INSTRUCTIONS

PEGylated DyLight Amine-Reactive Dyes

2498.1

Number	Description
62269	**DyLight 550-2xPEG NHS Ester,** 1mg
62274	**DyLight 650-4xPEG NHS Ester,** 1mg
46601	**DyLight 680-4xPEG NHS Ester,** 1mg
62277	**DyLight 755-4xPEG NHS Ester,** 1mg
46604	**DyLight 800-4xPEG NHS Ester,** 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.

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Introduction

The Thermo Scientific™ DyLight™ PEGylated Dyes display absorption maxima 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 for 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 amine-reactive dyes contain N-hydroxysuccinimide (NHS) esters, the simplest and most commonly used reactive group for labeling proteins. NHS esters react with primary amines on the protein, forming a stable, covalent amide bond and releasing the NHS group.

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Table 1. Properties of the PEGylated DyLight NHS-Ester Fluor.

DyLight Fluor	Ex/Em*	ε†	MW (g/mol)	Spectrally Similar Dyes
DyLight 550-2xPEG	557 / 571	150,000	1102	Alexa Fluor 555, Cy3, DyLight 550, CF555
DyLight 650-4xPEG	658 / 681	250,000	1425	Alexa Fluor 647, Cy5, DyLight 650, CF647
DyLight 680-4xPEG	684 / 706	180,000	1729	Alexa Fluor 680, Cy5.5, DyLight 680, CF680, IR Dye 680
DyLight 755-4xPEG	754 / 777	220,000	1451	Alexa Fluor 750, DyLight 755, CF750
DyLight 800-4xPEG	783 / 797	270,000	1685	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

- NHS ester-activated fluorophores are moisture-sensitive. Store product in the original pouch at -20°C. Avoid moisture condensation onto the product by equilibrating the vial to room temperature before opening. Prepare these labeling reagents immediately before use. Do not store NHS-ester reagents prepared in aqueous solutions.
- Use the following fluorescent lasers/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%) may not interfere with protein labeling, but will affect the stability during storage so we do not recommend the presence of sodium azide in the conjugation buffer; 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.
- 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).

Procedure for Protein Labeling

The following is an example method to label a protein with the DyLight Amine-Reactive Fluors. Specific applications will require further optimization.

A. Protein Preparation

The optimal labeling buffer is 0.05M sodium borate buffer at pH 8.5 (Thermo Scientific™ BupH™ Borate Buffer Packs, Product No. 28384). Buffers that contain primary amines (e.g., Tris or glycine) will interfere because they react with the NHS-ester dye moiety. Dissolve protein directly in the labeling buffer. For each labeling reaction, use 100-500µL of purified protein sample at 1-10mg/mL. After reconstitution, proceed to the Calculations for Labeling Section. If the protein is already in a buffer that is not suitable, exchange the buffer into a more optimal labeling buffer by dialysis or gel filtration.

Note: The following buffers may be substituted for borate buffer: 0.1M sodium phosphate, 0.15M NaCl at pH 7.2-7.5 (e.g., BupH Phosphate Buffered Saline Packs, Product No. 28372) or 0.1M sodium carbonate at pH 8.3-9.0. However, dye molar excesses will need to be optimized as labeling efficiencies with these buffers are typically lower (pH 7.2-7.5) or higher (pH 9.0) as compared to borate buffer (pH 8.5).

B. DyLight Dye Preparation

Note: The PEGylated DyLight NHS Ester reagents are moisture-sensitive. Store the reagent in the original container at -20°C with desiccant.

1. Equilibrate vial to room temperature before opening to avoid moisture condensation onto the reagent. Dissolve reagent in DMF at 10mg/mL. The reagent may also be dissolved at 1mg/mL to make pipetting small amounts more accurate; however, adjust for the concentration change when calculating the reagent amount added to the labeling reaction.

C. Calculations for Labeling

The amount of fluorescent-labeling reagent to use for each reaction depends on the amount of protein to be labeled and the specific fluorophore being used (Table 2). Generally, the more concentrated the protein, the more efficient the reaction.

Table 2. Amounts of amine-reactive dye to use.

