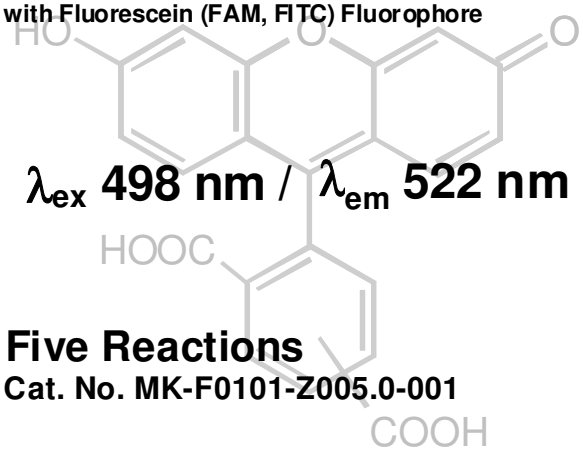
Fluoro•Spin 498

Protein Labeling & Purification Kit (for proteins >25kD)

with Fluorescein (FAM, FITC) Fluorophore



λ_{ex} 498 nm / λ_{em} 522 nm

...your bridge between

molecular biology

and organic chemistry

Five Reactions

Cat. No. MK-F0101-Z005.0-001

Fluoro•Spin 498 Protein Labeling Kit

Product Code: MK-F0101 **λ_{ex} 498 nm / λ_{em} 522 nm** **Five Reactions**

1. Introduction

Fluoro•Spin 498 Protein Labeling & Purification Kit is designed for the labeling of proteins with molecular weights greater than 25 kDa (in particular antibodies), using a reactive succinimidyl-ester of carboxy-fluorescein. The conjugates result from the formation of a stable covalent amide linkage. The protein-dye conjugates have fluorescence-excitation and fluorescence-emission maxima at around 498 nm and 522 nm, respectively.

Up to 15 nmol of protein (see note **1**) can be labeled using one vial (50 nMol) of the reactive Fluoro•Spin 498 dye.

This kit includes enough reactive dye for up to 5 labeling reactions and ten *Centri•Pure Mini* spin columns for rapid and efficient purification of your protein-dye conjugates. To get a quick start with IgG, use the *Optimized Protocol for Labeling 1 mg of IgG* or the *Optimized Protocol for Labeling 100 µg of IgG* (i.e. monoclonal antibodies). For proteins other than IgG or to raise or lower the degree of labeling, please carefully and thoroughly read the following instructions before starting.

Storage: **Store at room temperature (15 – 20 °C). For column storage, see note 6! Keep the reactive dye from light!** When stored properly, the kit components should be stable for at least six months.

2. Contents

Component 1 (blue caps)

Succinimidyl ester of Fluoro•Spin 498 (Carboxyfluorescein, mixed isomers), five vials; 50 nMol each

Component 2 (yellow caps)

Dimethylsulfoxide (DMSO), anhydrous, two vials; 1.5 mL each

Component 3 (white caps)

Sodium bicarbonate, five vials; 84 mg each

Component 4 (green caps)

Reaction tubes, five 0.5 mL

Component 5

Ten *Centri•Pure Mini* spin columns with collection tubes

Component 6

Ten washing tubes (without caps)

Caution: Keep the reactive dye away from light! Protect the reactive dye and DMSO from moisture!

3. Preliminary Work

3.1. Protein Preparation

A 100 µL volume of protein solution is used here for typical labeling reactions (see note **2**). Purified protein should be pre-prepared at a concentration of 2 – 10 mg/mL in buffer (not in serum). The buffer cannot contain ammonium ions or primary amines. The presence of low concentrations of sodium azide (≤ 3 mM) or thimerosal (≤ 1 mM) will not significantly affect the conjugation reaction. If the protein to be labeled is in an unsuitable buffer (e.g. Tris or glycine), the buffer must be replaced by either dialysis against PBS or by using the provided spin columns (see section 5).

