GE Healthcare

Amersham Cy™3B mono-reactive dye

Reagents for the labelling of biological compounds with Cy™3B monofunctional dyes

Product Booklet

Codes: PA63100
PA63101
PA63106
PA63120
PA63121
PA63126
PA63130
PA63131
PA63136

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 material safety data sheet(s) and/or safety statement(s) 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 Cy3B NHS esters and maleimides are readily hydrolysed back to the free acid. Therefore, do not store aliquots of aqueous Cy3B esters or maleimide solutions. Use immediately and discard residues. Aliquots of Cy3B

NHS esters, hybrazides and maleimide in anhydrous DMSO are more stable and may be stored at -20 °C, but for no longer than 2 weeks. Aliquots of Cy3B hydrazide in aqueous solutions appear to be fairly stable at neutral pH, therefore, it is recommended that aliquots of aqueous Cy3B hydrazide solutions can be stored for up to 2 weeks at -20 °C.Safety warnings and precautions

3. Components

Foil packs, each containing 1, 5, or 25 mg dried dye

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 -20°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, maleimides or hydrazides and are provided in a dried, pre-measured form ready for the labelling of compounds containing free amino groups, thiol groups or carbonyl groups. The Cy3B dye is orange fluorescing cyanine that produces an intense signal easily detected using most rhodamine filter sets in the appropriate instrumentation.

Figure 1. Cy3B NHS ester monofunctional dye

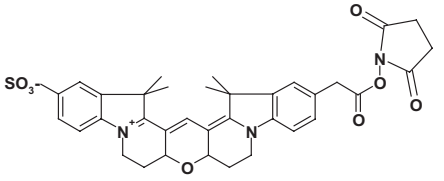


Figure 2. Cy3B maleimide monofunctional dye

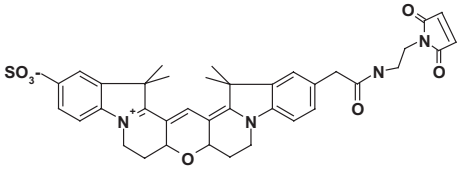
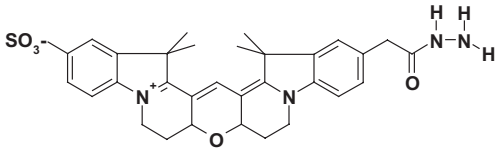


Figure 3. Cy3B hydrazide monofunctional dye



4.1. Labelling with Cy3B 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 ε-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 Cy3B NHS esters.

4.2. Labelling with CyDye hydrazides

A convenient and widely used functional group for the labeling of free carbonyl groups of glycoproteins and carbohydrates is the hydrazide. For maximum convenience, stable active hydrazide may be stored as solid materials. The labeling of glycoproteins and carbohydrates is generally performed in an aqueous buffer; hydrolysis of the hydrazide group is the major competing reaction.

4.3. Labelling with CyDye maleimides

A convenient and widely used functional group for the labeling of free thiol groups of peptides proteins, and oligonucleotides is the maleimide. Cysteine is the most common amino acid residue containing a thiol reactive group. For maximum convenience, stable active maleimides may be stored as solid materials.

Altering the protein concentration and reaction pH will change the labelling efficiency of the reaction. The optimal pH for the reaction of maleimides is near 7.0. In the pH range 7.0–7.5 the protein thiol groups are sufficiently nucleophilic so that they almost exclusively react with the maleimide dye in the presence of the more numerous protein amines, which are protonated and relatively unreactive.

4.4. Labelling of antibodies and biologically active proteins

When labelling antibodies or other proteins with NHS esters, maleimides or hydrazides 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 Cy3B 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. In a more recent study by the University of Linz, it has been found that a D/P ratio of 2:1 gives the brightest signal. The general comments given above about labeling with NHS esters, maleimides and hydrazides should be taken into consideration when the labeling 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 CyDye 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.

Protocol 1 has been designed for the preparation of Cy -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 with NHS esters 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,

Antibody (Ab) = 155 K Daltons and, Cy3B NHS ester = 766 Cy3B Hydrazide = 688

These figure are 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.

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.

For Example

% Chromophore =

% NHS ester by HPLC =

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

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 milligrams, 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.

