GE Healthcare

Amersham CyDye™ mono-reactive NHS Esters

Reagents for the labelling of biological compounds with Cy™ monofunctional dyes

Product Booklet

Codes: PA13101-PA13102
PA13104-PA13106
PA13601-PA13602
PA13604-PA13606
PA15100-PA15102
PA15104
PA15106
PA15601-PA15602
PA15604-PA15606
PA17101
PA17105-PA17106

Page finder

1. Legal

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2. Handling

2.1. Safety warnings and precautions

Warning: *For research use only*. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. (See safety data sheet for specific advice).

Caution: These dyes are intensely coloured and very reactive. Care should be exercised when handling the dye vials to avoid staining clothing, skin, and other items.

Note: This article contains example protocols. It is the responsibility of the user to design and optimise protocols that are appropriate for the target compound that is being labelled. These example protocols utilise chemicals that may be hazardous, and should only be performed by appropriately qualified and trained persons.

2.2. Storage

Store refrigerated at 2–8°C in the dark. Do not use if desiccant capsule in foil pack is either pink or green. Aqueous solutions of CyDye NHS esters are readily hydrolysed back to the free acid. Therefore, do not store aliquots of aqueous CyDye solutions. Use immediately and discard residues. Aliquots of CyDye NHS esters in anhydrous DMSO are more stable and may be stored at -20°C, but for no longer than 2 weeks.

3. Components

Foil packs, each containing 1, 5, 10, 25 or 50 mg dried dye (for Cy3, Cy3.5, Cy5 & Cy5.5).

Foil packs, each containing 1, 5 or 25 mg dried dye (for Cy7).

Product specification sheet with instructions for using the dye.

Reconstitute the material to 1 mg/ml in anhydrous DMF/DMSO. Reconstituted material may be stored for up to 2 weeks at -200°C in aliquots to avoid repeat freeze thaw cycles.

4. Introduction

Cyanine reagents have been shown to be useful as fluorescent labels for biological compounds (1,2). These dyes are intensely fluorescent and highly water soluble, providing significant advantages over other existing fluorophores (3).
The Cydye are fluorescent cyanine compounds that produce an intense signal easily detected using appropriate detection equipment. The Cydye supplied here are monofunctional NHS-esters, and are provided in a dried, pre-measured form ready for the labelling of compounds containing free amino groups.

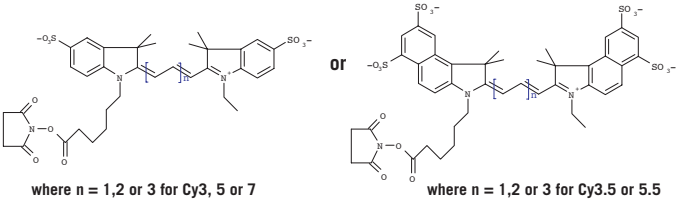


Figure 1. Cy monofunctional dye

4.1. Labelling with CyDye NHS esters

The most convenient and widely used functional group for the labelling of peptides and proteins is the primary amino group provided by the e-amino group of lysine or the N-terminal amino group. Lysine is a relatively common amino acid and most proteins will have at least one. In many cases one or more lysine residues will be accessible to labelling reagents (5). The most useful reaction for labelling at amino groups is acylation. For maximum convenience, stable active esters that may be stored as solid materials, particularly NHS esters, have been extensively used over many

years for the acylation of amino groups. The labelling of proteins is generally performed in an aqueous buffer; hydrolysis of the NHS ester is the major competing reaction of the acylation reaction. The rate of hydrolysis can be increased by raising the pH and by using dilute solutions of proteins. The pH affects the balance of the NHS ester hydrolysis rate versus the rate of reaction with primary amines. However, pH values between 7 and 9 are commonly used for most protein labelling reactions, together with phosphate, bicarbonate/carbonate and borate buffers. Others may be employed, but they should not contain a source of primary or secondary amines, e.g. Tris. These general principles apply to labelling reactions when using CyDye NHS esters.

