Protocol

TD-P Revision 2.0

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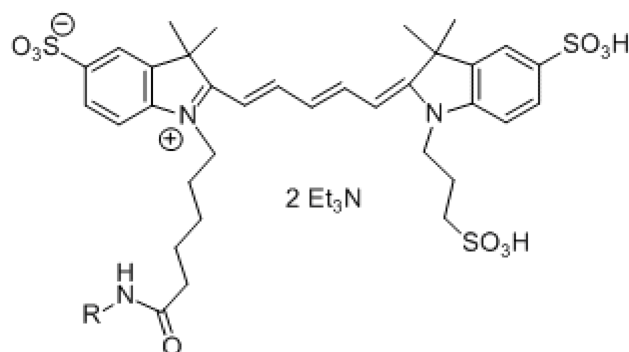
Revision Date: 3/5/2019

Cy5-NHS Ester Technical Information

Product No.:	B-430
Product Name:	CY5-NHS Ester
Chemical Structure:	
Chemical Composition:	$C_{38}H_{46}N_3O_{11}S_3$
Molecular Weight:	1050.97 g/mol (triethylammonium salt)
Solubility:	Water, DMF, DMSO
Spectral Properties:	
Absorbance/Emissions	649/670 nm
ε_{max}	250,000 $M^{-1}cm^{-1}$
CF_{280}	0.05
CF_{260}	0.05
Storage:	Store at -20°C. (Product shipped at ambient temperature).

Introduction

Cy5-NHS Ester is a bright, photostable, and pH insensitive fluorescent dye that enables simple and efficient Cy5 fluorescent labeling of any primary amine-containing macromolecule. They are excited to about 98% of maximum with a krypton/argon laser (647 nm line) or to about 63% of maximum with a helium/neon laser (633 nm line). NHS activated esters react efficiently with primary amino groups (-NH2) forming stable amide bonds. When labeling antibodies or other

proteins with Cy dyes, the brightest conjugates have dye/protein ratios ranging from 4-12(1), depending on the specific applications. High ratios can increase non-specific background, loss of antibody binding affinity, and/or lead to aggregation. For this reason, optimal labeling ratios should be experimentally verified for any given application.

Materials

Important Product Information

- NHS esters are moisture-sensitive. To avoid moisture condensation onto the product always let vial come to room temperature before opening; be careful to limit exposure to. The NHS-ester moiety readily hydrolyzes and becomes non-reactive; therefore, prepare stock solutions immediately before use. Prepare stock solutions in anhydrous solvents (e.g. DMSO or DMF) immediately before use.
- Hydrolysis of the NHS ester is a competing reaction in aqueous buffers. Conjugation with primary amines of proteins/peptides (i.e., acylation) is favored in the pH range 7-9 with concentrated protein solutions (e.g. 1-10 mg/ml). Use non-amine-containing buffers at pH 7-9, e.g. 100mM sodium phosphate, 150mM sodium chloride, pH 7.5, 100mM HEPES, pH 7.5, 100mM carbonate/bicarbonate, pH 8-9 or 100mM borate buffer, pH 8-9.
- Do not use buffers that contain primary amines, (e.g., Tris, glycine).

Additional Materials Required

- Water-miscible organic solvent such as dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF)
- Reaction buffer: Sodium phosphate buffer (100mM sodium phosphate, 150mM sodium chloride, pH 7.5).
- Desalting Spin Columns or Gravity Flow Desalting SEC Columns (e.g. Sephadex G-25).

Method

Procedure for Labeling an Antibody with Cy5-NH2

1. Dissolve antibody into a suitable amine-free buffer (e.g. 100mM sodium phosphate, 150mM sodium chloride, pH 7.5) to obtain a 1-5 mg/ml solution. If necessary buffer exchange/desalt into 100mM sodium phosphate, 150mM sodium chloride, pH 7.5 prior to labeling if residual traces of Tris or glycine contaminants are present.

2. Immediately before use, dissolve Cy5-NHS Ester reagent into a suitable volume of DMSO or aqueous buffer in order to achieve a reagent concentration 400-fold greater than the initial concentration of antibody. For example, if the IgG to be labeled is at 1 mg/ml (i.e. 6.7µM) dissolve the Cy5-NHS Ester reagent at 2.7mM in DMSO, if IgG is at 2.5 mg/ml dissolve the reagent at 6.75mM, etc.

3. Add the appropriate volume of NHS reagent to the antibody in order to achieve the desired molar excess of Cy5-NHS Ester reagent. To target a specific degree of labeling, refer to Figure 1 under calculations.

