PEGylated DyLight Sulfhydryl-Reactive Dyes

2500.1

Number	Description
62292	**DyLight 550-2xPEG Maleimide,** 1mg
62294	**DyLight 650-4xPEG Maleimide,** 1mg
46626	**DyLight 680-4xPEG Maleimide,** 1mg
62297	**DyLight 755-4xPEG Maleimide,** 1mg
46627	**DyLight 800-4xPEG Maleimide,** 1mg

Storage: Upon receipt store at -20°C. Products shipped with an ice pack. Store all dyes in the foil pouch with desiccant to protect from light and moisture.

Table of Contents

Introduction 1
Important Product Information 2
Example Procedure for IgG Reduction and Labeling 2
A. Additional Materials Required 3
B. Material Preparation 3
C. Reduction of IgG Disulfide Bonds 3
D. Labeling of Reduced IgG 3
E. Calculate the Degree of Labeling 4
Troubleshooting 5
Additional Information 5
Related Thermo Scientific Products 5
Reference 6

Introduction

The Thermo Scientific™ DyLight™ PEGylated Dyes display absorption spectra ranging from 550nm to 783nm (Table 1). These reagents fluoresce over a broad pH range, are more intense than Alexa Fluor™ or Cy™ Dyes in many applications and match the output wavelengths of common fluorescence instrumentation. Additionally, the water solubility of the DyLight Reagents allows a high dye-to-protein ratio without precipitation during conjugation.

The PEGylated DyLight Dyes are derivatives of corresponding high-performance non-PEGylated DyLight Dyes, which are used to fluorescently label antibodies and proteins. Conjugates made with PEGylated DyLight Dyes are effective molecular probes that can be used in cellular imaging and other fluorescence detection methods. These DyLight Dyes contain two to four non-toxic polyethylene glycol (PEG) chains. The PEG chains enhance fluorescence and reduce nonspecific binding of conjugates, improve solubility of the dyes and labeled molecules in aqueous solution, aid in cell permeability, and improve retention, notably in tumors.[1] The near-infrared (NIR) to far-red fluorescence properties of some of the PEGylated dyes such as DyLight 680-4xPEG and DyLight 755 make them useful in biological, chemical and pharmaceutical applications, including *in vivo* imaging.

The sulfhydryl-reactive fluors contain maleimide groups that react predominantly with free –SH groups at pH 6.5-7.5, forming a stable thioether bond. At pH 7, the maleimide group is ~1000 times more reactive toward a free sulfhydryl than to an amine. At pH values > 7.5, reactivity towards primary amines increases and hydrolysis of the maleimide group can occur.

Table 1. Properties of the PEGylated DyLight Maleimide Fluors.

DyLight Fluor	Ex/Em*	ε†	MW (g/mol)	Spectrally Similar Dyes
DyLight 550-2xPEG	557 / 571	150,000	1127	Alexa Fluor 555, Cy3, DyLight 550, CF555
DyLight 650-4xPEG	656 / 675	250,000	1450	Alexa Fluor 647, Cy5, DyLight 650, CF647
DyLight 680-4xPEG	684 / 706	180,000	1754	Alexa Fluor 680, Cy5.5, DyLight 680, CF680, IR Dye 680
DyLight 755-4xPEG	757 / 778	220,000	1476	Alexa Fluor 750, DyLight 755, CF750
DyLight 800-4xPEG	784 / 798	270,000	1710	Alexa Fluor 790, Cy7, DyLight 800, CF790, IR Dye 800

* Excitation and emission maxima in nanometers
†Molar extinction coefficient (M^{-1} cm^{-1})

