GENAXXON
bioscience

X-Rhodamine Protein Labeling Kit

Catalog No.: S5258; S5250; S5251 λ_{abs} 585 nm / λ_{em} 597 nm

Store at room temperature (15 – 20 °C). Keep the reactive dye from light!

1. Introduction

The X-Rhodamine Protein Labeling Kit is designed for the labeling of proteins with molecular weights greater than 25 kD (in particular antibodies), using a reactive succinimidylester of carboxy-X-rhodamine. The conjugates result from the formation of a stable covalent amide linkage. The protein-dye conjugates have absorption and fluorescence-emission maxima at around 585 nm and 597 nm, respectively.

Up to 50 nmol (see note 1) of protein can be labeled using one vial (0.5 mg) of activated carboxy-X-rhodamine. 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: When stored properly, the kit components should be stable for at least four months.

2. Contents

Component 1 (blue caps)	-	Succinimidyl ester of carboxy-X-rhodamine, five vials; 0.5 mg each
Component 2 (yellow caps)	-	Dimethylsulfoxide (DMSO), anhydrous, two vials; 0.5 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, five 2 mL and five 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 dye stock solution to be added can be calculated as follows:

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

- $c_{m,\,\text{Protein}}$ is the mass concentration of protein solution in mg/mL.
- $c_{m,\,\text{act. dye}}$ is 10 mg/mL, the mass concentration of activated dye solution.
- 100 µL is the recommended volume of protein solution to be used in this reaction.
- $MW_{\text{act. dye}} = 668$, the molecular mass of the active dye.
- 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 15 for labeling reactions with IgGs. You may choose other MR values, from less than 10 to over 20, 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. Pipet 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 prior to starting the reaction: Add 50 µL of Component 2 (DMSO) to a Component 1 vial of reactive carboxy-X-rhodamine. Pipet up and down to completely dissolve the contents of the vial.

4.4. Add the calculated volume of reactive 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 two 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**).

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 pipet 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 pipet tip, since this can reduce the purification efficiency.

5.9. Place each column into a Component 6 collection tube 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 its absorbance in a cuvette (see note **8**) with a 1 cm pathlength at both 280 nm (A_{280}) and 585 nm (A_{585}).

6.2. Calculate the protein concentration:

$$c_{m,\,protein}\ [mg/mL] = \frac{[A_{280} - (A_{585} \times K)] \times \text{dilution factor}}{\varepsilon} \times MW_{protein}$$

- K is a correction factor, which compensates the absorption of the dye at 280 nm (see note **9**). For most IgGs: K is 0.3 and for other proteins: K is 0.4.
- ε is the molar extinction coefficient of the protein at 280 nm. For most IgGs, $\varepsilon = 203{,}000\ M^{-1}cm^{-1}$.
- For most IgGs, $MW_{protein} = 145{,}000$

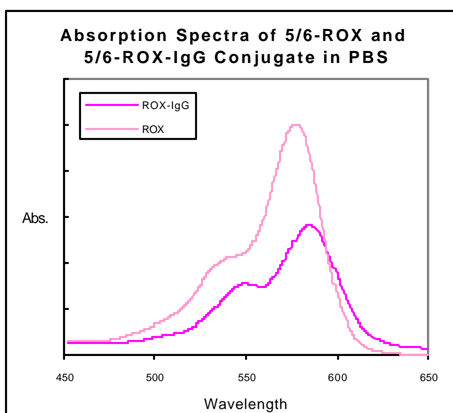
6.3. Calculate the degree of labeling:

$$\text{dye per protein molecule} = \frac{A_{585} \times \text{dilution factor}}{63{,}000 \times c_{m,\,protein}} \times M_{Protein}$$

- 63,000 is the molar extinction coefficient of the dye at 585 nm..

7. Absorption and Fluorescence Properties of Conjugates

7.1. Absorption properties: The absorption maxima of X-Rhodamine-protein conjugates in PBS are between 583 – 585 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 (63,000) is also about 30% lower from that of the free dye (see example shown below of the absorption spectra of a X-Rhodamin-IgG conjugate vs. free dye). Absoption properties of conjugates with other IgGs or proteins will vary.



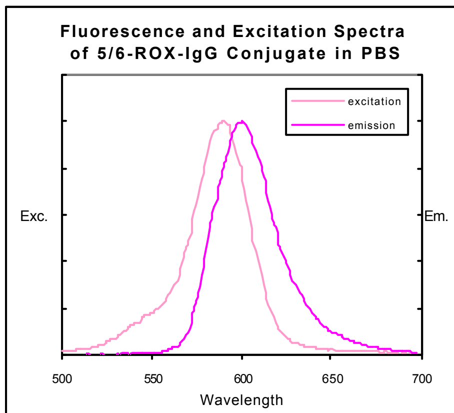
5/6-Carboxy-X-rhodamine in PBS

$\lambda_{abs} = 578$ nm $\quad \varepsilon = 95{,}000\ M^{-1}cm^{-1}$

5/6-Carboxy-X-rhodamine-IgG conjugate in PBS

$\lambda_{abs} = 585$ nm $\quad \varepsilon = 53{,}000\ M^{-1}cm^{-1}$

7.2. Fluorescence properties: As in the case of absorption, the maxima of the fluorescence and excitation spectra of X-Rhodamine-protein conjugates in PBS (λ_{em} 597-603 nm / λ_{exc} 584-590 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 X-Rhodamine-IgG conjugate is shown.



5/6-Carboxy-X-rhodamine in PBS

λ_{em} = 594 nm λ_{exc} = 581 nm

5/6-Carboxy-X-rhodamine-IgG conjugate in PBS

λ_{em} = 601 nm λ_{exc} = 590 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. <u>Avoid repeated freezing and thawing! Protect from light!</u>

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 subsititute 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 Centri Sep Accessory Kit (catalog no. S5300.0020).

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 exeeds 180 µL, two spin columns will not adaquately 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 Centri Sep Accessory Kit (catalog no. S5300.0020).

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

6 Maximum yield and efficiency are obtained with the horizontal or swinging-bucket rotors. However, fixed-angle-rotor microcentrifuges provide acceptable performance and save time. On a variable speed microcentrifuge, <u>do not</u> 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 = 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 585 nm should fall between 0.2 and 1.0.

8 In order to avoid 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 accessable amino groups) and on 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:

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

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

10. Related Products

Genaxxon biotech offers the following protein labeling kits for different detection wavelenghts:

S5256; S5246; S5247	Fluorescein Protein Labeling Kits	λabs 498 nm / λem 520 nm
S5257; S5248; S5249	Tetramethylrhodamine Protein Labeling Kit	λabs 555 nm / λem 575 nm
S5258; S5250; S5251	X-Rhodamine Protein Labeling Kit	λabs 585 nm / λem 597 nm
S5253	DY-633	λabs 645 nm / λem 660 nm
S5254	DY-675	λabs 662 nm / λem 669 nm
S5252	N-Methylanthranilic acid (MANT)	λabs 325 nm / λem 415 nm
S5300.0020	CentriSep Accessory Kit (dye removel)	20 spin columns

For any product and technical question

Please call us at: +49 (0) 7357 916377
or send a fax: +49 (0) 7357 916378
or send an e-mail: info@genaxxon.com