

# Fluorescein Protein Labelling & Purification Kit

For proteins >25 kDa.  
With Carboxy-fluorescein as Fluorophore

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Product	Cat#	Package size
Fluorescein Labelling & Purification Kit	S5256.0005	5 reactions
Fluorescein Labelling & Purification Kit	S5256.0010	10 reactions

## Related products / overview

- MANT Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  331 nm/ $\lambda_{\text{exc}}$ . 426 nm) S5252
- 5/6 FAM/FITC Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  498 nm/ $\lambda_{\text{exc}}$ . 522 nm) S5256
- 5 FAM/FITC Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  498 nm/ $\lambda_{\text{exc}}$ . 522 nm) S5246
- 6 FAM/FITC Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  498 nm/ $\lambda_{\text{exc}}$ . 522 nm) S5247
- 5/6 TAMRA Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  557 nm/ $\lambda_{\text{exc}}$ . 574 nm) S5257
- 5 TAMRA Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  557 nm/ $\lambda_{\text{exc}}$ . 574 nm) S5248
- 6 TAMRA Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  557 nm/ $\lambda_{\text{exc}}$ . 574 nm) S5249
- 5/6 ROX Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  587 nm/ $\lambda_{\text{exc}}$ . 599 nm) S5258
- 5 ROX Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  587 nm/ $\lambda_{\text{exc}}$ . 599 nm) S5250
- 6 ROX Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  587 nm/ $\lambda_{\text{exc}}$ . 599 nm) S5251
- DY-633 Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  635 nm/ $\lambda_{\text{exc}}$ . 654 nm) S5253
- DY-675 Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  673 nm/ $\lambda_{\text{exc}}$ . 699 nm) S5254
- DY-565 Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  565 nm/ $\lambda_{\text{exc}}$ . 580 nm)
- DY-700 Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  709 nm/ $\lambda_{\text{exc}}$ . 737 nm)
- DY-651 Protein Labelling&Purification Kit ( $\lambda_{\text{ex}}$  651 nm/ $\lambda_{\text{exc}}$ . 666 nm) S5255
- 
- Centrispin 10 columns S5301.10xx
- Centrispin 20 columns S5301.20xx
- Centrisep Dye Terminator Removal Kit S5300

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## Notes on Warranties and Disclaimer

Genaxxon is dedicated to your success and every batch of this product is tested with an extensive routine procedure to make sure that it meets all your needs. However, it has neither been developed nor tested for a specific application.

### **This product is for research use only. For *in vitro* use only**

Genaxxon's liability with respect to any product is limited to the replacement of the product. No other warranties are provided by Genaxxon. Genaxxon is not liable to any direct, indirect, incidental or consequential damage arising out of or in connection with the use of any of Genaxxon products.

## Notes (continued)

8. In order to avoid the using large volumes of conjugate for absorption measurements, we recommend the use of 50µL Eppendorf “Uvettes” 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:

$$C_{m, \text{protein}} = (C_{m, \text{protein}, 0} \times V_{\text{protein}} / (V_{\text{protein}} + V_{\text{NaHCO}_3} + V_{\text{HA}} + V_{\text{DYE}})) \times 0.9$$

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

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

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

$V_{\text{NaHCO}_3}$  [µL] is the volume of added sodium bicarbonate solution.

$V_{\text{HA}}$  [µL] is the volume of added hydroxylamine solution.

$V_{\text{DYE}}$  [µL] is the volume of added dye stock solution.

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## Kit Contents & Storage Information

ITEM	Content
Component 1 (blue caps) Succinyl ester of carboxy-fluorescein (mixed isomer, 1µMol each)	5 vials
Component 2 (yellow caps) Dimethylsulfoxide (DMSO), anhydrous (1.5mL each)	2 vials
Component 3 (white caps) Sodium bicarbonate (84mg each)	5 vials
Component 4 (red caps) Hydroxylamine, buffered	5 vials
Component 5 (green caps) Reaction tubes (0.5mL each)	5 tubes
Component 6 CentriSep spin columns	10 columns
Component 7 Washing tubes without caps	10 tubes
Manual	1
Optimised Protocol for Labelling 100µg and 1mg of IgG	1