Important Product Information

- The maleimide-activated fluors are moisture-sensitive. Store product in the original container at -20°C with desiccant.
- Equilibrate vial to room temperature before opening to avoid moisture condensation onto the product.
- Prepare this reagent immediately before use. Do not store these reagents in aqueous solutions.
- To remove excess non-reacted DyLight Fluor, use a dialysis membrane with a molecular weight cutoff ≥ 10kDa, an optimized gel filtration matrix or a purification resin (Thermo Scientific™ Pierce™ Dye Removal Columns, Product No. 22858).
- Molecules to be labeled with DyLight Maleimide Fluors must have free –SH group(s) available. Some sulfhydryl-containing peptides and proteins may oxidize in solution to form disulfide bonds, which cannot react with maleimides. Disulfide bonds may be reduced to produce free sulfhydryls. After reduction, most reducing reagents must be removed before conjugation. Thermo Scientific™ Pierce™ Immobilized TCEP Disulfide Reducing Gel (Product No. 77712) enables peptide or protein reduction while recovering the sample in the absence of reducing agents.
- As an alternative to disulfide reduction, sulfhydryls can be introduced via amine modification using N-succinimidyl S-acetylthioacetate (Thermo Scientific™ Pierce™ SATA, Product No. 26102) or 2-iminothiolane•HCl (Thermo Scientific™ Pierce™ Traut's Reagent, Product No. 26101). Avoid sulfhydryl-containing components during conjugation; these components will react with the maleimide portion of the reagent, thereby inhibiting and reducing conjugation efficiency of the intended molecule.
- Use the following fluorescent imagers:
  - 550-2xPEG Dye: Green (526) laser
  - 650-4xPEG Dye: Red (633) laser
  - 680-4xPEG, 755-4xPEG and 800-4xPEG Dyes: laser- and filter-based instruments (e.g., LI-COR Odyssey™ and Aerius™ Infrared Imaging Systems) that emit in the 700nm and 800nm region of the spectrum, respectively.
- Low concentrations of sodium azide (≤ 3mM or 0.02%) or thimerosal (≤ 0.02mM or 0.01%) will not significantly interfere with protein labeling; however, 20-50% glycerol will reduce labeling efficiency.
- The use of sodium azide is not recommended for the PEGylated dyes especially for DyLight 800-4xPEG dye as it will affect the stability of the conjugates.

Example Procedure for IgG Reduction and Labeling

The following protocol is an example method to label a protein with the DyLight Maleimide Fluors. Specific applications may require optimization. In this method, whole IgG is reduced with 2-MEA, which is used to cleave disulfide bonds between the antibody heavy chains while preserving the disulfide linkages between the heavy and light chains. During reduction, the absolute concentration of 2-MEA is more critical than the antibody concentration, as 1-10mg of IgG may be

effectively reduced with 50mM 2-MEA. To prevent metal-catalyzed oxidation of sulfhydryls, EDTA is included in the buffers. The protocol may be modified for other proteins, peptides and molecules.

A. Additional Materials Required

- 2-Mercaptoethylamine•HCl (Thermo Scientific™ Pierce™ 2-MEA, Product No. 20408)
- 0.5M EDTA
- 1M sodium phosphate, pH 6.0
- Phosphate-buffered saline (PBS, 0.1M phosphate, 0.15M sodium chloride; pH 7.2; Product No. 28372) or other buffer at pH 6.5-7.5
- 37°C incubator or water bath
- Dextran Desalting Columns (Product No. 43230) for removing 2-MEA
- Dialysis cassette with a molecular weight cutoff of ≥ 10kDa for removing excess fluor (e.g., Thermo Scientific™ Slide-A-Lyzer™ Dialysis Cassettes or Pierce Dye Removal Columns, Product No. 22858)

B. Material Preparation

Reducing Buffer	Prepare 1mL of Reducing Buffer by combining 100µL of 1M sodium phosphate, pH 6.0, 5µL of 0.5M EDTA and 900µL of ultrapure water
Conjugation Buffer	Add 20µL of 0.5M EDTA to 10mL of PBS for each 10mL of Conjugation Buffer required
IgG Solution	Dissolve 2.5mg of IgG in 1mL of Reducing Buffer

C. Reduction of IgG Disulfide Bonds

1. Add 1mL of IgG Solution to a 6mg vial of 2-MEA. Gently shake vial to dissolve. Incubate reaction for 90 minutes at 37°C.
2. Cool the solution to room temperature.
3. Remove 2-MEA from the reduced antibody using a desalting column equilibrated with Conjugation Buffer. After the antibody solution has entered the gel bed, add additional Conjugation Buffer and collect 500µL fractions.

