INSTRUCTIONS

Thermo SCIENTIFIC

DyLight™ Sulfhydryl-Reactive Dyes

1964.10

Number	Description
46622	**DyLight 350 Maleimide,** 1mg
46600	**DyLight 405 Maleimide,** 1mg
46602	**DyLight 488 Maleimide,** 1mg
62290	**DyLight 550 Maleimide,** 1mg
46608	**DyLight 594 Maleimide,** 1mg
46613	**DyLight 633 Maleimide,** 1mg
62295	**DyLight 650 Maleimide,** 1mg
46618	**DyLight 680 Maleimide,** 1mg
62298	**DyLight 755 Maleimide,** 1mg
46621	**DyLight 800 Maleimide,** 1mg

Storage: Upon receipt store at -20°C in foil pouch with desiccant to protect from light and moisture. Product shipped at ambient temperature.

Introduction

The Thermo Scientific™ DyLight™ Dyes have absorption spectra ranging from 350 to 770nm (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 fluor-to-protein ratio without precipitation during conjugation.

The sulfhydryl-reactive dyes 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.[1] At pH values > 7.5, reactivity toward primary amines increases and hydrolysis of the maleimide group can occur.

Table 1. Properties of the Thermo Scientific DyLight Maleimide Dyes.

DyLight Dye	Ex/Em*	ε†	MW (g/mol)	Spectrally Similar Dyes
350	353 / 432	15,000	899	AMCA, Alexa 350
405	400 / 420	30,000	818	Alexa Fluor 405
488	493 / 518	70,000	800	Alexa Fluor 488, Cy2
550	557 / 572	150,000	1065	Alexa Fluor 555, Cy3
594	593 / 618	80,000	1059	Alexa Fluor 594
633	638 / 658	170,000	1091	Alexa Fluor 633
650	654 / 672	250,000	1091	Alexa Fluor 647, Cy5
680	682 / 715	140,000	972	Alexa Fluor 680, Cy5.5
755	754 / 776	220,000	1117	Alexa Fluor 750
800	770 / 794	270,000	1075	IRDye 800

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

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Important Product Information

- The maleimide-activated dyes 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.
- Molecules to be labeled with DyLight Maleimide Dyes 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. The Thermo Scientific 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 (SATA, Product No. 26102) or 2-iminothiolane•HCl (Traut's Reagent, Product No. 26101).
- Avoid sulfhydryl-containing components during conjugation, as these will react with the maleimide portion of the reagent, thereby inhibiting and reducing conjugation efficiency of the intended molecule.
- Use the following fluorescent imagers:
  - 350 dye: UV argon-ion laser at 351-363nm
  - 405 dye: Spectral line of the blue diode laser
  - 488 dye: Green (526) laser
  - 550 and 594 dyes: Green (532) laser
  - 633 and 650 dyes: Red (633) laser
  - 680, 755 and 800 dyes: laser- and filter-based instruments that emit in the 700nm and 800nm region of the spectrum, respectively; these dyes are well-suited for the 700 and 800 channels of the LI-COR Odyssey™ and the LI-COR Aerius™ Infrared Imaging Systems.
- To remove excess non-reacted DyLight Dye, use a dialysis membrane with a molecular-weight cutoff ≥ 10K.

Example Procedure for IgG Reduction and Labeling

The following protocol is an example application for DyLight Maleimide and, therefore, specific applications 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 antibody concentration, as 1-10mg IgG can be effectively reduced with 50mM 2-MEA. To prevent metal-catalyzed oxidation of sulfhydryls, EDTA is included in buffers. The protocol can be modified for other proteins, peptides and molecules.

A. Additional Materials Required

- 2-Mercaptoethylamine•HCl (2-MEA, Product No. 20408)
- 0.5M EDTA
- 1M Sodium phosphate, pH 6.0
- Dimethylformamide (DMF)
- 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
- Thermo Scientific Slide-A-Lyzer Dialysis Cassette with a molecular-weight cutoff of ≥ 10K or Thermo Scientific Fluorescent Dye Removal Column (Product No. 22858) for removing excess dye

B. Material Preparation

- Reducing Buffer: Prepare 1mL of 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 IgG in 1mL of Reducing Buffer.