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

Example

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

5. Example protocols

5.1. Labelling of 1 mg Goat IgG Antibody with Cy3B NHS ester (see note)

Antibody Labelling

1. Prepare a solution of Goat IgG antibody at 1 mg/ml in 0.1 M sodium borate buffer at pH 8.3.
2. Prepare a 10 mg/ml solution of Cy3B monofunctional NHS ester (MW 761) in Dimethylsulfoxide (DMSO)/Dimethylformamide (DMF) [1:1] by adding 300 µl DMSO/DMF solution to 3 mg Cy3B mono NHS ester. This will give the desired ratio of CyDye NHS ester to antibody of 3:1.
3. Add this solution gradually to the antibody solution while stirring. Stir the solution for a further 60 minutes at room temperature in the dark.
4. The reaction is stopped by the addition of 1.3 ml of Sodium Dihydrogen orthophospate buffer pH<<7
5. Purify the labelled antibody by gel chromatography.

Purification of Labelled Antibody

1. Prewash a PD-10 column with 25 ml buffer pH 7.5.
2. Load sample onto column.
3. Add a further 2 x 1 mls buffer.
4. Elute the labelled IgG from the column with water (2 ml).
5. The unbound dye is the eluted from the column with a further 10 ml water.

• IgG concentration is measured at 280 nm

$$[\mathbf{IgG}] = \{\mathbf{A}_{280} - \mathbf{k}_{corr\ dye} * \mathbf{A}_{dye\ max}\}/\mathbf{210\ 000}$$

• Dye concentration is measured by $A_{dye\ max}$

$$[\mathbf{dye}] = (\mathbf{A}_{dye\ max}/\varepsilon_{dye})$$

D/P_{final} = [Cy3B]/[antibody]

Notes

a. Any Cy3B NHS ester solution should be used immediately. Do not aliquot Cy3B NHS esters in aqueous solutions for storage, always use immediately and discard any residues.

b. This protocol is designed to give one label per antibody. As stated above, two dye labels per antibody gives the best signal. To achieve this it is suggested that the dye to antibody in the labelling reaction should be increased to 6:1.

5.2. Labelling of an Antibody with Cy3B Hydrazide (see note)

Material supplied

Foil packs, each containing dried dye to label 1 mg of antibody
Product specification sheet with instructions for using the dye

Material required but not supplied

Conjugation buffer: phosphate buffer pH 7.0

Notes

1. All solvents and buffers should be degassed

This protocol has been designed for the preparation of Cy3B-labelled IgG antibodies. It is designed to label 1 mg. This assumes an average protein molecular weight of 155 K Daltons.

Oxidation of antibody

1. Dissolve the antibody in coupling buffer (10 mg/ml). Place 1 mg of antibody to be labelled (0.1 ml) in a 0.5 ml microfuge tube and flush with nitrogen.
2. Add 150 µl Sodium Periodate solution (0.1 M) to the antibody, seal tightly and mix thorough for 30–45 minutes in the absence of light at ambient temperature.
3. While the oxidation reaction is incubating, equilibrate the gel filtration column in coupling buffer.

Place the periodate reaction on the column: collect and combine the fractions containing antibody

Coupling of the antibody with a Cy3B hydrazide

1. Dissolve the Cy3B hydrazide in coupling buffer to afford a 0.02 M solution.
2. Combine equal volumes of antibody to Cy3B hydrazide solution and gently mix for 3 hours at ambient temperature or overnight at 4°C.
3. Purify the product using a 10 ml gel filtration column that has been pre-treated with PBS. Elute the product with PBS.
4. Isolate the first coloured fractions and scan sample from 250–800 nm against a PBS blank.
5. Record the wavelength at the maximum absorbance 548 nm and the 280 nm absorbance.
6. Determine the dye/protein ratio using the calculation below.

$$\textbf{Dye/protein} = \frac{(\mathbf{A}_{max})\,(\textbf{Antibody Extinction Coefficient})}{\{(\mathbf{A}_{280})-(\textbf{correction factor})(\mathbf{A}_{max})\}\,(\textbf{CyDye Extinction Coefficient}}$$

Correction factor for Cy3B = 0.08

The dye/protein ratio may vary depending on the antibody and equivalence of CyDye used. Increased reaction time could also lead to saturated labeling. Incubation times and dye/protein ratios must be

determined for each biomolecule used.

Note:

1. Unconjugated dye can also be separated from the labelled antibody by dialysis. Dialysis does not give as efficient and rapid separation as gel filtration. It is recommended that gel filtration be used whenever possible.

