GE Healthcare

Amersham Cy5 Maleimide Mono-Reactive Dye 5-pack

Reagents for the labelling of biological compounds with Cy™5 monofunctional dye

Product Specification Sheet

Code: PA25031

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.

Storage

Store refrigerated at 2–8°C in the dark. Do not use if desiccant capsule in foil pack is either pink or green.

Expiry

See outer packaging.

Components

Five foil packs: each containing dried dye to label 1 mg of protein.

Product specification sheet: with instructions for using the dye.

Other materials required

- Conjugation buffer: 10–100 mM Phosphate (such as Phosphate-Buffered 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.

Safety warnings and precautions

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: This dye is intensely colored and very reactive. Care should be exercised when handling the dye vial to avoid staining clothing, skin, and other items.

Introduction

Cyanine reagents have been shown to be useful as fluorescent labels for biological compounds (1, 2).

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 mono-functional maleimide ready for the labelling of compounds containing free sulphydryl groups (3).

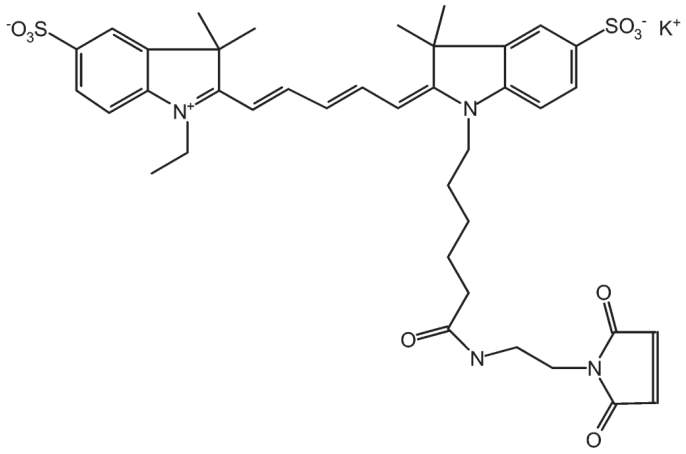

Figure 1. Cy5 monofunctional dye

Recommended procedure for use

This protocol has been designed for the preparation of Cy5-labelled IgG antibodies. It is designed to label 1 mg protein to a final molar dye/protein (D/P) ratio between 0.5 and 3.5. 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 labelled, 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).

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.

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.

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. Leave the solution for 30 minutes at room temperature. 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.

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 Dimethylformamide to one pack of dye. Flush the vial with Nitrogen gas, cap the vial, and mix thoroughly. 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. Then leave the reaction overnight at 2–8°C.

Separation of protein from free dye

Labelled antibody can be separated from the excess, unconjugated dye by gel permeation chromatography. It is convenient to pre-equilibrate 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-labelled antibody while the slower band is free dye. For precise separation 1 ml fractions should be collected, analysed and the desired fractions pooled. Many Cy5-labelled proteins can be stored at 2–8°C without further manipulation.

Labelled 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

Dilute a portion of the labelled 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.

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).

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

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

$(D/P)_{final}=[\text{dye}] / [\text{antibody}]$

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

Cy5 bisfunctional dye characteristics

Formula weight	817.0
Absorbance max	649 nm
Extinction max	250 000 M^{-1} cm^{-1}
Emission max	670 nm

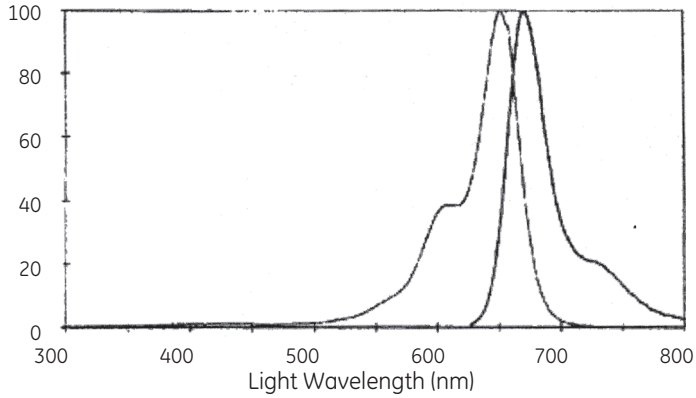

Figure 2. Cy5 dye absorption and fluorescence spectra.

Reference

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4. Aslam, M., and Dent, A., *Bioconjugation*, Macmillan Reference Ltd, (1998).

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PA25031PS Rev D2007