



NHS LABELLING KITS MANUAL

| BY FIDA BIOSYSTEMS

FIDA 1 PROTEIN LABELLING KITS

FL 480, ALC 480 AND ALC 640

Table 1. Labelling kit contents.

Reagent	Amount	Cap
Reactive dye: <ul style="list-style-type: none">• FAM NHS ester (FL 480 Kit)• AF 488 NHS ester (ALC 480 Kit)• AF 647 NHS ester (ALC 640 Kit)	3 x 100 µg	Green cap
Sodium bicarbonate	3 x 84 mg	White cap
DMSO	3 x 250 µL	Red cap
Resin for purification	5 mL	Transparent cap
Reaction tube	3 x 1 tube	Blue cap
Yellow wash tube	3 x 1 tube	
Collection tube	6 x 1 tube	
Spin columns	6 x 1	

Storage: 2 °C – 8 °C. **Do not freeze!**

Table 2. Properties of FL 480, ALC 480 and ALC 640 dyes.

Catalogue no.	Dye	Molecular weight (g/mol)	Ex/Em maxima (nm)	ϵ^*	Fluorescence quantum yield	CF260**	CF280**
410-003	FL 480	473.39	494/517	74,000	0.93	0.22	0.17
420-003	ALC 480	732.74	495/519	71,800	0.91	0.16	0.10
430-003	ALC 640	1,056.33	655/680	191,800	0.15	0.09	0.08

* Excitation coefficient in $\text{L mol}^{-1} \text{cm}^{-1}$

** Correction factor for absorption readings (A_{260}/A_{280}) at 260 nm or 280 nm.

For example, $A_{260, \text{actual}} = A_{260, \text{observed}} - (CF_{260} \times \lambda_{\text{max}})$

Introduction

The FL 480, ALC 480 and ALC 640 Fida 1 Protein Labelling Kits offer the ideal way to label proteins with the FL 480, ALC 480 or ALC 640 dyes for a FIDA experiment. The Fida 1 Protein Labelling Kits each hold everything that is required for 3 labelling reactions and purification of the formed conjugates. The reactive dyes are based on NHS coupling chemistry and are thus optimal for labelling primary amines (N-terminus and lysines). The ALC 480 and ALC 640 are not sensitive to changes in pH (4-9), whereas FL 480 exhibits pH-dependent fluorescence and is sensitive to binding related fluorescence changes.

Before starting the labelling reaction

Allow all reagents to reach room temperature. Thereafter the steps are:

01



Prepare 1 M Sodium Bicarbonate Buffer

To do so, add 1 mL of deionised water to the tube containing 84 mg of sodium bicarbonate (white cap) and dissolve completely by vortexing. Store the solution at 4°C and use within one week.

02



Prepare the Protein Solution

The standard labelling protocol requires 75 μ L of protein solution. Purified protein should be at a concentration of 1–5 mg/mL in buffer. For smaller volumes/concentrations the protocol should be adjusted. The buffer cannot contain ammonium ions or primary amines (NO Tris-HCl buffer, Ammonium acetate, Ammonium carbonate, Glycine or Histidine buffer etc.). The presence of low concentrations of sodium azide (≤ 3 mM) or thimerosal (≤ 1 mM) will not significantly affect the conjugation reaction. Glycerol concentration higher than 5 % will significantly hamper the conjugation reaction.

The presence of TCEP (tris(2-carboxyethyl)phosphine) or DTT (Dithiothreitol) in the labelling buffer have a negative impact on the degree of labelling.

TCEP and DTT should be removed prior to labelling.

If the protein is in an unsuitable buffer (e.g., Tris or glycine), the buffer should be replaced by dialysis against PBS, or other means of buffer exchange e.g., Size Exclusion Chromatography.



Calculations

Perform the following calculations before beginning the conjugation reaction outlined under the “Conjugation Reaction” paragraph.

The amount of reactive dye to be used for each reaction depends upon the concentration of protein to be labelled. In the labelling procedure, a small volume of 4 mg/mL reactive dye stock solution (prepared in step 1.3 re the Experimental Protocol below) is added to 75 μ L of protein solution. Calculate the volume of the 4 mg/mL reactive dye stock solution to be added (in step 1.4 re the Experimental Protocol below) using the following formula:

$$\mu\text{L of reactive dye stock solution} = \frac{\text{mg/mL protein} \times V \mu\text{L}}{\text{MW}_{\text{protein}} \times C \text{ mg/mL}} \times \text{MW} \times \text{MR}$$

- V is the volume of protein solution, V = 75 μ L
- MW is the molecular weight of the reactive dye,
 $\text{MW}_{\text{FL480}} = 473.39 \text{ gr/mol}$, $\text{MW}_{\text{ALC480}} = 732.74 \text{ gr/mol}$, $\text{MW}_{\text{ALC640}} = 1,056.33 \text{ gr/mol}$
- C is the concentration of the dye DMSO solution, C=4 mg/ml.
- For most whole IgGs, $\text{MW}_{\text{protein}} = 145,000 \text{ gr/mol}$
- MR is the molar ratio of dye to protein in the reaction mixture. For IgG labelling reactions, we recommend an MR of 5. For other proteins, the MR usually ranges from 5 to 10. You can choose to perform two reactions at different MRs to better ensure optimal labelling.

