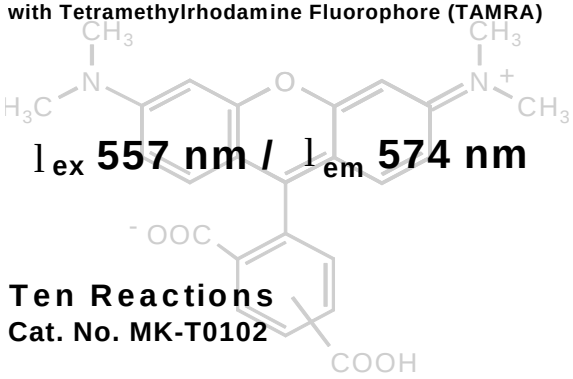
Fluoro•Spin 557

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

with Tetramethylrhodamine Fluorophore (TAMRA)



λ_{ex} 557 nm / λ_{em} 574 nm

Ten Reactions

Cat. No. MK-T0102

emp Biotech GmbH

...your bridge between molecular biology and organic chemistry

Fluoro•Spin 557 Protein Labeling Kit

Catalog No.: MK-T0102 **λ_{ex} 557 nm / λ_{em} 574 nm** **Ten Reactions**

1. Introduction

Fluoro•Spin 557 Protein Labeling & Purification Kit is designed for the labeling of proteins with molecular weights greater than 25 kD (in particular antibodies), using a reactive succinimidyl-ester of carboxy-tetramethylrhodamine (TAMRA). 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 557 nm and 574 nm, respectively.

Up to 50 nmol of protein (see note **1**) can be labeled using one vial (1 µMol) of the reactive Fluoro•Spin 557 dye. In order to avoid unspecific interactions or unstable ester bond formation between dye and protein, this kit provides hydroxylamine for use as a stop reagent.

This kit includes enough reactive dye for up to 10 labeling reactions and twenty *Centri•Sep* 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). 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 557 (carboxy-tetramethylrhodamine, mixed isomers), five vials; 1 µMol 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 (red caps)
Hydroxylamine, buffered, five vials

Component 5 (green caps)
Reaction tubes, ten 0.5 mL

Component 6
Twenty *Centri•Sep* purification columns with collection tubes

Component 7
Twenty washing tubes (without caps)

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

3. Preliminary Work

3.1. Protein Preparation

A 100 µL volume of protein solution is used here for standard labeling reactions (see note **2**). Purified protein should be pre-prepared at a concentration of 1 – 15 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:

$$V\,[\mu L]_{\text{ of dye stock solution}} = \frac{c_{m,\text{ protein}} \times 100\,\mu L \times 1000}{c_{\text{act. dye}} \times MW_{\text{ protein}}} \times MR$$

- $c_{m,\text{ protein}}$ is the mass concentration of protein solution in mg/mL.
- $c_{\text{act. dye}}$ is 20 µMol/mL, the concentration of activated dye solution.
- 100 µL is the recommended volume of protein solution to be used in the reaction.
- 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 5 reaction tube (0.5 mL tube with green cap, use the 2 mL tube for larger volumes).

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 50 µL of Component 2 (DMSO) to a Component 1 of reactive Fluoro•Spin 557 dye. Pipette up and down to completely dissolve the contents of the vial.

4.4. Add the calculated volume of reactive Fluoro•Spin 557 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 1 hour at room temperature, protected from light. During this time you should prepare the hydration of the Centri•Sep purification columns (section 5.1. to 5.3.).

4.6. Add 100 µL of deionized water to one vial of Component 4 (hydroxylamine). Transfer 10 µL of the hydroxylamine solution to the conjugation reaction. Vortex the mixture gently. Hydroxylamine solution not used within one hour should be discarded.

4.7. Let the mixture react for 30 minutes and then proceed with the purification below.

5. Purification

5.1. Prepare <u>two</u> spin columns (see notes **3**, **4**): Gently tap the columns to insure that the dry gel has settled in the bottom of the spin column.

5.2. Remove the top column caps and reconstitute the columns by adding 0.8 mL of buffer of choice (e.g. PBS with 2 mM sodium azide) to each. Replace the column cap and vortex vigorously for about 5 seconds. Remove air bubbles by sharply tapping the bottom of the columns. It is important to hydrate all of the dry gel.

5.3. Allow at least 30 minutes of room temperature hydration time before using the columns (see note **5**).

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5.4. After 30 minutes of hydration, is complete, remove first the top column caps, and then remove the column end stoppers from the bottom.

5.5. Allow excess column fluid to drain (via gravity) into a Component 7 wash tube. If the fluid does not begin to flow immediately through the end of the column, use a 2 mL latex pipette bulb to gentle apply air pressure to the top of the column to force the fluid to start through the column filter. The column will stop draining on its own. Approximately 200 – 250 µL will drain from the column. Discard this fluid.