3.2. Calculations

The amount of reactive dye to be used for each reaction depends on the concentration of protein to be labeled and on the desired dye-protein molar ratio (MR). The following calculation must be performed *before* beginning your conjugation reaction. In the labeling procedure, a small volume of a dye stock solution (step 4.3 in *Conjugation Reaction*) is added to 100 µL of protein solution. The volume of the dye stock solution to be added can be calculated as follows:

$$V\,[\mu L] \text{ of dye stock solution} = \frac{c_{m,\ protein} \times V_{protein} \times 1000}{c_{act.\ dye} \times MW_{protein}} \times MR$$

- $c_{m,\ protein}$ is the mass concentration of protein solution in mg/mL.
- $c_{act.\ dye}$ is 2 µMol/mL, the concentration of activated dye solution.
- $V_{protein}$ is the volume of protein solution. The recommended volume of protein solution to be used in the reaction is 100 µL.
- 1000 is an unit correction factor
- $MW_{protein}$ is the molecular mass of the protein to be labeled. For most IgGs, this is 145,000.
- MR is the molar ratio of activated dye to protein *in the reaction mixture*. This will NOT be the end Molar Ratio of conjugated dye-protein, which can be substantially less. We recommend a MR of 5 for labeling reactions with IgGs. You may choose other MR values, from less than 5 to over 10, based on your individual labeling requirements.

4. Conjugation Reaction

4.1. Transfer 100 µL of your pre-prepared protein solution to a Component 4 reaction tube (0.5 mL tube with green cap).

4.2. Add 1 mL deionized water to one vial Component 3 (sodium bicarbonate) and dissolve it completely by vortexing. Pipette 20 µL of this solution to the protein vial. The remaining sodium bicarbonate solution can be stored at 4°C for one week.

4.3. Prepare the reactive dye stock solution just before starting the reaction: Add 25 µL of Component 2 (DMSO) to a Component 1 of reactive Fluoro•Spin 498 dye. Pipette up and down to completely dissolve the contents of the vial.

4.4. Add the calculated volume of reactive Fluoro•Spin 498 dye (section 3.2.) to the protein solution in the reaction tube. Vortex the mixture gently until thoroughly mixed. Dye stock solution not used within one hour should be discarded.

4.5. Let the mixture react for 5 minutes at room temperature, protected from light (see note **3**).

5. Purification

5.1. Take two spin columns (see notes **4, 5, 6**): Gently tap or briefly vortex the columns to resuspend gel and remove air bubbles.

5.2. Remove the bottom caps and then remove the top caps.

5.3. Place the column into a wash tube (Component 6) and centrifuge at 1000 x g for two minutes to remove interstitial fluid (see note **7**). If you use a fixed-angle microcentrifuge, keep track of the position of the columns using the orientation mark molded into the columns.

5.4. If there is a drop at the end of the columns, blot it dry. Discard the wash tubes and the interstitial fluid. Do not allow the gel material to dry excessively. Process the samples within the next few minutes.

5.5. Hold the columns up to a source of light. Transfer half of the labeling reaction mixture (65 µL) to the top of the gel of each column. Carefully dispense the sample directly onto the center of the gel bed at the top of the column, without

disturbing the gel surface. Do not contact the sides of the columns with the reaction mixture or the sample pipette tip, since this can reduce the efficiency of purification.

5.6. Place the column into the collection tubes (Component 5) and place both into the rotor. Maintain proper column orientation. The highest point of the gel media in the column should always point towards the outside of the rotor. Spin the columns and collection tubes at 1000 x g for two minutes. The purified protein conjugate (~130 µL together in both columns) will collect in the bottom of the collection tubes. Discard the spin columns.

6. Determination of Degree of Labeling

6.1. Dilute an equivalent of the purified conjugate into PBS or other suitable buffer (see note **8**) and measure the absorbance in a cuvette (see note **9**) with a 1 cm pathlength at both 280 nm (A_{280}) and 498 nm (A_{498}).

6.2. Calculate the protein concentration:

$$c_{m,\,protein}\,[mg/mL] = \frac{[A_{280} - (A_{498} \times K)] \times \text{dilution factor}}{\varepsilon} \times MW_{protein}$$

- K is a correction factor, which compensates the absorption of the dye at 280 nm (see note **10**). For IgGs: K = 0.4 and for other proteins: K = 0.3 (0.3 for avidin and 0.25 for ovalbumin)
- ε is the molar extinction coefficient of the protein at 280 nm. For most IgGs, ε = 203,000 $M^{-1}cm^{-1}$.
- For most IgGs, $MW_{protein}$ = 145,000

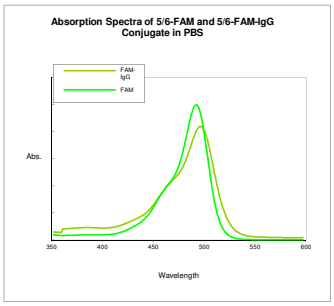
6.3. Calculate the degree of labeling:

$$\text{dye per protein molecule} = \frac{A_{498} \times \text{dilution factor}}{60{,}000 \times c_{m,\,protein}} \times MW_{protein}$$

- 60,000 is the molar extinction coefficient of the dye in the conjugate at 498 nm.

