Amersham FluoroLink **Cy**5.5 Monofunctional Dye 5-pack

Product Specification Sheet

Introduction

Product code

PA25501

About

Reagents for the labeling of biological compounds with Cy™5.5 Monofunctional Dye

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

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

Storage

Store refrigerated at 2°C–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:** 0.1 M Sodium Carbonate buffer (pH 9.3).
- **Separation column:** containing a permeation gel (Sephadex™ G-50, or Bio-Gel™ P-10, minimum of 1 cm diameter and 12 cm length packed volume).
- **Separation buffer:** Phosphate-Buffered Saline, pH 7.2, containing 0.1% Sodium Azide.
- **Test tubes.**
- **Transfer pipettes.**
- **Glassware.**

Background

Cyanine reagents have been shown to be useful as fluorescent labels for biological compounds (1, 5). These dyes are intensely fluorescent and highly water soluble, providing significant advantages over other existing fluorophores (4).

The Cy5.5 produces an intense signal in the near IR 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 Cy5.5 dye supplied here is a monofunctional NHS-Ester, and is provided in a dried, pre-measured form ready for the labeling of compounds containing free amino groups.

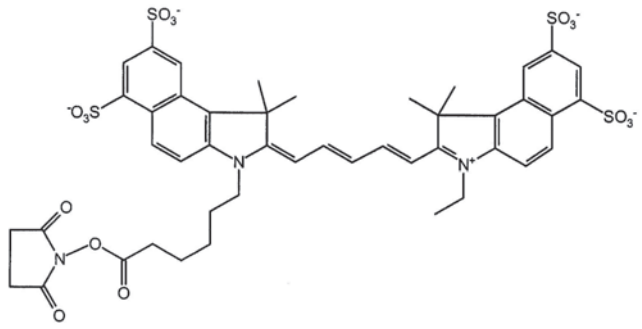

Figure 1. Cy5.5 Monofunctional Dye

Recommended procedure for use

This protocol has been designed for the preparation of Cy5.5-labeled IgG antibodies. It is designed to label 1 mg protein to a final molar dye/protein (D/P) ratio between 2 and 4. This assumes an average protein molecular weight of 155 000 daltons. Other D/P ratios can be obtained by using different amounts of protein.

Note: *The following materials and procedures have been optimized for 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.*

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

Conjugation of dye to antibody

Step	Action
1	Antibody to be conjugated should be dissolved at 1 mg/mL in Sodium Carbonate-Sodium Bicarbonate buffer (2).
2	Add the protein solution (1 mL) to the dye vial, cap the vial, and mix thoroughly. Care should be taken to prevent foaming of the protein solution.
3	Incubate the reaction at room temperature for 30 minutes with additional mixing approximately every 10 minutes. ***Note:*** *Buffers containing primary amino groups such as TRIS and Glycine will inhibit the conjugation reaction.*

The presence of low concentrations (<2%) of biocides such as Azide or Thimerosal do not affect protein labeling.

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 bluish-green bands should develop during elution.

The faster moving band is Cy5.5-labeled antibody while the slower band is free dye. Many Cy5.5-labeled proteins can be stored at 2°C–8°C without further manipulation.

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

Step	Action
1	Dilute a portion of the labeled protein solution so that the maximum absorbance is 0.5 to 1.5 AU.
2	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 678 nm for the Cy5.5 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 18% of the absorbance at 678 nm).

$[Cy5.5\ dye]=(A_{678}) / 250\,000$

$[antibody]=[A_{280} - (0.18 \bullet A_{678})] / 170\,000$

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

$(D/P)_{final}=[0.68 \bullet (A678) / [A_{280} - (0.18 \bullet A_{678})]$

Conjugation of dye to oligonucleotides

Modified oligonucleotides containing alkyl amino groups can be labeled with cyanine dye. Synthetic oligonucleotides must be deprotected before conjugation. Procedures that use concentrated Ammonium Hydroxide require the following pretreatment to remove all traces of Ammonia.

Step	Action
1	Pretreatment: **a.** Concentrate the sample until it is dry (a vacuum concentrator works effectively). **b.** Dissolve the sample in 0.25 mL of a 0.5 M Sodium Chloride solution and separate using an appropriate desalting column (Bio-Gel P-4 or equivalent) equilibrated with a 5.0 mM Borate buffer solution adjusted to a pH of 8.0. **c.** Elute the sample with above Borate buffer solution. **d.** Concentrate the sample until it is dry. Dissolve the dry sample in a 0.1 M Carbonate buffer (pH 8.5-9.0).
2	Conjugation is carried out by adding 30 nmoles of oligonucleotide sample is approximately 0.5 mL of Carbonate buffer to the dye vial.
3	Cap the vial and mix thoroughly.
4	Incubate the reaction at room temperature for 60 minutes with additional mixing at 15 minute intervals.

Separation of labeled oligonucleotides

Conjugated oligonucleotides can be separated from free dye using the same gel filtration procedures listed for separating conjugated antibody. A gel with a smaller exclusion size (such as Bio-Gel P-4) and a longer column length must be used with shorter oligonucleotides in order to ensure complete separation. Cy5.5-labeled oligonucleotides can be separated from unconjugated oligonucleotides using RP-HPLC. The general procedure listed in reference 3 may be optimized for the specific nucleotide sequence and HPLC configuration.

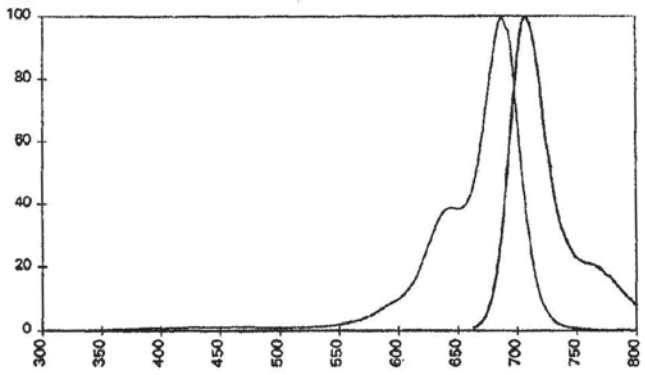

Figure 2. Cy5.5 dye absorption and fluorescence spectra

Cy5.5 Monofunctional Dye characteristics

Formula weight	1128.41
Absorbance max	675 nm
Extinction max	250 000 M^{-1} cm^{-1}
Emission max	694 nm
Quantum yield	>0.28[1]

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

References

1. Mujumdar, R.B. *et al., Bioconjugate Chemistry*, **7**(3), 356-362, (1996).

2. Southwick, P.L. *et al., Cytochemistry*, **11**, 418-430, (1990).
3. Smith, L.M. *et al., Nucleic Acids Research*, **13**, 2399-2412, (1985).
4. Wessendorf, M.W. and Brelje, T.C., *Histochemistry*, **98**(2), 81-85, (1992).
5. Yu, H. *et al., Nucleic Acids Research*, **22**(15), 3226-3232, (1994).

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