4.2. Labelling of antibodies and biologically active proteins

When labelling antibodies or other proteins with NHS esters, the optimum conditions have to be established experimentally. The extent of labelling to give maximum fluorescence between the different CyDye vary and should be taken into account, as shown by Gruber et al (2); it will be necessary to optimise the ratio of CyDye NHS ester to protein and pH to give the final dye to protein (D/P) ratio that is required.

Waggoner and co-workers (4) investigated the labelling of antibodies using CyDye NHS esters. They found that the brightest antibodies had D/P ratios between 4 and 12; at higher D/P ratios self-quenching was observed. Labelling reactions could be carried out with either monofunctional or bisfunctional CyDye NHS esters; cross-linking of proteins was not observed using the bisfunctional ester under typical labelling conditions (4). The labelling of antibodies with CyDye NHS esters in the pH range 7.0–9.4 has been studied (1), D/P ratios of 5–6 were obtained after ten minutes using a higher pH (8.5–9.4). Lower

D/P ratios were observed at pH 7.0, and longer reaction times were also required.

The general comments given above about labelling with NHS esters should be taken into consideration when the labelling protocol is being designed. Biologically active proteins will vary greatly in terms of their properties (size, morphology, solubility etc.) and these should be taken into account. These properties may affect the choice of separation method of the labelled protein from free dye. Each case has to be considered on its own merits. Methods of separating excess free dye from labelled antibody other than gel filtration (e.g. dialysis) may be used.

At GE Healthcare, an anti-glutathione-S-transferase (GST) polyclonal antibody has been labelled using different Cy3 NHS ester to antibody ratios. Ratios of 1:1, 5:1, 10:1 and 20:1 gave final D/P ratios of 0.28:1, 1.16:1, 2.3:1 and 4.6:1 respectively; these results are in general agreement with data previously reported by Waggoner and co-workers (4).

The scale of the labelling reaction is another factor that will affect the degree of labelling obtained; with small-scale labellings (100 µg or less) poor recoveries obtained during the purification processing can be a significant problem. The dye should be accurately aliquoted in anhydrous DMSO solution for use in small scale labellings. It is important that the biological properties of the labelled protein are maintained, and there should be some way of determining this. Information in the literature may provide guidance on the particular protein being used. Generally, higher degrees of labelling are more likely to have an effect on the biological properties of the protein. In some cases, a lysine residue accessible to the labelling reagent may be critical for the biological properties of the protein. When are bis-functional dyes used as opposed to mono-functional dyes? During labelling of larger proteins such as antibodies. The mono-functional

dye is used when there is by design, a single amino group available for modification e.g. N-terminus of peptide or amino modified oligo.

The Cy3 dye is an orange fluorescing cyanine that produces an intense signal easily detected using most rhodamine filter sets in the appropriate instrumentation. The Cy3 dye supplied here is a monofunctional NHS-ester, and is provided in a dried, pre-measured form ready for the labelling of compounds containing free amino groups.

Cy5 dye produces an intense signal in the far-red region of the spectrum. Though not recommended for visual applications, this dye is ideally suited for detection using CCD cameras, PMT's and some red-sensitive film. The Cy5 dye supplied here is a monofunctional NHS-ester, and is provided in a dried, pre-measured form ready for the labelling of compounds containing free amino groups.

Protocol 1 has been designed for the preparation of CyDye -labelled IgG antibodies. It is designed to utilise CyDyes in the quantities provided in the bulk pack sizes, 1–25 mg, and label protein to a final molar dye/protein (D/P) ratio between 4 and 12. This assumes an average protein molecular weight of 155 K daltons. Other D/P ratios can be obtained by using different amounts of protein and/or dye.

Note. The following materials and procedures have been optimised for IgG antibodies. Other proteins may also be readily labelled, however, choice of buffers, separation media, and technique may need to be varied in order to produce optimal results.

Altering the protein concentration and reaction pH will change the labelling efficiency of the reaction. Optimal labelling generally occurs at pH 9.3. Proteins have been successfully labelled with this dye at a pH as low as 7.3, however, labelling times must be significantly longer at lower pH. Higher protein concentrations usually increase labelling efficiency. Solutions of up to 10 mg/ml protein have produced good conjugation reactions.