   Note: The antibody generally emerges when one void volume of buffer has been added to the column after the antibody has been applied. Molecules smaller than the column's exclusion limit, such as 2-MEA, emerge from the column in subsequent fractions, which can be discarded after confirming that all fractions containing protein have been collected.
4. Determine antibody location by measuring the absorbance of each fraction at 280nm. Pool fractions containing reduced antibody. To minimize sulfhydryl oxidation, proceed immediately to Section D.

D. Labeling of Reduced IgG

Note: Upon reduction or modification of the protein, remove excess reducing or modification reagent by desalting before reaction with the maleimide-activated dyes.

1. Tap the bottom of the dye vial against a hard surface to ensure the dye is at the bottom of the tube. Add 100µL of DMF to the vial.
2. Vortex the vial for 30 seconds. Incubate the vial at room temperature for 5 minutes. Pipette the solution up and down to completely dissolve.
3. Add 20µL of dye to the tube containing the reduced IgG Solution and mix well.
4. Allow the reaction to proceed for 2 hours to overnight at room temperature.
5. Remove non-reacted dye from the antibody using a dialysis cassette or a Pierce Dye Removal Column (Product No. 22858).
6. Store labeled antibody protected from light at 4°C for up to one month. Alternatively, store labeled antibody in single-use volumes at -20°C.

E. Calculate the Degree of Labeling

1. Remove excess fluor reagent from the sample using a dialysis membrane with a molecular weight cutoff ≥ 10kDa.

   Note: The non-reacted fluor must be completely removed for optimal results and accurate determination of the fluor-to-protein ratio. For best results, remove excess non-reacted fluor by dialyzing for ~4 hours using three dialysis buffer changes. Gel filtration (e.g., desalting column) is typically not as effective as dialysis.
2. Dilute a small amount of labeled, purified protein in PBS.
3. Using a 1cm path length cuvette, measure the absorbance at 280nm and the A_{max} of the specific fluor (Table 2).

Table 2. Properties of the PEGylated DyLight Fluor.

DyLight Fluor	A_{max}*	ε†	CF‡
DyLight 550-2xPEG	557	150,000	0.080
DyLight 650-4xPEG	656	250,000	0.037
DyLight 680-4xPEG	684	180,000	0.090
DyLight 755-4xPEG	757	220,000	0.030
DyLight 800-4xPEG	784	270,000	0.020

* Excitation wavelength in nanometers – note that upon protein conjugation the absorption maximum shifts to the right of the spectra
†Molar extinction coefficient (M^{-1} cm^{-1}) at A_{max}
‡Correction factor (A_{280}/A_{max})

4. Calculate protein concentration as follows:

$$\textbf{Protein concentration (M)} = \frac{[\mathbf{A_{280} - (A_{max} \times CF)}]}{\boldsymbol{\varepsilon}_{\mathbf{protein}}} \times \textbf{dilution factor}$$

$\varepsilon_{protein}$ = protein molar extinction coefficient (e.g., the molar extinction coefficient of IgG is ~210,000 M^{-1} cm^{-1})

$$\text{CF = correction factor} = \frac{\mathbf{A_{280}\ of\ the\ fluor}}{\mathbf{A_{max}\ of\ the\ fluor}} \text{ (see Table 2)}$$

5. Calculate the degree of labeling:

$$\textbf{Moles dye per mole protein} = \frac{\mathbf{A_{max}\ of\ the\ labeled\ protein \times dilution\ factor}}{\boldsymbol{\varepsilon}_{\mathbf{fluor}} \times \textbf{protein concentration (M)}}$$

ε_{dye} = see Table 2

Example calculations for DyLight 680-4xPEG Dye conjugated to antibodies:

