

Optimised Labelling Protocol for Fluorescein Labelling Kit for 100µg IgG ≈ 2 - 4 Labels per IgG

λ_{ex} 492nm - λ_{em} 514nm

Preparation of Materials

Buffer Preparation

1. Make a fresh 1M sodium bicarbonate solution (pH ca. 8.5) by dissolving Component 3 in 1mL deionised water. This solution is stable at 4°C for a maximum on one week.
2. Dissolve the antibody (100µg per 100µL) in a buffer, e.g. PBS. 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 antibody 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 CentriSep spin columns.

Conjugation Reaction

3. Transfer 100µL of protein solution to a Component 5 reaction tube (0.5mL tube with green cap).
4. Add 20µL of 1M sodium bicarbonate (see step 1) to the protein containing reaction tube.
5. **Prepare the reactive dye stock solution just prior to starting the reaction:**
Add 500µL of Component 2 (DMSO) to a Component 1 vial or reactive carboxy-fluorescein. Pipette up and down to completely dissolve the contents of the vial.
6. Add 5µL of reactive dye to the protein solution in the reaction tube. Vortex the mixture gently until thoroughly mixed. The remaining dye stock solution should be discarded.
7. Let the mixture react 1 hour at room temperature, protected from light.
8. During this time, prepare 2 spin columns: Gently tap the columns to insure that the dry gel has settled in the bottom of the spin column.
9. 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.
10. Allow at least 30 minutes at room temperature hydration time before using the columns
11. After the 1 hour labelling reaction is over, add 100µL of deionised water to one vial of Component 4 (hydroxylamine, stop solution). Transfer 10µL of this solution to the conjugation reaction. Vortex the mixture gently. Remaining hydroxylamine should be discarded.
12. Let the stop mixture react for 30 minutes.

Purification

13. After hydration, first remove the top columns caps, and then remove the column end stoppers from the bottom.
14. Allow excess column fluid to drain (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.
15. Spin the columns and wash tubes in a variable speed centrifuge at **750xg for 2 minutes** 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.
16. 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.**
17. Hold the columns up to the light. Transfer half of the labelling reaction mixture (up to but not more than 68µL) to the top of the gel of each column. Carefully dispense the sample **directly onto the centre 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.
18. 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.

Degree of Labelling Determination

19. Measure the absorbance of the purified conjugate without dilution in a cuvette with a 1cm pathlength at both 280nm (A_{280}) and 498nm (A_{498}).

20. Calculate the protein concentration:

$$C_{m, \text{protein}} [\text{mg/mL}] = [A_{280} - (A_{498} \times 0.4)] \times 0.71$$

21. Calculate the degree of labelling:

$$\text{dye per protein molecule} = (A_{498} \times 2.4) / C_{m, \text{protein}}$$

Storage

22. Store the labelled protein at 4°C, protected from light. 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!

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Preparation of Materials

Buffer Preparation

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2. Dissolve the antibody (1mg per 100 μ L) in a buffer, e.g. PBS. 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 antibody 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 CentriSep spin columns.

Conjugation Reaction

3. Transfer 100 μ L of protein solution to a Component 5 reaction tube (0.5mL tube with green cap).

4. Add 20 μ L of 1M sodium bicarbonate (see step 1) to the protein containing reaction tube.

5. Prepare the reactive dye stock solution just prior to starting the reaction:

Add 100 μ L of Component 2 (DMSO) to a Component 1 vial or reactive carboxy-fluorescein. Pipette up and down to completely dissolve the contents of the vial.

6. Add 5 μ L of reactive dye to the protein solution in the reaction tube. Vortex the mixture gently until thoroughly mixed. The remaining dye stock solution should be discarded.

7. Let the mixture react 1 hour at room temperature, protected from light.

8. During this time, prepare 2 spin columns: Gently tap the columns to insure that the dry gel has settled in the bottom of the spin column.

9. 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.

10. Allow at least 30 minutes at room temperature hydration time before using the columns

11. After the 1 hour labelling reaction is over, add 100 μ L of deionised water to one vial of Component 4 (hydroxylamine, stop solution). Transfer 10 μ L of this solution to the conjugation reaction. Vortex the mixture gently. Remaining hydroxylamine should be discarded.

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15. Spin the columns and wash tubes in a variable speed centrifuge at 750 \times g for 2 minutes 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.

16. 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.

17. Hold the columns up to the light. Transfer half of the labelling reaction mixture (up to but not more than 68 μ L) to the top of the gel of each column. Carefully dispense the sample directly onto the centre 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.

18. 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 \times g for 2 minutes. The purified protein conjugate (approx. 135 μ L total both columns) will collect in the bottom of the collection tubes. Discard the spin columns.

Degree of Labelling Determination

19. Dilute the purified conjugate into PBS or other suitable buffer and measure the absorbance in a cuvette with a 1 cm pathlength at both 280nm (A_{280}) and 498nm (A_{498}).

20. Calculate the protein concentration:

$$C_{\text{m, protein}} [\text{mg/mL}] = [A_{280} - (A_{498} \times 0.4)] \times \text{dilution factor} \times 0.71$$

21. Calculate the degree of labelling:

$$\text{dye per protein molecule} = (A_{498} \times \text{dilution factor} \times 2.4) / C_{\text{m, protein}}$$

Storage

22. Store the labelled protein at 4°C, protected from light. 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!