Amersham
CyDye Maleimides

Reagents for the labeling of biological compounds with Cy monofunctional maleimide dyes

Product Booklet

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1 Introduction

Product codes

PA13131

PA13136

PA15130

PA15131

PA15136

PA15630

PA15631

PA15636

Important

Read these instructions carefully before using the products.

Intended use

The products are intended for research use only, and shall not be used in any clinical or *in vitro* procedures for diagnostic purposes.

Safety

The example protocols utilize chemicals that may be hazardous.

NOTICE

These dyes are intensely colored 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 user's responsibility to design and optimize protocols that are appropriate for the protein that is being labeled. These*

For use and handling of the products in a safe way, refer to the Safety Data Sheets.

Storage

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

2 Components

- Foil packs, each containing 1, 5 & 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.

3 Background

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 fluorescing cyanine compounds that produce an intense signal easily detected using appropriate filter sets. The CyDye supplied here are monofunctional maleimide esters, and are provided in a dried, pre-measured form ready for the labeling of compounds containing free thiol groups.

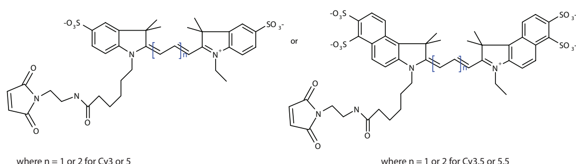

Fig 1. Cy monofunctional maleimide dye

Labeling with

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

Altering the protein concentration and reaction pH will change the labeling 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.

Labeling of antibodies and biologically active proteins

When labeling antibodies or other proteins with maleimide esters, the optimum conditions have to be established experimentally. The extent of labeling 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 optimize the ratio of CyDye ester to protein and pH to give the final dye to protein (D/P) ratio that is required.

The general comments given above about labeling with maleimide esters 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 labeled protein from free dye. Each case has to be considered on its own merits. Methods of separating excess free dye from labeled antibody other than gel filtration (e.g. dialysis or HPLC) may be used.

The scale of the labeling reaction is another factor that will affect the degree of labeling obtained; with small-scale labelings (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 labeling reactions. It is important that the

biological properties of the labeled 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 labeling are more likely to have an effect on the biological properties of the protein. In some cases, a thiol residue accessible to the labeling reagent may be critical for the biological properties of the protein.

4 Protocol: 1. Labeling of an oligonucleotide with Cy3 maleimide

Material supplied

- Foil packs, each containing dried dye 1 mg of CyDye maleimide is used to label 100–150 nmoles of thio modifed oligonucleotide.
- Product specification sheet with instructions for using the dye.

Materials required but not supplied

- 0.17 M phosphate buffer, pH 8.0, containing 0.04 M Dithiothreitol (DTT) (USB 15397 or equivalent)
- NAP™-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

Note: *pH will change the labeling efficiency of the reaction. Optimal labeling occurs at pH 7.2 in degassed buffer.*

Note: *To minimize 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 CyDye maleimide reagent.

Reduction of oligonucleotide disulphide linkage

Step	Action
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-ionized 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.
5	Proceed with the next part of the protocol.

Conjugation of a Cy3 to an oligonucleotide

Step	Action
1	To the flask, containing the oligonucleotide, add 375 µL sterile, de-ionized water and 125 µL of labeling buffer (0.2 M sodium phosphate, pH 7.2) mix gently and transfer to screw top vial.
2	To 1 mg Cy™3 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 labeling is 10:1.
3	Separation of the product from the free Cy3. Precipitate oligonucleotide by adding 60 µL 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-ionized water and purify labeled fraction by HPLC. Monitor at the absorbance wavelength of Cy3, 548 nm.

5 Protocol 2: Labeling of an antibody with Cy5 maleimide

Materials required but not supplied

- Conjugation buffer 10–100 mM phosphate (such as phosphatebuffered saline (PBS)), Tris or HEPES buffer with pH between 7.0–7.5.
- Dimethylformamide, anhydrous (DMF) for preparing dye solutions.
- Tris-(2-carboxethyl)phosphine(TCEP) for reducing disulphide bonds. Separation column containing a permeation gel (such as Bio-Gel™ P-6 gel, minimum of 10 mL bed volume and 6 cm packed length).
- Separation buffer: phosphate-buffered saline, pH 7–7.5, containing 0.1% Sodium Azide.
- Test tubes, transfer pipettes, glassware.

Recommended procedure for use

This protocol has been designed for the preparation of Cy5-labeled IgG antibodies. It is designed to label 1 mg protein to a final molar dye/protein (D/P) ratio between 0.5 and 3. This assumes an average protein molecular weight of 155 000 daltons.

Note: *The following materials and procedures are used in a functional test of the dye to label reduced IgG antibodies. Other proteins may also be readily labeled, however, choice of buffers, separation media, and technique may vary in order to produce optimal results. TCEP is used to reduce the IgG. Other reducing reagents such as 2-Mercaptoethylamine Hydrochloride may give a more selective reduction of disulphide bonds (4).*

Conjugation of dye to antibody

To minimize oxidation of thiols, carry out thiol modifications in an oxygen-free environment, that is under Nitrogen, using degassed solvents/buffers.

Step	Action
1	Antibody to be conjugated should be dissolved at 1 mg/mL in degassed PBS buffer being careful not to introduce air bubbles into the solution.
2	Leave the solution for 30 minutes at room temperature.
3	Add a 100 molar excess of TCEP (180 µg, 10 µL of a 18 mg/mL TCEP solution in PBS, per 1 mg of IgG). Flush the vial with Nitrogen gas, cap the vial, and mix thoroughly.