C. Reduction of IgG Disulfide Bonds

1. Add the 1mL 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, it is essential to remove the excess reducing or modification reagent by desalting before reaction with the maleimide-activated dyes.

1. Tap the bottom of the vial of dye 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 vial at room temperature for 5 minutes. Pipette the solution up and down to completely dissolve.
3. Add 20µL of the 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 Thermo Scientific Fluorescent 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 dye reagent from the sample using a Fluorescent Dye Removal Column or a dialysis membrane with a molecular-weight cutoff ≥ 10K.

   Note: The non-reacted dye must be completely removed for optimal results and accurate determination of the dye-to-protein ratio. For best results when using dialysis, dialyze for ~4 hours using three dialysis buffer changes. Desalting columns are not effective for dye removal.
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 dye (Table 2).

Table 2. Properties of the Thermo Scientific DyLight Dyes.

DyLight Dye	A_{max}*	ε†	CF‡
350	353	15,000	0.144
405	405	30,000	0.564
488	493	70,000	0.147
550	557	150,000	0.081
594	595	80,000	0.585
633	627	170,000	0.110
650	655	250,000	0.037
680	684	140,000	0.128
755	755	220,000	0.030
800	777	270,000	0.045

* 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:

$$\text{Protein concentration (M)} = \frac{[A_{280} - (A_{max} \times CF)]}{\varepsilon_{protein}} \times \text{dilution factor}$$

- $\varepsilon_{protein}$ = protein molar extinction coefficient (e.g., the molar extinction coefficient for IgG is ~210,000 M^{-1} cm^{-1})
- $\text{CF = Correction factor} = \frac{A_{280} \text{ of the fluor}}{A_{max} \text{ of the fluor}}$ (see Table 2)

5. Calculate the degree of labeling:

$$\text{Moles dye per mole protein} = \frac{A_{max} \text{ of the labeled protein} \times \text{ dilution factor}}{\varepsilon_{fluor} \times \text{protein concentration (M)}}$$

- ε_{dye} = See Table 2

Example calculations for DyLight 488-Maleimide conjugated antibody:

- Dilution factor = 20
- A_{280} = 0.072
- A_{max} at 493nm = 0.053

$$\text{Protein concentration (M)} = \frac{[0.072 - (0.053 \times 0.147)]}{210{,}000} \times 20 = 0.000006115\ \text{M}$$

$$\text{Moles dye per mole protein} = \frac{0.053 \times 20}{70{,}000 \times 0.000006115} = 2.5$$

Troubleshooting

Problem	Cause	Solution
The application in which the dye-labeled protein was used was unsuccessful	The protein was not 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 dye-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

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Additional Information

Please 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

46426 **DyLight 350 NHS Ester,** 1mg
46400 **DyLight 405 NHS Ester,** 1mg
46402 **DyLight 488 NHS Ester,** 1mg
62262 **DyLight 550 NHS Ester,** 1mg
46412 **DyLight 594 NHS Ester,** 1mg
46414 **DyLight 633 NHS Ester,** 1mg
62295 **DyLight 650 NHS Ester,** 1mg
46418 **DyLight 680 NHS Ester,** 1mg
62278 **DyLight 755 NHS Ester,** 1mg
46421 **DyLight 800 NHS Ester,** 1mg
66382 **Slide-A-Lyzer Dialysis Cassette Kit, 10K MWCO,** for 0.5-3mL samples, 10 units, buoys and syringes
66807 **Slide-A-Lyzer Dialysis Cassette Kit, 10K MWCO,** for 3-12mL samples, 10 units, buoys and syringes

General Reference

1. Hermanson, G.T. (1996). Bioconjugate Techniques, Academic Press.

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Current product instructions are available at *www.thermoscientific.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.

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