5.6. Spin the columns and wash tubes in a variable speed centrifuge at 750 x g for two minutes (see note **6**) to remove interstitial fluid. If you use a fixed-angle microcentrifuge, keep track of the position of the columns using the orientation mark molded into the columns.

5.7. 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.

5.8. Hold the columns up to the light. Transfer half of the labeling reaction mixture (up to but not more than 90 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 purification efficiency.

5.9. Place each column into Component 6 collection tubes and place together 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 750 x g for 2 minutes. The purified protein conjugate (approx 135 µL total 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 **7**) and measure the absorbance in a cuvette (see note **8**) with a 1 cm pathlength at both 280 nm (A_{280}) and 555 nm (A_{555}).

6.2. Calculate the protein concentration:

$$c_{m,protein}\ [mg/mL] = \frac{[A_{280} - (A_{555} \times K)] \times \text{dilution factor}}{\varepsilon} \times MW_{protein}$$

- K is a correction factor, which compensates the absorption of the dye at 280 nm (see note **9**). For IgGs: K = 0.6 and for other proteins: K = 0.4 (0.45 for avidin and 0.4 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

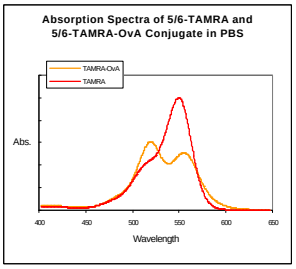
6.3. Calculate the degree of labeling:

$$\text{dye per protein molecule} = \frac{A_{555} \times \text{dilution factor}}{42{,}000 \times c_{m,\,protein}} \times MW_{Protein}$$

- 42,000 is the molar extinction coefficient of the dye at 555 nm.

7. Absorption and Fluorescence Properties of Conjugates

7.1. Absorption properties: The absorption maxima of Fluoro•Spin 557 (tetramethylrhodamine, TAMRA) dye-protein conjugates in PBS are between 553 – 555 nm. This is a bathochrome shift of about 5 to 7 nm from that of the free dye. The absorption coefficient of the conjugated dye (42,000) is also about 45% lower from that of the free dye (see example shown below of the absorption spectra of a TAMRA-IgG conjugate vs. free dye). Absorption properties of conjugates with other IgGs or proteins will vary.



Carboxy-tetramethylrhodamine (TAMRA) in PBS

λ_{abs} = 548 nm

ε= 78,000 $M^{-1}cm^{-1}$

Carboxy-tetramethylrhodamine-IgG conjugate in PBS

λ_{abs} = 553 nm

ε = 40,000 $M^{-1}cm^{-1}$

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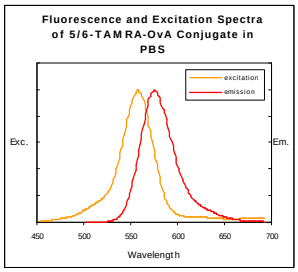
7.2. Fluorescence properties: As in the case of absorption, the maxima of the fluorescence and excitation spectra of TAMRA-protein conjugates in PBS (λ_{em} 573-575 nm / λ_{exc} 557-558 nm) are red-shifted in comparison with unconjugated dye. The fluorescence intensity of conjugates varies between 20% and 50% of the free dye. In the figure below a typical fluorescence and excitation spectrum of a TAMRA-IgG conjugate is shown.



Carboxy-teramethylrhodamine in PBS

λ_{em} = 570 nm λ_{exc} = 554 nm

Carboxy-tetramethylrhodamine-ovalbumin conjugate in PBS

λ_{em} = 575 nm λ_{exc} = 558 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 50 nmol „protein“ are equivalent to 7.25 mg IgG, 3.3 mg BSA or Avidin and 2.2 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.). The amount of hydroxylamine solution must be scaled up or down using a hydroxylamine volume 1/10th of your volume of protein, in µL. For larger scale reactions, purification methods such as dialysis, column chromatography, or multiple *Centri•Sep* spin columns (each column has a maximum sample volume of 90 µL) must be used. Additional *Centri•Sep* columns are available in a 20 column *Fluoro•Spin Accessory Kit* (Catalog No. MK-Z0105).

3 If the molecular weight of the protein is less than 25,000, then the provided *Centri•Sep* columns should not be used. Free dye can be removed from the conjugate either by using *Centri•Spin 10* columns (20 column kit, Catalog No. CS-100, size exclusion ≥ 5 kD) or by extensive dialysis.

4 If the volume of the reaction exceeds 180 µL, two spin columns will not adequately separate the conjugate from the free dye. The reaction can be divided into aliquots of <90 µL and applied to multiple spin columns. Again, additional *Centri•Sep* columns are available in a 20 column *Fluoro•Spin Accessory Kit* (catalog no. MK-Z0105).

5 Reconstituted columns may be stored at 4°C for several days. Longer storage can be accomplished in 10 mM sodium azide. *Allow refrigerated columns to warm to room temperature before use*.