### 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.

### Notes

- 50nmol “protein” are equivalent to 7.25mg IgG, 3.3mg BSA or Avidin and 2.2mg Ovalbumin.
- 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 2.2. and substitute your volume for 100µL). The amount of hydroxylamine solution must be scaled up or down using a hydroxylamine volume 1/10<sup>th</sup> of your volume of protein, in µL. For larger scale reactions, purification methods such as dialysis, column chromatography, or multiple CentriSep spin columns (each column has a maximum sample volume of 90µL) must be used. Additional CentriSep columns are available (S5300).
- If the molecular weight for the protein is less than 25,000 Da, then the provided CentriSep columns should not be used. Free dye can be removed from the conjugate either by using CentriSpin 10 columns (20 column kit, (S5301.1020) size exclusion >5 kDa) or by extensive dialysis.
- If the volume of the reaction exceeds 180µL, 2 spin columns will not adequately separate the conjugate from the free dye. The reaction can be divided into aliquots of < 90µL and applied to multiple spin columns. Additional CentriSep columns can be ordered: S5300.
- Reconstituted columns may be stored at 4°C for several days. Longer storage can be accomplished in 10mM sodium azide. **Allow refrigerated columns to warm to room temperature before use.**
- 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:

$$\text{rpm} = \sqrt{\text{RCF} / (1.119 \times 10^{-5}) r(\text{cm})}$$

Where: rpm = revolutions per minute  
RCF = Relative Centrifugal Force  
r = radius (cm) measured from centre of spindle to bottom of rotor bucket.

**Example:** RCF = 750 and r = 7.5 cm

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

- For protein concentrations between 1 to 5mg/mL a 5- to 20-fold dilution is recommended. For protein concentrations between 10-15mg/mL a 50- to 100-fold dilution is recommended. The relative intensity at 280 nm and 498 nm should fall between 0.2 and 1.0.

## 6. Absorption and Fluorescence Properties of Conjugates

**6.1.** Absorption properties: The absorption maxima of carboxy-fluorescein-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) is also about 25% lower from that of the free dye. Absorption properties of conjugates with other IgGs or proteins will vary.

**6.2.** Fluorescence properties: As in the case of absorption, the maxima of the fluorescence and excitation spectra of fluorescein-protein conjugates in PBS ( $\lambda_{em}$  518-523 nm /  $\lambda_{exc}$  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.

## 7. Storage of Conjugates

**7.1.** Store the labelled protein at 4°C, protected from light. If the final concentration of purified protein conjugate is less than 1mg/mL (see step 5.2, page 4), add bovine serum albumin (BSA) or other stabilising proteins at 1-10mg/mL. In the presence of 2mM 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!**

## Introduction

This Fluorescein Protein Labelling & Purification Kit is designed for the labelling 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 498nm and 522nm, respectively.

Up to 15nmol of protein (see note 1, page 6) can be labelled using one vial (1µMol) of the reactive carboxy-fluorescein. 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 5 labelling reactions and ten CentriSep spin columns (S5300) for rapid and efficient purification of your protein-dye conjugates. To get a quick start with IgG, use the “**Optimised Protocol for Labelling 1mg of IgG**” or the “**Optimised Protocol for Labelling 100µg of IgG**” (i.e. monoclonal antibodies). Both protocols are printed on a separate sheet (part of this kit). For proteins other than IgG or to raise or lower the degree of labelling, please carefully and thoroughly read the following instructions before starting.

**CAUTION:**                    **Store at RT**  
                                      **Keep the reactive dye away from light!**  
                                      **Protect the reactive dye, DMSO and hydroxylamine from moisture!**

## Getting started

### 1. Protein Preparation

A 100µL volume of protein solution is used here for standard labelling reactions (see note 2, page 6). Purified protein should be pre-prepared at a concentration of 1 – 15mg/mL in buffer (not in serum). The **buffer cannot contain** ammonium ions or primary amines. The presence of low concentrations of sodium azide (< 3mM) or thimerosal (< 1mM) will not significantly affect the conjugation reaction. If the protein to be labelled 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 4, page 3).

### 2. Calculations

The amount of reactive dye to be used for each reaction depends on the concentration of protein to be labelled and on the desired dye-protein molar ratio (MR). **The following calculation must be performed before beginning your conjugation reaction.** In the labelling procedure, a small volume of a dye stock solution (step 3.3 in Conjugation Reaction, page 3) is added to 100µL of protein solution. The volume of the dye stock solution to be added can be calculated as follows.