7. Absorption and Fluorescence Properties of Conjugates

7.1. Absorption properties: The absorption maxima of Fluoro•Spin 498 (fluorescein, FAM) dye-protein conjugates in PBS are between 496 – 500 nm. This is a bathochrome shift of about 4 to 8 nm from that of the free dye. The absorption coefficient of the conjugated dye (60,000 $M^{-1}cm^{-1}$) is also about 25% lower from that of the free dye (see example shown below of the absorption spectra of a FAM-IgG conjugate vs. free dye). Absorption properties of conjugates with other IgGs or proteins will vary.



Carboxyfluorescein (FAM) in PBS

λ_{abs} = 498 nm

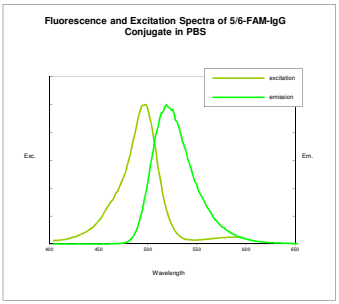
ε= 81,000 $M^{-1}cm^{-1}$

Carboxyfluorescein-IgG conjugate in PBS

λ_{abs} = 498 nm

ε = 60,000 $M^{-1}cm^{-1}$

7.2. Fluorescence properties: As in the case of absorption, the maxima of the fluorescence and excitation spectra of fluorescein-protein conjugates in PBS (λ_{em} 518-523 nm / λ_{exc} 495-499 nm) are red-shifted in comparison with unconjugated dye. The fluorescence intensity of conjugates varies between 30% and 50% of the free dye. In the figure below a typical fluorescence and excitation spectrum of a fluorescein-IgG conjugate is shown.



Carboxyfluorescein (FAM) in PBS

λ_{em} = 514 nm λ_{exc} = 494 nm

Carboxyfluorescein-IgG conjugate in PBS

λ_{em} = 519 nm λ_{exc} = 498 nm

8. Storage of Conjugates

Store the labeled protein at 4 °C, protected from light. If the final concentration of purified protein conjugate is less than 1 mg/mL (see step 6.2.), add bovine serum albumin (BSA) or other stabilizing proteins at 1-10 mg/mL. In the presence of 2 mM sodium azide, the conjugate should be stable at 4°C for several months. For longer storage, divide the conjugate into small aliquots and freeze at –20°C. Avoid repeated freezing and thawing! Protect from light!

9. Notes

1 15 nmol „protein“ are equivalent to 2.18 mg IgG, 1.0 mg BSA or Avidin and 0.68 mg Ovalbumin.

2 The reaction can be scaled to accommodate other volumes of protein. However, the amount of reactive dye must be calculated to reflect your desired reaction volume (see section 3.2 and substitute your volume for 100 µL.). For larger scale reactions, purification methods such as dialysis, column chromatography, or multiple *Centri•Pure Mini* spin columns (each column has a maximum sample volume of 80 µL) must be used. Additional *Centri•Pure Mini* spin columns are available in a 20 column *Fluoro•Spin Accessory Kit* (Catalog No. MK-Z0105).

3 Optional: In order to avoid unspecific interactions or unstable ester bond formation between dye and protein, use hydroxylamine as a stop reagent.

4 If the molecular weight of the protein is less than 25 kDa, then the provided *Centri•Pure Mini* spin columns should not be used. Free dye can be removed from the conjugate either by using *CENTRI•Pure P2* columns (Catalog No. CP-0110, size exclusion ≥ 5 kDa) or by extensive dialysis.

5 If the volume of the reaction exceeds 160 µL, two spin columns will not adequately separate the conjugate from the free dye. The reaction can be divided into aliquots of ≤80 µL and applied to multiple spin columns. Again, additional *Centri•Pure Mini* spin columns are available in a 20 column *Fluoro•Spin Accessory Kit* (Catalog no. MK-Z0105).