Dilution factor = 20
A_{280} = 0.081
A_{max} at 684 nm = 0.065

$$\textbf{Protein concentration (M)} = \frac{[\mathbf{0.081 - (0.065 \times 0.09)}]}{\mathbf{210{,}000}} \times \mathbf{20} = \mathbf{0.000007157M}$$

$$\textbf{Moles dye per mole protein} = \frac{\mathbf{0.526 \times 20}}{\mathbf{180{,}000 \times 0.000007157}} = \mathbf{1.009}$$

Troubleshooting

Problem	Cause	Solution
Detection of the fluor-labeled protein was unsuccessful	Instrument configuration and/or detection procedure were not optimal for specific fluorophores	Optimize the detection method
		Check the instrument manual and optimize configuration for the fluorophore
	The protein was not labeled or was weakly labeled	Before troubleshooting, determine if the protein is labeled by calculating the A_{max}:A_{280} ratio; determine this ratio after thorough desalting or dialysis **Note:** For fluor-labeled antibodies the A_{max}:A_{280} ratio should be > 1
The protein was not labeled	Substance interfered with the reaction or incorrect reaction conditions	Ensure that the Conjugation Buffer is at pH 6.5-7.5 and does not contain free thiols, such as reducing agents
	There are no free sulfhydryls available on the protein	Reduce existing disulfide bonds to generate free sulfhydryls, or introduce sulfhydryls with Traut's Reagent or SATA

Additional Information

Visit our website for additional information including the following items:

- Tech Tip #43: Protein stability and storage
- Tech Tip #3: Determine reactivity of NHS-ester biotinylation and crosslinking reagents
- Tech Tip #30: Modify and label oligonucleotide 5´ phosphate groups

Related Thermo Scientific Products

22858	**Pierce Dye Removal Columns**
46646-53067	**DyLight Near Infrared Specialty Dyes**
62262	**DyLight 550 NHS Ester,** 1mg
62263	**DyLight 550 NHS Ester,** 5 × 50µg
84530	**DyLight 550 Antibody Labeling Kit**
84531	**DyLight 550 Microscale Antibody Labeling Kit**
62265	**DyLight 650 NHS Ester,** 1mg
62266	**DyLight 650 NHS Ester,** 5 × 50µg
84535	**DyLight 650 Antibody Labeling Kit**
84536	**DyLight 650 Microscale Antibody Labeling Kit**
46418	**DyLight 680 NHS Ester,** 1mg
46419	**DyLight 680 NHS Ester,** 5 × 50µg
53056	**DyLight 680 Antibody Labeling Kit**
53057	**DyLight 680 Microscale Antibody Labeling Kit**
46601	**DyLight 680-4xPEG NHS Ester,** 1mg
46603	**DyLight 680-4xPEG NHS Ester,** 5 × 65µg
53076	**DyLight 680-4xPEG Antibody Labeling Kit**
53077	**DyLight 680-4xPEG Microscale Antibody Labeling Kit**

46626	**DyLight 680-4xPEG Maleimide,** 1mg
62278	**DyLight 755 NHS Ester,** 1mg
62279	**DyLight 755 NHS Ester,** 5 × 50µg
84538	**DyLight 755 Antibody Labeling Kit**
84539	**DyLight 755 Microscale Antibody Labeling Kit**
62298	**DyLight 755 Maleimide,** 1mg
46421	**DyLight 800 NHS Ester,** 1mg
46422	**DyLight 800 NHS Ester,** 5 × 50µg
53062	**DyLight 800 Antibody Labeling Kit**
53063	**DyLight 800 Microscale Antibody Labeling Kit**
46621	**DyLight 800 Maleimide**
46645	**Pierce Immunostain Enhancer,** 2mL
46644	**Pierce Immunostain Enhancer,** 20mL
62247	**DAPI Nuclear Counterstain**
62248	**DAPI Solution**
62249	**Hoechst 3342 Solution**
62254	**DRAQ5™ Fluorescent Probe**
20036	**Bioconjugate Techniques, 2nd Edition**

Reference

1. Knop, K., *et al.* (2010). Poly(ethylene glycol) in drug delivery: Pros and cons as well as potential alternatives. *Angew Chem Int Ed* **49:**6288-308.