Step	Action
4	Incubate the reaction at room temperature for 10 minutes. It is not necessary to remove excess TCEP before conjugation. While the IgG reduction is taking place prepare a dye solution by adding 50 µL of anhydrous DMF to one pack of dye. If using a larger pack size a small quantity (0.25-1 mg) of the dye should be weighed into a suitable screw cap vial and dissolved in anhydrous DMF. Flush the vial with Nitrogen gas, cap the vial, and mix thoroughly.
5	Add the dye solution (50 µL) to 1010 µL of reduced IgG. Flush the vial with Nitrogen gas, cap the vial and mix thoroughly. Incubate the reaction at room temperature for two hours with additional mixing every 30 minutes.
6	Leave the reaction overnight at 2°C to 8°C.

Separation of protein from free dye

Labeled antibody can be separated from the excess, unconjugated dye by gel permeation chromatography. It is convenient to preequilibrate the column with phosphate-buffered saline and to elute the protein using the same buffer. Two blue bands should develop during elution. The faster moving band is Cy5-labeled antibody while the slower band is free dye. For precise separation 1 mL fractions should be collected, analyzed and the desired fractions pooled. Many Cy5-labeled proteins can be stored at 2–8°C without further manipulation.

Note: *Labeled antibody can also be separated from unconjugated dye by dialysis. Dialysis does not give as efficient and rapid a separation as gel filtration. We therefore recommend that protein purification by gel filtration be used.*

Estimation of final dye/protein (D/P) ratio

1. Dilute a portion of the labeled protein solution so that the maximum absorbance is 0.5 to 1.5 AU. Molar concentrations of dye and protein are calculated, and the ratio of these values is the average number of dye molecules coupled to each protein molecule. 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. For a different Cy dye label then the extinction co-efficient for that dye given in table in section *Monofunctional Cy Dye characteristics, on page 15* is substituted into the calculation. 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 650 nm). (the absorbance correction factor for Cy3 is approximately 8% at 280nm).

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

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

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

(D/P)final = $[0.68\,(A_{650})]\,/\,[A_{280}-(0.05\,A_{650})]$

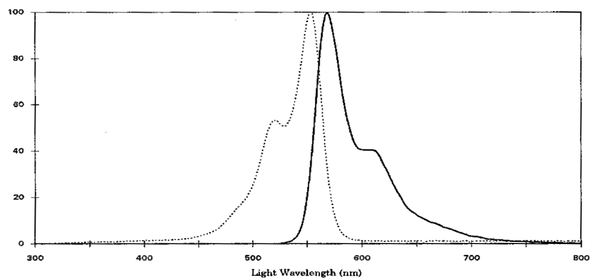

Fig 2. Cy3 dye absorption and fluorescence spectra

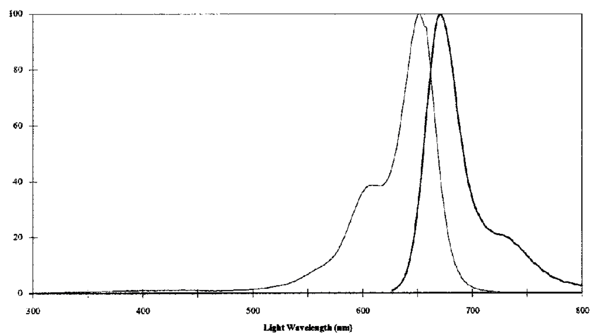

Fig 3. Cy5 dye absorption and fluorescence spectra

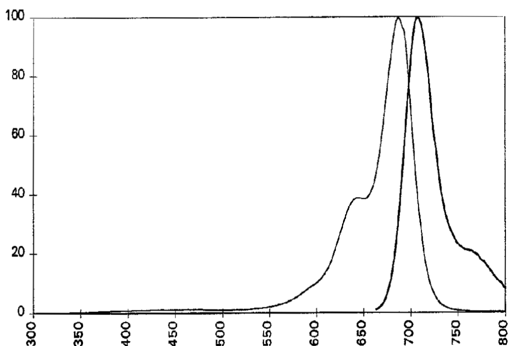

Fig 4. Cy5.5 dye absorption and fluorescence spectra

Monofunctional Cy Dye characteristics

	Cy3	**Cy5**	**Cy5.5**
Formula weight	791	817	1153.5
Absorbance max	550 nm	649 nm	675 nm
Extinction max	150 000 $M^{-1}cm^{-1}$	250 000 $M^{-1}cm^{-1}$	250 000 $M^{-1}cm^{-1}$
Emission max	570 nm	670 nm	694 nm
Quantum yield>	0.15[1]	>0.28[1]	>0.28[1]

[1] for labeled proteins, D/P=2

6 References

1. Mujumdar, R.B., Ernst, L.A., Mujumdar, S.R., Lewis, C.J. and Waggonner, A.S. *Cyanine dye labeling 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 labeling reagents - carboxymethylindocyanine succinimidyl esters. Cytometry*, **11(3)**, 418-430, (1990).
5. Yu, H. *et al., Nucleic Acids Research*, **22(15)**, 3226-3232, (1994).
6. Neblock, D.S. *et.al., Bioconjugate Chemistry*, **3(2)**, 126-131, (1992).
7. Aslam, M., and Dent, A., *Bioconjugation, Macmillan Reference Ltd*, (1998).

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