$V (\mu\text{L}) \text{ of dye stock solution} = (C_{m, \text{protein}} \times 100\mu\text{L} \times 1000 / C_{\text{act. dye}} \times \text{MW protein}) \times \text{MR.}$

(Explanations for abbreviations are given on page 3)

## Getting started (continued)

- $C_{m, \text{protein}}$  is the mass concentration of protein solution in mg/mL
- $C_{\text{act, dye}}$  is 2μMol/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.
- $MW_{\text{protein}}$  is the molecular weight of the protein to be labelled. For most IgGs, this is 145000
- 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 3 for labelling reactions with IgGs. You may choose other MR values, from less than 3 to over 5, based on your individual labelling requirements.

## 3. Conjugation Reaction

- 3.1. Transfer 100μL of your pre-prepared protein solution to a Component 5 reaction tube (0.5mL tube with green cap).
- 3.2. Add 1mL deionised water to one vial Component 3 (sodium bicarbonate) and dissolve it completely by vortexing. Pipette 20μL of this solution to the protein vial. The remaining sodium bicarbonate solution can be stored at 4°C for one week.
- 3.3. Prepare the reactive dye stock solution just before starting the reaction: Add 50μL of Component 2 (DMSO) to a Component 1 of reactive carboxy-fluorescein. Pipette up and down to completely dissolve the contents of the vial.
- 3.4. Add the calculated volume of reactive carboxy-fluorescein (section 2, page 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.
- 3.5. Let the mixture react for 1 hour at room temperature, protected from light. During this time you should prepare the hydration of the CentriSep purification columns (S5300) (section 4.1 to 4.3, page 3).
- 3.6. Add 100μL of deionised water to one vial of Component 4 (hydroxylamine). Transfer 10μL of the hydroxylamine solution to the conjugation reaction. Vortex the mixture gently. Hydroxylamine solution not used within one hour should be discarded.
- 3.7. Let the mixture react for 30 minutes and then proceed with the purification below.

## 4. Purification

- 4.1. Prepare two spin columns (see notes 3 and 4, page 6): Gently tap the columns to insure that the dry gel has settled in the bottom of the spin column.
- 4.2. Remove the top column caps and reconstitute the columns by adding 0.8mL of buffer of choice (e.g. PBS with 2mM 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.
- 4.3. Allow at least 30 minutes at room temperature hydration time before using the columns (see note 5, page 6).
- 4.4. After 30 minutes of hydration, is complete, remove first the top column caps, and then remove the column end stoppers from the bottom.

4.5. 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 2mL latex pipette bulb to gently 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 – 250μL will drain from the column. Discard the fluid.

4.6. Spin the columns and wash tubes in a variable speed centrifuge at **750xg for 2 minutes** (see note 6, page 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.

4.7. 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.**

4.8. Hold the columns up to the light. Transfer half of the labelling reaction mixture (up to but not more than 90μL) 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.

4.9. 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 750xg for 2 minutes. The purified protein conjugate (approx 135μL total both columns) will collect in the bottom of the collection tubes. Discard the spin columns.

## 5. Determination of Degree of Labelling

5.1. Dilute an equivalent of the purified conjugate into PBS or other suitable buffer (see note 7, page 6) and measure the absorbance in a cuvette (see note 8, page 7) with a 1 cm pathlength at both 280nm ( $A_{280}$ ) and 498nm ( $A_{498}$ ).

5.2. Calculate the protein concentration:

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

- K = is a correction factor, which compensates the absorption of the dye at 280 nm (see note 9, page 7). For IgGs: K=0.4 and for other proteins: K=0.3 for avidin and K=0.25 for ovalbumin.
- $\epsilon$  is the molar extinction coefficient of the protein at 280 nm. For most IgGs,  $\epsilon = 203,000 \text{ M}^{-1} \text{ cm}^{-1}$ .
- For most IgGs,  $MW_{\text{protein}} = 145,000 \text{ Da}$ .

5.3. Calculate the degree of labelling:

$$\text{dye per protein molecule} = (A_{498} \times \text{dilution factor} / 60,000 \times C_{m, \text{protein}}) \times MW_{\text{protein}}$$

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