INSTRUCTIONS

Thermo
SCIENTIFIC

DyLight™ Sulfhydryl-Reactive Fluors

Number	Description
46622	**DyLight 350 Maleimide,** 1 mg
46600	**DyLight 405 Maleimide,** 1 mg
46602	**DyLight 488 Maleimide,** 1 mg
46607	**DyLight 549 Maleimide,** 1 mg
46608	**DyLight 594 Maleimide,** 1 mg
46613	**DyLight 633 Maleimide,** 1 mg
46615	**DyLight 649 Maleimide,** 1 mg
46618	**DyLight 680 Maleimide,** 1 mg
46620	**DyLight 680B Maleimide,** 1 mg
46619	**DyLight 750 Maleimide,** 1 mg
46621	**DyLight 800 Maleimide,** 1 mg

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 Fluors have absorption spectra ranging from 350 to 770 nm (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 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 ~1,000 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 DyLight Maleimide Fluors.

DyLight Fluor	Ex/Em*	ε†	MW (g/mol)	Spectrally Similar Dyes
350	353/432	15,000	899.15	AMCA, Alexa 350
405	400/420	30,000	818	Alexa Fluor 405
488	493/518	70,000	800	Alexa Fluor 488, Cy2
549	562/576	150,000	1,007	Alexa Fluor 555, Cy3
594	593/618	80,000	1,059	Alexa Fluor 594
633	638/658	170,000	1,091	Alexa Fluor 633
649	646/674	250,000	1,033	Alexa Fluor 647, Cy5
680	682/715	140,000	972	Alexa Fluor 680, Cy5.5
680B	679/698	180,000	1221.21	Alexa Fluor 680, Cy5.5
750	752/778	210,000	1,059	Alexa Fluor 750
800	770/794	270,000	1,075	IRDye 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.
- 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. The 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 fluor: UV argon-ion laser at 351-363 nm
    - 405 fluor: Spectral line of the blue diode laser
    - 549 and 594 fluors: Green (532) laser
    - 633 and 649 fluors: Red (633) laser
    - 488 fluor: Green (526) laser
    - 680, 680B (B=brighter), 750 and 800 fluors: laser- and filter-based instruments that emit in the 700 nm and 800 nm region of the spectrum, respectively; these fluors 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 Fluor, use a dialysis membrane with a molecular-weight cutoff ≥ 10 K.

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 1-10 mg IgG can be effectively reduced with 50 mM 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)
- 0.5 M EDTA
- 1 M Sodium phosphate, pH 6.0
- Phosphate-buffered saline (PBS): 0.1 M phosphate, 0.15 M 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 ≥ 10 K for removing excess fluor

B. Material Preparation

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

C. Reduction of IgG Disulfide Bonds

1. Add the 1 ml IgG Solution to a 6 mg 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 500 µl 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 280 nm. 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. Dissolve the 1 mg DyLight Maleimide in 100 µl of DMF.

   Note: Dissolve DyLight 750 Maleimide in 50 µl of DMF and 50 µl of ultrapure water.
2. Add 20 µl of the dye to the tube containing the reduced IgG solution and mix well.
3. Allow the reaction to proceed for 2 hours to overnight at room temperature.
4. Remove nonreacted dye from the antibody by desalting or dialysis.
5. Store labeled antibody protected from light at 4°C for up to one month. Alternatively, store labeled antibody in single-use aliquots 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 ≥ 10 K.

   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, such as desalting columns, is typically not as effective as dialysis.
2. Dilute a small amount of labeled, purified protein in PBS.
3. Using a 1 cm path length cuvette, measure the absorbance at 280 nm and the A_{max} of the specific fluor (Table 2).

Table 2. Properties of the DyLight Fluors.

DyLight Fluor	A_{max}*	ε†	CF‡
350	353	15,000	0.144
405	405	30,000	0.564
488	493	70,000	0.147
549	562	150,000	0.081
594	595	80,000	0.585
633	627	170,000	0.110
649	654	250,000	0.037
680	684	140,000	0.128
608B	681	180,000	0.090
750	755	210,000	0.020
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})

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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})
- 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_{fluor} = See Table 2

Example calculations for DyLight 488-Maleimide conjugated antibody:

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

Please visit our web site for additional information including the following items:

- Tech Tip #43: Protein stability and storage
- Tech Tip #6: Extinction coefficients guide
- 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, 1 mg**
46400	**DyLight 405 NHS Ester,** 1 mg
46402	**DyLight 488 NHS Ester,** 1 mg
46407	**DyLight 549 NHS Ester,** 1 mg
46412	**DyLight 594 NHS Ester,** 1 mg
46414	**DyLight 633 NHS Ester,** 1 mg
46415	**DyLight 649 NHS Ester,** 1 mg
46418	**DyLight 680 NHS Ester,** 1 mg
53068	**DyLight 680B NHS Ester,** 1 mg
46420	**DyLight 750 NHS Ester,** 1 mg
46421	**DyLight 800 NHS Ester,** 1 mg
66382	**Slide-A-Lyzer Dialysis Cassette Kit, 10 K MWCO,** for 0.5-3 ml samples, 10 units, buoys and syringes
66807	**Slide-A-Lyzer Dialysis Cassette Kit, 10 K MWCO,** for 3-12 ml samples, 10 units, buoys and syringes

Reference

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

Patents pending on DyLight™ 488, 549, 649 and 750 Dyes

Slide-A-Lyzer Dialysis Cassettes are protected by U.S. Patent # 5,503,741 and 7,056,440; CA 2,170,738; and EP0 720 508 B1.

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

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