Conjugation of dye to antibody

Empirically we have determined the reaction molar stoichiometry required to produce a dye protein ratio in the range 6–12. This is based on mW values of,

Ab = 155 K daltons and, Cy3™ NHS ester = 766 Cy5™ NHS ester = 792.

This figure is used to determine an adjusted labelling factor, relating the required stoichiometry by weight, which can then be used to determine the quantity of Ab (in mg) required for labelling with the amount of dye reagent (also in mg).

Adjusted dye labelling factor.

Dye	ADJUSTED DYE LABELLING FACTOR
Cy3	0.089
Cy5	0.071

It is also necessary to calculate a dye purity factor which takes into account the % chromophore and NHS ester content of a specific batch of reagent. This information can be found on the Batch Analysis sheet supplied with the product.

% Chromophore =

% NHS ester by HPLC =

$$\text{Dye Purity Factor} = \frac{(\%\ \text{Chromphore})\ (\%\text{NHS ester HPLC})}{100 \qquad 100}$$

Example

% Chromophore = 95%

% NHS ester by HPLC = 82.9%

Dye Purity Factor = (95/100) (82.9/100) = 0.787

Now calculate the amount of antibody to use for the preparation based on the amount of dye in mgs, the adjusted dye labelling factor

(this figure is product specific please see table at the beginning of the dye calculation section) and the dye purity NHS ester to be used.

$$\text{Quantity of Ab} = \frac{\text{mgs of dye x dye purity factor}}{\text{Dye labelling factor}}$$

Example

$$\text{Quantity of Ab} = \frac{8.62 \times 0.787}{0.071} = 95.5 \text{ mgs}$$

5. Protocol

5.1. Labelling of anti-GST polyclonal antibody with Cy3™ Mono NHS ester

1. Dialyse the anti-GST antibody (at a concentration of 0.5 ml at 3 mg/ml) against 1 L of 0.15 M sodium chloride for 4 hours at room temperature.

2. Repeat this dialysis overnight using a fresh litre of 0.15 M sodium chloride at +4°C.

3. The next day dialyse the antibody against 1 L of 0.1 M $NaHCO_3$ (pH 8.3) for a maximum of 4 hours.

4. Filter the antibody solution using a 0.22 µm syringe filter.

5. Dilute a small sample of the antibody solution with 0.1 M $NaHCO_3$ so that the absorbance at 280 nm can be measured. Calculate the total amount of antibody required for labelling (the molar extinction coefficient of IgG antibody is 170 000 M^{-1} cm^{-1} at 280 nm).

6. Prepare a 10 mg/ml solution of Cy3 monofunctional NHS ester (MW 766) in dimethyl sulfoxide (DMSO). Calculate the volume needed to give the desired ratio of CyDye NHS ester to antibody (e.g. 20 : 1), and add this gradually to the antibody solution while stirring. Stir the solution for a further 45 minutes at room temperature in the dark.

7. To separate the free dye, dialyse against 1 L of 0.15 M sodium chloride for 4 hours at room temperature. NB. Perform all dialyses in the dark following labelling.

8. Repeat this dialysis using a fresh litre of 0.15 M sodium chloride overnight at +4°C.

9. Dialyse the antibody against 1 L of 0.01 M PBS/ 0.01% sodium azide for 4 hours at room temperature, repeating this step as before using an overnight incubation at +4°C.
10. Filter the labelled antibody solution through a 0.22 µm syringe filter.
11. Dilute an aliquot of labelled antibody solution with 0.01 M PBS/0.01 % sodium azide for the dual absorbance measurements at 280 nm (for protein) and at 552 nm (for Cy3; the molar extinction coefficient is 150 000 M^{-1} cm^{-1} at this wavelength). Correct the calculation for the absorbance of CyDye at 280 nm; this is approximately 8 % of the absorbance at 552 nm. (see below)

For Cy3

Molar extinction coefficients of 150 000 $M^{-1}cm^{-1}$ at 552 nm for the Cy3 dye and 170 000 $M^{-1}cm^{-1}$ at 280 nm for the protein are used in this example. The extinction coefficient will vary for different proteins. The calculation is corrected for the absorbance of the dye at 280 nm (approximately 8% of the absorbance at 552 nm).