4. Incubate the reaction at room temperature for 60 minutes (or longer).

5. Remove excess non-reactive reagent by dialysis or desalting spin column into buffer of choice.

6. Measure absorbance of Cy5-antibody conjugate at 280 nm and 550 nm.

7. Determine Cy5 DOL and conjugate concentration. See Calculations.

Calculations

Calculate degree of labeling (DOL) and Cy5 conjugate concentration (mg/ml) with the following equations:

Eq. 1 $$number\ of\ Cy5\ dye\ per\ protein = \frac{molarity\ Cy5\ dye}{molarity\ protein}$$

Eq. 2 $$[molarity\ of\ Cy5\ dye] = \frac{A_{650}}{\varepsilon_{650}}$$

Eq. 3 $$[molarity\ of\ protein] = \frac{A_{280C}}{\epsilon_{280}}$$

Eq. 4 $$mg/ml = \frac{A_{280} - (650 \times 0.08)}{\left(\frac{E1\%}{10}\right)} \times dilution\ factor$$

A_{550} = *conjugate absorbance at* 650 ± 2 *nm*
$\mathcal{E}_{350}$ = *molar extinction coefficient Cy5 dye* = 150,000 $M^{-1}cm^{-1}$
A_{280} = *conjugate absorbance at 280 nm*
A_{280c} = *corrected conjugate absorbance at 280 nm* = A_{280} - (A_{650} x (0.05))
ϵ_{280} = *molar extinction coefficient protein* ($M^{-1}cm^{-1}$) = $\frac{MW_p\ x\ E1\%}{10}$

Example 1. Determine Cy5 DOL and conjugate concentrations as follows:
A Goat IgG antibody 0.1 ml at 1.0 mg/ml was labeled using a 20-fold molar excess Cy5-NHS reagent. After removal of excess dye with a spin column, the conjugate's A_{280} and A_{650} were determined in PBS (1:5 dilution), A_{280} = 0.2906 and A_{650} = 0.9825. Goat IgG M. W. 150 kDa, E1% = 13.6 or 204,000 $M^{-1}cm^{-1}$.

Calculate DOL (# Cy3/protein) as follows:

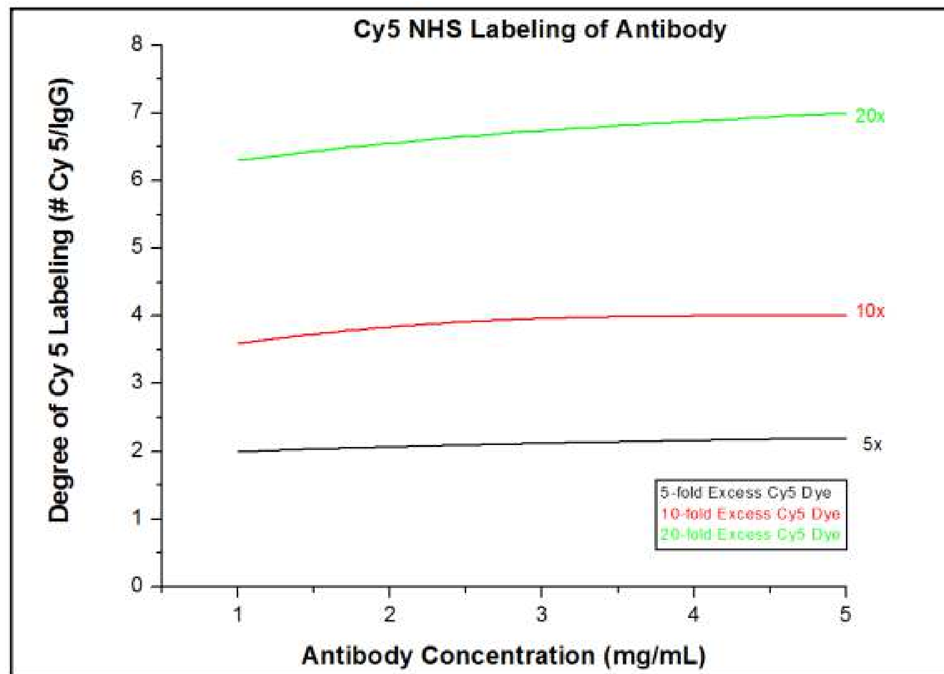
By Equation 2 $$molarity\ of\ Cy5 = \frac{1.3783}{250{,}000\ M^{-1}\ cm^{-1}} = 5.51\ \mu M$$

By Equation 3 $$molarity\ of\ IgG = \frac{0.2680 - (1.3783 \times 0.05)}{204{,}000\ M^{-1}\ cm^{-1}} = 0.877\ \mu M$$

By Equation 1 $$Number\ of\ Cy5\ per\ IgG = \frac{5.51\ \mu M}{0.877\ \mu M} = 6.28$$

Calculate Cy3-IgG (mg/ml)

By Equation 4 $$mg/ml = \frac{0.2680 - (1.3783 \times 0.05)}{1.36} \times 5 = 0.066\ mg/ml$$



References

Southwick, P.L., et al. (1990). Cyanine dye labeling reagents-carboxymethylindocyanine succinimidyl esters. Cytometry. 11(3): 418-430.

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