6 For longer storage columns must be stored at 4°C. *Allow refrigerated columns to warm to room temperature before use.*

7 Maximum yield and efficiency are obtained with the horizontal or swinging-bucket rotors. However, fixed-angle-rotor microcentrifuges provided acceptable performance and save time. On a variable speed microcentrifuge, *do not* use the pulse button, which overrides the speed setting and takes the rotor to maximum g-force. If you are not sure of the g-force generated by your centrifuge at specific speeds, calculate the correct speed by using the following formula:

$$rpm = \sqrt{\left(\frac{RCF}{1.119 \times 10^{-5} \times r}\right)}$$

- rpm is revolutions per minute
- RCF is relative centrifugal force
- r is the radius (cm) measured from center of spindle to bottom of rotor bucket

Example: for RCF = 1000 and r = 7.5 cm

$$rpm = \sqrt{\left(\frac{1000}{1.119 \times 10^{-5} \times 7.5}\right)} = 3450 \text{ rpm}$$

8 To achieve a relative intensity at 280 nm and 498 nm between 0.2 and 1.0 a 4 to 20-fold dilution is recommended.

9 In order to avoid the using large volumes of conjugate for absorption measurements, we recommend the use of 50 µL- Eppendorf „Uvette“ microcuvettes.

10 The given correction factors are averaged. Depending upon the type of protein (surface structure, number of accessible amino groups) and of the number of coupled dye molecules, your correction factor may be quite different. In this case, we recommend the estimation of protein concentration based on actual dilution using our kit and from an estimated 90% recovery during spin column purification:

$$c_{m,\,protein} = \frac{c_{m,\,protein,\,0} \times V_{protein}}{(V_{protein} + V_{NaHCO3} + V_{dye})} \times 0.9$$

$c_{m,\,protein}$ [mg/mL] is the mass concentration of the calculated protein solution.

$c_{m,\,protein,\,0}$ [mg/mL] is the mass concentration in mg/mL of the starting protein solution.

$V_{protein}$ [µL] is the volume of used protein solution.

V_{NaHCO3} [µL] is the volume of added sodium bicarbonate solution.

V_{dye} [µL] is the volume of added dye stock solution.

Related Products

emp Biotech offers protein labeling kits for different detection wavelengths, quenching and redox applications (more at www.empbiotech.com):

Fluoro•Spin 331
Protein Labeling & Purification Kit
with N-Methylanthranilic acid (MANT) Fluorophore
λ_{ex} 331 nm / λ_{em} 426 nm

10 reactions	5 reactions
MK-F0108-10	MK-F0108-05

Fluoro•Spin 557
Protein Labeling & Purification Kit
with Tetramethylrhodamine (TAMRA) Fluorophore
λ_{ex} 557 nm / λ_{em} 574 nm

10 reactions	5 reactions
MK-T0102-10	MK-T0102-05

Fluoro•Spin 565
Protein Labeling & Purification Kit
with DYOMICS DY-555 Fluorophore

λ_{ex} 565 nm / λ_{em} 580 nm

10 reactions	5 reactions
MK-D0128-10	MK-D0128-05

Fluoro•Spin 587
Protein Labeling & Purification Kit
with X-Rhodamine (ROX) Fluorophore

λ_{ex} 587 nm / λ_{em} 599 nm

10 reactions	5 reactions
MK-R0103-10	MK-R0103-05

Fluoro•Spin 635
Protein Labeling & Purification Kit
with DYOMICS DY-633 Fluorophore

λ_{ex} 635 nm / λ_{em} 654 nm

10 reactions	5 reactions
MK-D0104-10	MK-D0104-05

Fluoro•Spin 673
Protein Labeling & Purification Kit
with DYOMICS DY-675 Fluorophore
λ_{ex} 673 nm / λ_{em} 699 nm

10 reactions	5 reactions
MK-D0106-10	MK-D0106-05

Fluoro•Spin 709
Protein Labeling & Purification Kit
with DYOMICS DY-700 Fluorophore

λ_{ex} 709 nm / λ_{em} 737 nm

10 reactions	5 reactions
MK-D0130-10	MK-D0130-05

Fluoro•Spin 661Q
Protein Labeling & Purification Kit
with DYOMICS DYQ-661 Quencher
λ_{ex} 665 nm / λ_{em} none

10 reactions	5 reactions
MK-D0105-10	MK-D0105-05

Fluoro•Spin 667E
Protein Labeling & Purification Kit
with Methylene Blue Redoxlabel
λ_{ex} 667 nm / λ_{em} 696 nm

10 reactions	5 reactions
MK-E0150-10	MK-E0150-05

Fluoro•Spin Accessory Kit

Extra spin columns for protein purifications	20 columns *Contains 20* ***Centri•Pure Mini*** spin columns	MK-Z0105