DyLight fluor	Protein (mg/mL)	Molar-fold Excess	Protein (mg/mL)	Molar-fold Excess
DyLight 550-2xPEG	< 5	10-15	≥ 5	8
DyLight 650-4xPEG	< 5	6	≥ 5	4
DyLight 680-4xPEG	< 5	5-8	≥ 5	6-7
DyLight 755-4xPEG	< 5	5-10	≥ 5	6-7
DyLight 800-4xPEG	< 5	5-8	≥ 5	6-7

2. Calculate amount (mg) of DyLight NHS-Ester Dye to be added to the labeling reaction:

$$\frac{\textbf{amount of protein (mg)}}{\textbf{MW of protein}} \times \mathbf{5} \times \textbf{MW of fluor} = \_\_\_\_\_ \textbf{ mg of fluor}$$

5 = molar-fold excess of the NHS-Ester dye to protein

3. Calculate microliters of NHS-Ester dye solution to add to the reaction:

$$\textbf{mg of fluor (calculation \#1)} \times \frac{\mathbf{100\,\mu L}}{\mathbf{1\,mg}} = \_\_\_\_\_ \textbf{ µL NHS - ester fluor solution at 10mg/mL}$$

100µL = solvent volume in which the 1mg of NHS-Ester dye is dissolved

Example Calculation:

For 1mL of a 2mg/mL solution of IgG (150,000 MW), 11.52µL of DyLight 680-4xPEG NHS-Ester (10mg/mL) will be used.

$$\frac{\mathbf{2\,mg\ IgG}}{\mathbf{150{,}000\ \ MW}} \times \mathbf{5} \times \mathbf{1729} = \mathbf{0.1153\ mg\ of\ DyLight\ 680\ -\ 4xPEG\ \ NHS\ -\ Ester}$$

$$\mathbf{0.1153mg\ \ of\ DyLight\ 680\ -\ 4xPEG\ NHS\ -\ Ester} \times \frac{\mathbf{100\,\mu L}}{\mathbf{1mg}} = \mathbf{11.53\mu 1\ \ of\ DyLight\ 680\ -\ 4xPEG\ NHS\ -\ Ester}$$

D. Labeling Reaction

1. To prevent condensation, allow dye and DMF to equilibrate to room temperature before opening vials.
2. Transfer the protein solution to a reaction tube. Add 100µL of DMF to the 1mg dye to prepare a 10mg/mL solution. Pipette up and down or vortex until completely dissolved.

   Note: To ensure complete dissolution, allow the dye to completely dissolve for 5 minutes and then vortex again.
3. Transfer the appropriate amount of dye solution (based on calculations) to the reaction tube containing the protein. Mix well and incubate at room temperature for 1 hour.
4. Remove non-reactive excess fluor reagent from the protein using a dye removal column or a dialysis membrane with a molecular weight cutoff ≥ 10kDa.

Note: Non-reacted fluor must be completely removed for optimal results and accurate determination of the fluor-to-protein ratio. For best results, use a dye removal column or dialyze for ~4 hours using three dialysis buffer changes. Gel filtration, such as with a desalting column, is not typically as effective as dialysis.

5. Store labeled protein protected from light at 4°C for up to one month. We do not recommend the use of sodium azide when storing conjugates made with the PEGylated dyes; especially for DyLight 800-4xPEG Dye, as it will affect the long term stability. Alternatively, store labeled protein in single-use volumes in 50% Glycerol at -20°C.

E. Calculate the Degree of Labeling

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

   Note: The non-reacted dye must be completely removed for optimal results and accurate determination of the dye-to-protein ratio.

2. Dilute a small amount of labeled, purified protein in PBS. Using a 1cm path length cuvette, measure the absorbance at 280nm and the A_{max} of the specific dye (Table 3).

Table 3. Properties of the PEGylated DyLight Fluor.

DyLight Fluor	A_{max}*	ε†	CF‡
DyLight 550-2xPEG	557	150,000	0.080
DyLight 650-4xPEG	658	250,000	0.037
DyLight 680-4xPEG	684	180,000	0.090
DyLight 755-4xPEG	754	220,000	0.030
DyLight 800-4xPEG	783	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})

3. 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})

$$\text{CF = correction factor} = \frac{\mathbf{A_{280}\ of\ the\ fluor}}{\mathbf{A_{max}\ of\ the\ fluor}} \text{ (see Table 3)}$$

4. 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 3

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

Dilution factor = 10
A_{280} = 0.177
A_{max} at 684 nm = 0.526

$$\textbf{Protein concentration (M)} = \frac{[0.177 - (0.526 \times 0.09)]}{210{,}000} \times 10 = 0.000006174\ \textbf{M}$$

$$\textbf{Moles dye per mole protein} = \frac{0.526 \times 10}{150{,}000 \times 0.000006174} = 5.67$$

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	Conjugation buffer contained primary amines (e.g., Tris or glycine) that interfered with the reaction	Use a conjugation buffer free of primary amines such as borate, carbonate or PBS
	The NHS-ester has hydrolyzed and is non-reactive	Prepare labeling reagent immediately before use; do not store NHS-ester reagents in aqueous solutions

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.