Protocol

TD-P Revision 2.0

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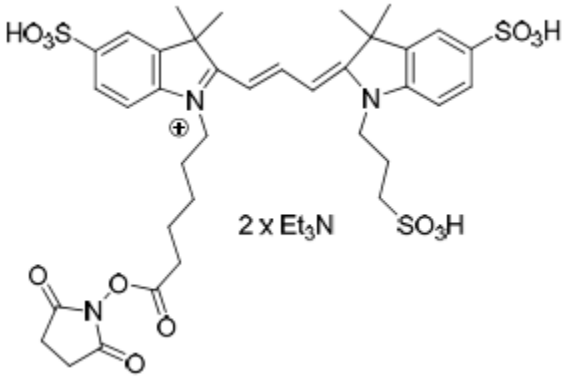
Revision Date: 3/5/2019

CY3-NHS Ester Technical Information

Product No.: B-230

Product Name: CY3-NHS Ester

Chemical Structure:



Chemical Composition: $C_{36}H_{44}N_3O_{13}S_3$

Molecular Weight: 1024.94 g/mol (triethylammonium salt)

Solubility: Water, DMF, DMSO

Spectral Properties:

Absorbance/Emissions	550/570 nm
ε_{max}	150,000 $M^{-1}cm^{-1}$
CF_{280}	0.08
CF_{260}	0.08

Storage: Store at -20°C. (Product shipped at ambient temperature).

Introduction

Cy3-NHS Ester is a bright, photostable, and pH insensitive fluorescent dye that enables simple and efficient Cy3 fluorescent labeling of antibodies, proteins and any other primary amine-containing macromolecule. Cy3 labeled antibodies, proteins, peptides or other primary amine-containing biopolymers give less background than TAMRA or most other fluorophores. N-Hydroxysuccinimide (NHS) activated esters react efficiently with primary amino groups (-NH2)

forming stable amide bonds. Most proteins can be labeled at multiple amine containing sites, including the epsilon amino group of lysine or the N-terminus. 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 Cy3-NHS Ester

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 Cy3-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 Cy3-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 Cy3-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 Cy3-antibody conjugate at 280 nm and 550 nm.

7. Determine Cy3 DOL and conjugate concentration. See Calculations.

Calculations

Calculate the degree of labeling (DOL) and Cy3 conjugate concentration (mg/ml) with the following equations:

Eq. 1 $$number\ of\ Cy3\ dye\ per\ protein\ = \frac{molarity\ Cy3\ dye}{molarity\ protein}$$

Eq. 2 $$[molarity\ of\ Cy3\ dye]\ = \frac{A_{550}}{\varepsilon_{550}}$$

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

Eq. 4 $$mg/ml\ \ = \frac{A_{280}\ -\ (550\ \times\ 0.08)}{\left(\frac{E1\%}{10}\right)}\ \times\ dilution\ factor$$

A_{550} = *conjugate absorbance at* 550 ± 2 *nm*
ε_{350} = *molar extinction coefficient Cy3 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_{550} x (0.08))
ϵ_{280} = *molar extinction coefficient protein* ($M^{-1}cm^{-1}$) = $\frac{MW_p\ x\ E1\%}{10}$

Example 1. Determine Cy3 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 Cy3-NHS reagent. After removal of excess dye with a spin column, the conjugate's A_{280} and A_{550} were determined in PBS (1:5 dilution), A_{280} = 0.2906 and A_{550} = 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 $$molarity\ of\ Cy3 = \frac{0.9825}{150{,}000\ M^{-1}\ cm^{-1}} = 6.55\ \mu M$$

By Equation 3 $$molarity\ of\ IgG = \frac{0.2906 - (0.9825 \times 0.08)}{204{,}000\ M^{-1}\ cm^{-1}} = 1.039\ \mu M$$

By Equation 1 $$Number\ of\ Cy3\ per\ IgG = \frac{6.55\ \mu M}{1.039\ \mu M} = 6.3$$

Calculate Cy3-IgG (mg/ml)

By Equation 4 $$mg/ml = \frac{0.2906 - (0.9825 \times 0.08)}{1.36} \times 5 = 0.78\ mg/ml$$

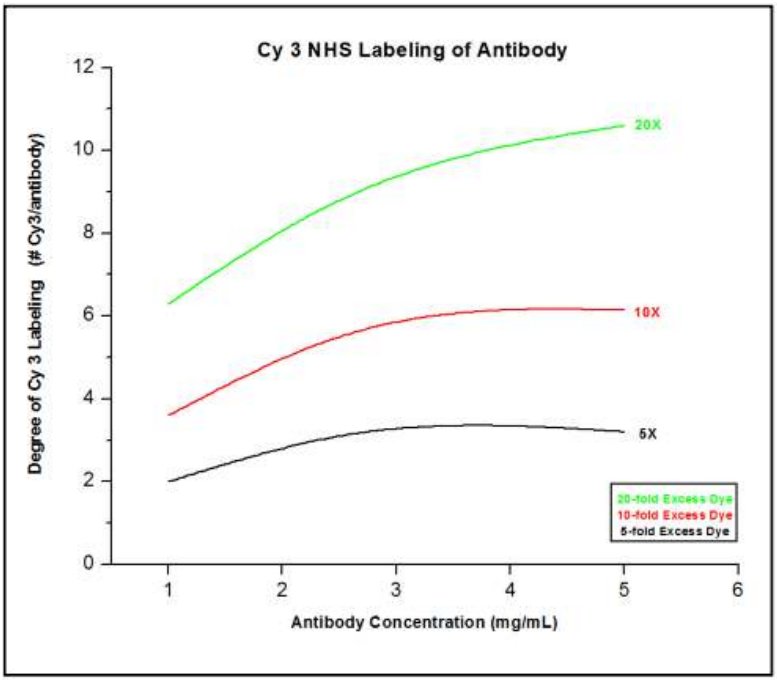


Figure 1. Goat IgG samples (100mM sodium phosphate, 150mM sodium chloride, pH 7.5) at various concentrations were labeled with 5, 10, or 20-fold excess Cy3-NHS Ester for 1 hour at room temp. Resulting conjugates generated

the curves seen above. These can be used to target other dye levels under the buffer, pH, and solvent (DMSO) conditions used in this protocol.

References

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

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