6 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:

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

- 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 = 750 and r = 7.5 cm

$$\text{rpm} = \sqrt{\left(\frac{750}{1.119 \times 10^{-5} \times 7.5}\right)} = 2990 \text{ rpm}$$

7 For protein concentrations between 1 to 5 mg/mL a 5- to 20-fold dilution is recommended. For protein concentrations between 10-15 mg/mL a 50- to 100-fold dilution is recommended. The relative intensity at 280 nm and 557 nm should fall between 0.2 and 1.0.

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

9 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:

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

$c_{m,\,Protein}$ [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_{HA} [µL] is the volume of added hydroxylamine solution.
V_{DYE} [µL] is the volume of added dye stock solution.

Related Products

emp Biotech offers the following protein labeling kits for different detection wavelengths:

Product Name	Cat. No. 5-reaction kit	Cat. No. 10-reaction kit	Product Name	Cat. No. 5-reaction kit	Cat. No. 10-reaction kit
Fluoro•Spin 331 Protein Labeling & Purification Kit *with N-Methylanthranilic acid (MANT) Fluorophore* λ_{ex} 331 nm / λ_{em} 426 nm	MK-D0108-05	MK-D0108-10	**Fluoro•Spin 557** Protein Labeling & Purification Kit *with mixed 5/6 isomer carboxy-X-Rhodamine (5/6-ROX) Fluorophore* λ_{ex} 587 nm / λ_{em} 599 nm	MK-R0103-05	MK-R0103-10
Fluoro•Spin 498 Protein Labeling & Purification Kit *with mixed 5/6 isomer carboxy-Fluorescein (5/6-FAM, FITC) Fluorophore* λ_{ex} 498 nm / λ_{em} 522 nm	MK-F0101-05	MK-F0101-10	**Fluoro•Spin 557 Single 5** Protein Labeling & Purification Kit *with Single isomer 5-carboxy-X-Rhodamine (5-ROX) Fluorophore* λ_{ex} 587 nm / λ_{em} 599 nm	MK-R0113-05	MK-R0113-10
Fluoro•Spin 498 Single 5 Protein Labeling & Purification Kit *with Single isomer 5-carboxy-Fluorescein (5-FAM) Fluorophore* λ_{ex} 498 nm / λ_{em} 522 nm	MK-F0109-05	MK-F0109-10	**Fluoro•Spin 557 Single 6** Protein Labeling & Purification Kit *with Single isomer 6-carboxy-X-Rhodamine (6-ROX) Fluorophore* λ_{ex} 587 nm / λ_{em} 599 nm	MK-R0114-05	MK-R0114-10
Fluoro•Spin 498 Single 6 Protein Labeling & Purification Kit *with Single isomer 6-carboxy-Fluorescein (6-FAM) Fluorophore* λ_{ex} 498 nm / λ_{em} 522 nm	MK-F0110-05	MK-F0110-10	**Fluoro•Spin 635** Protein Labeling & Purification Kit *with DYOMICS DY-633 Fluorophore* λ_{ex} 635 nm / λ_{em} 654 nm	MK-D0104-05	MK-D0104-10
Fluoro•Spin 557 Protein Labeling & Purification Kit *with mixed 5/6 isomer carboxy-Tetramethylrhodamine (5/6-TAMRA) Fluorophore* λ_{ex} 557 nm / λ_{em} 574 nm	MK-T0102-05	MK-T0102-10	**Fluoro•Spin 651** Protein Labeling & Purification Kit *with EVOblue30 Fluorophore* λ_{ex} 651 nm / λ_{em} 666 nm	MK-D0107-05	MK-D0107-10
Fluoro•Spin 557 Single 5 Protein Labeling & Purification Kit *with Single isomer 5-carboxy-Tetramethylrhodamine (5-TAMRA) Fluorophore* λ_{ex} 557 nm / λ_{em} 574 nm	MK-T0111-05	MK-T0111-10	**Fluoro•Spin 673** Protein Labeling & Purification Kit *with DYOMICS DY-633 Fluorophore* λ_{ex} 673 nm / λ_{em} 699 nm	MK-D0106-05	MK-D0106-10
Fluoro•Spin 557 Single 6 Protein Labeling & Purification Kit *with Single isomer 6-carboxy-Tetramethylrhodamine (6-TAMRA) Fluorophore* λ_{ex} 557 nm / λ_{em} 574 nm	MK-T0112-05	MK-T0112-10	**Fluoro•Spin 709** Protein Labeling & Purification Kit *with DYOMICS DY-700 Fluorophore* λ_{ex} 709 nm / λ_{em} 737 nm	MK-D0130-05	MK-D0130-10
Fluoro•Spin 565 Protein Labeling & Purification Kit *with DYOMICS DY-555 Fluorophore* λ_{ex} 565 nm / λ_{em} 580 nm	MK-D0128-05	MK-D0128-10	**Fluoro•Spin Accessory Kit** Extra spin columns for protein purifications *Contains Twenty* ***Centri•Sep*** *Spin Columns*	20 columns MK-Z0105	

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