$[Cy3] = A_{552}/150\,000$

$[antibody] = \{A_{280} - (0.08 \times A_{552})\}/170\,000$

$D/P_{final} = [Cy3]/[antibody]$

$D/P_{final} = \{1.13 \times A_{552}\}/ \{A_{280} - (0.08 \times A_{552})\}$

Notes

- Any dissolved CyDye NHS ester powder should be used immediately. Do not aliquot CyDye NHS esters in aqueous solutions for storage, always use immediately and discard any residues.
- An appropriate wavelength should be used for measuring the absorbance of other CyDye fluors.
- The absorbance of Cy3.5, Cy5 , Cy5.5 and Cy7 at 280 nm are 24%, 5%, 18% and 11% of their absorbance at their excitation wavelengths of 581, 650, 678 and 750nm respectively.

5.2. N- Terminal labelling of (D-ser^2)-leucine-enkephalin with Cy5™ Mono NHS ester

1. Dissolve Cy5 NHS ester (1.0 mg) in DMSO (400 µl) and add this to (D-ser^2)-leucine-enkephalin acetate (YSGFLT, 0.75 mg) followed by DMSO (400 µl) in a 1 ml capacity Sarstedt vial.

2. Add triethylamine (15 µl) to the solution, and roll the reaction mixture overnight in the dark at room temperature.

3. Purify the crude product by HPLC on a protein and peptide C18 column (25 cm x 10 mm) as 2 x 400 µl injections, using a gradient from water containing 0.1% TFA (Trifluoroacetic acid) to acetonitrile:water (70 : 30) containing 0.1% TFA over 30 minutes at a flow rate of 4 ml per minute.

4. Collect the appropriately coloured peak. NB. The retention time of the dye labelled peptide will be longer than that of the unlabelled peptide.

5. Lyophilise the purified product, or store as aliquots at -20°C.

6. Characterise the product appropriately (e.g. Mass Spectrometry).

Notes

The starting ratio of dye to peptide was 1:1

Select a HPLC gradient appropriate for the labelled peptide being purified.

Any dissolved CyDye NHS ester powder should be used immediately. Do not aliquot CyDye NHS esters in aqueous solutions for storage, always use immediately and discard any residues.

For Cy5

Molar extinction coefficients of 250 000 $M^{-1}cm^{-1}$ at 650 nm for the Cy5 dye and 170 000 $M^{-1}cm^{-1}$ at 280 nm for the protein are used in this example. The extinction coefficient will vary for different proteins. The calculation is corrected for the absorbance of the dye

at 280 nm (approximately 5% of the absorbance at 650nm).

[Cy5 dye] = $(A_{650})/250\ 000$

[antibody] = $[A_{280}-(0.05 \times A_{650})]/170\ 000$

(D/P)final = [dye]/[antibody]