5.3. Labelling of an oligonucleotide with Cy3B Maleimide (see note)

Material supplied

Foil packs, each containing dried dye to label 1 mg of antibody
Product specification sheet with instructions for using the dye

Material required but not supplied

0.17 M phosphate buffer, pH 8.0

0.04 M Dithiothreitol (DTT) (USB 15397)

NAPTM-10 column (Sephadex™ G-25 DNA Grade contains 0.15% Kathron CG as preservative

0.2 M Sodium phosphate, pH 7.2

Anhydrous DMSO

3 M Sodium acetate

0.2 M Sodium phosphate, pH 7.2, containing 0.04 M DDT

Note:

1. pH will change the labelling efficiency of the reaction. Optimal labeling occurs at pH 7.2 in degassed buffer.

2. To minimise oxidation of the thiol, carry out in an oxygen free environment , i.e under nitrogen, using degassed solvents/buffers

This protocol was used to label ~120 nmol of thiol modified oligonucleotide with Cy3B maleimide reagent.

Reduction of oligonucleotide disulphide linkage

1. Incubate oligonucleotide in 0.17 M phosphate buffer, pH 8.0, containing 0.04 M DTT for 16 hours at ambient temperature to reduce the disulphide linkage.
2. Remove DTT and thiol by-products using a NAP-10 column and following manufacturer's instructions. During this procedure, sterile de-ionised water should be used in place of the recommended equilibration buffer.
3. Monitor each eluent fraction for the presence of DNA at 260 nm.
4. Isolate the appropriate fractions and concentrate in vacuo.

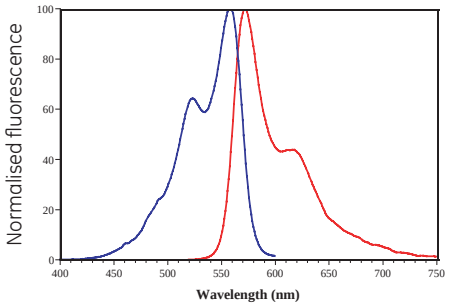
Conjugation of a Cy3B to an oligonucleotide

1. To the flask add 375 µl sterile, de-ionised water and 125 µl of labelling buffer (0.2 M sodium phosphate, pH 7.2, containing 0.04 M DDT) mix gently and transfer to screw top vial.
2. To 1 mg Cy3B maleimide reagent add 120 µl anhydrous DMSO, then transfer 100 µl to reaction vial and incubate, with gentle mixing, at ambient temperature for 120 minutes. The dye/oligonucleotide ratio during labelling is ~20:1.
3. Separation of the product from the free Cy3B. Precipitate oligonucleotide by adding 60 ml 3 M sodium acetate buffer, pH 5.2, and 1.65 ml ice cold ethanol and incubate on dry ice or at -80°C for 60 minutes.
4. Centrifuge in a microfuge for 15 minutes at 14 000 rpm.
5. Remove supernatant and wash pellet with 70% (v/v) ice-cold ethanol.
6. Dissolve pellet in 500 µl sterile, de-ionised water and purify labelled fraction by HPLC. Monitor at the absorbance wavelength of Cy3B, 559 nm.

5.3.1. Monofunctional dye characteristics

	Cy3B NHS ester	Cy3B Hydrazide	Cy3B Maleimide
Formula weight	771	688	796
Absorbance max	559 nm	559 nm	559 nm
Extinction coefficient in max Methanol	130000 $M^{-1}cm^{-1}$	130000 $M^{-1}cm^{-1}$	130000 $M^{-1}cm^{-1}$
Emission max	570 nm	570 nm	570 nm
Quantum yield in Methanol	>0.7	>0.7	>0.7

Figure 1. Cy3B dye absorption and fluorescence spectra



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).

2. Gruber, H.J., Hahn, C., D., Kada, G., Riener, C., K., Harms, G., S., Ahrer, W., Dax, T., G., Knaus, H-G. *Anomalous fluorescence enhancement of Cy3 and Cy3.5 versus anomalous fluorescence loss of Cy5 and Cy7 upon covalent linking to IgG and noncovalent binding to Avidin. Bioconjugate Chem.* **11**, 696-704 (2000).

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

4. Southwick, P.L., Ernst, L.A., Tauriello, E.W., Parker, S.R., Mujumdar, R.B., Mujumdar, S.R., Clever, H.A., and Waggoner, A.S. *Cyanine dye labelling reagents - carboxymethylindocyanine succinimidyl esters*. *Cytometry,* **11**(3), 418-430, (1990).

5. "Introduction to Molecular Biology" G.H.Haggis, D.Michie, A.R.Muir,K. B.Roberts & PMB Walker, Longmans (Bristol) Green & Co. LTD, 1965.

6. At the University of Linz, an independent labelling study on a range of dye reagents has recently been concluded. The complete results of this study will be available from GE Healthcare at a later date.

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