(D/P)final = $[0.68 \bullet (_{A650})] / [A_{280}-(0\text{-}05 \times A_{650})]$

Figure 2. Cy3 dye absorption and fluorescence spectra

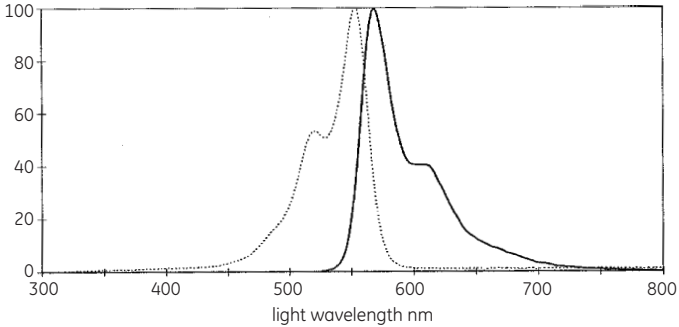


Figure 3. Cy3.5 dye absorption and fluorescence spectra

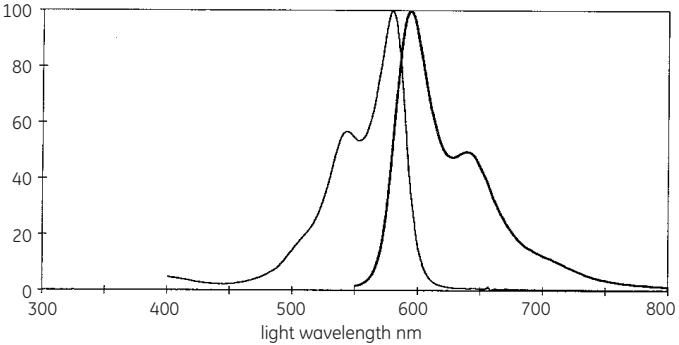


Figure 4. Cy5 dye absorption and fluorescence spectra

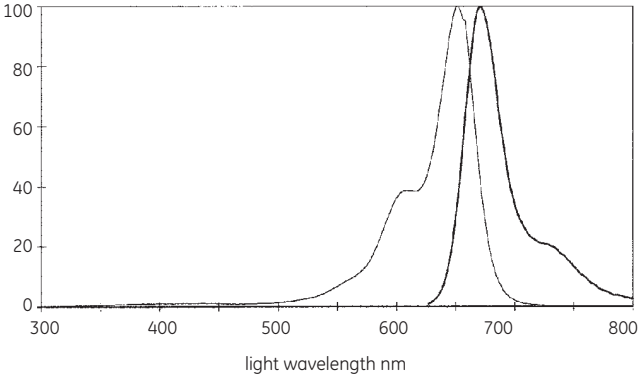


Figure 5. Cy5.5 dye absorption and fluorescence spectra

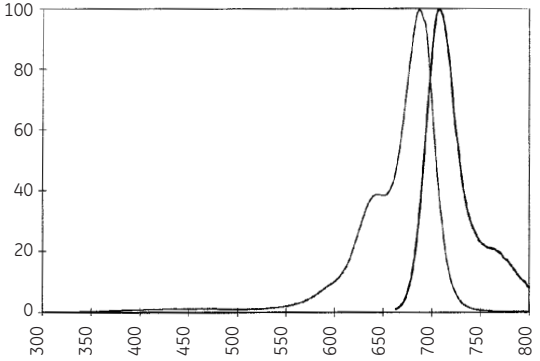
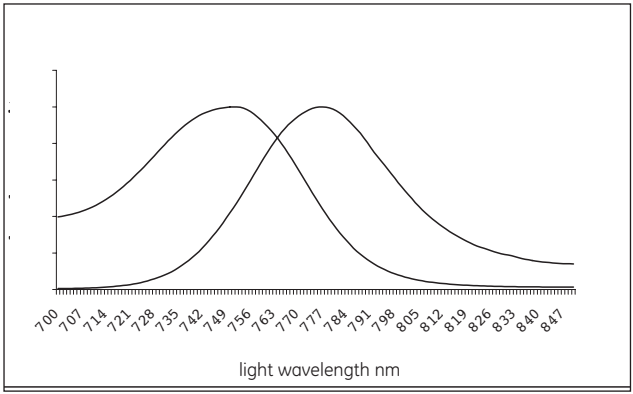


Figure 6. Cy7 dye fluorescence spectra



5.2.1. Monofunctional dye characteristics

	Cy3	Cy3.5	Cy5	Cy5.5	Cy7
Formula weight	765.95	1102.37	791.99	1128.42	818.00
Absorbance max	550 nm	581 nm	649 nm	675 nm	747 nm
Extinction max	150 000 $M^{-1}cm^{-1}$	150 000 $M^{-1}cm^{-1}$	250 000 $M^{-1}cm^{-1}$	250 000 $M^{-1}cm^{-1}$	200 000 $M^{-1}cm^{-1}$
Emission max	570 nm	596 nm	670 nm	694 nm	776 nm
Quantum yield	>0.15*	>0.15*	>0.28*	>0.28*	–

* for labelled proteins, D/P=2

6. References

1. Mujumdar, R.B., Ernst, L.A., Mujumdar, S.R., Lewis, C.J. and Waggoner, A.S. *Cyanine dye labelling reagents: sulfoindocyanine succinimidyl esters. Bioconj. Chem.,* **4(2)**, 105-111 (1993).

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3. Wessendorf, M.W. and Brelje, T.C. *Histochemistry,* **98(2)**, 81-85, (1992).

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