Experimental protocol

Conjugation reaction

- 1.1 Transfer 75 μ L of protein solution to a 500 μ L reaction tube (blue cap).
- 1.2 Add 7.5 μ L of freshly prepared 1 M sodium bicarbonate solution to the protein containing reaction tube (1/10 of the protein sample solution).
- 1.3 Prepare the 4 mg/mL reactive dye stock solution. Immediately before starting the reaction, add 25 μ L of DMSO (red cap) to the vial of reactive dye (green cap, 100 μ g dry dye). Pipet up and down to completely dissolve the contents of the vial.
- 1.4 Add the appropriate amount of dye (see calculations). Discard the remaining reactive dye stock solution.
- 1.5 The reaction mixture is incubated at room temperature for at least 30 minutes, protected from light.

Labelled protein purification

- 2.1 Shake the bottle with resin to homogenise the resin. Mix the resin by pipetting up and down with an appropriate pipette i.e., 1,000 μL and transfer 400 μL of the resin to the spin column.
- 2.2 Snap off the bottom stopper and place the spin column in a yellow wash tube.
- 2.3 Spin the column for 60 seconds at $0.8 \times 1,000$ RCF. NOTE! If you use a fixed angle microcentrifuge, put a mark at the top of the spin column to keep track of the position of the column. Discard the wash tube. Do not allow the resin bed to dry out. Process the sample (2.4 and 2.5) within the next few minutes.
- 2.4 Place the spin column in a collection tube (colourless eppendorf tube) careful (drop-wise) apply ≈ 75 μL of the sample directly to the centre of the resin bed without touching the resin bed or the sides of the column since this can reduce the efficiency of the purification.
- 2.5 Spin the column for 120 seconds at $0.8 \times 1,000$ RCF. Collect the labelled protein in the flow-through.

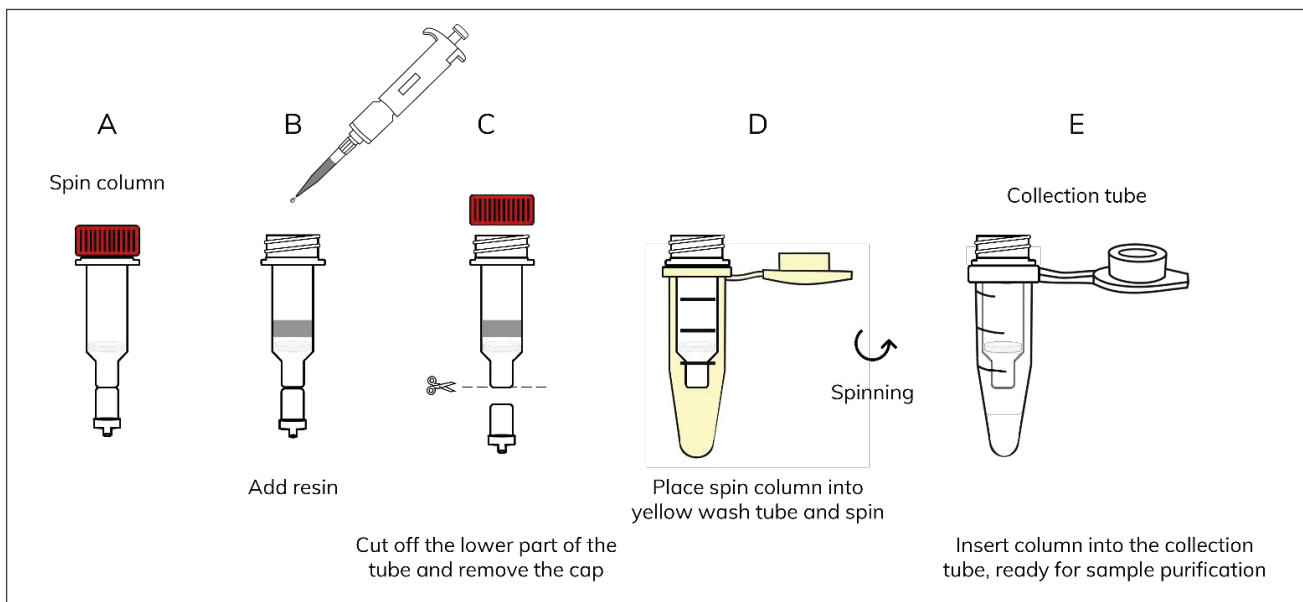


Figure 1. A. Spin column, B-D. Buffer removal from resin, C. Assembly of resin tube with collection tube.

Degree of Labelling (DOL) determination

Note: Before determining the Degree of Labelling (DOL) perform a test on the Fida 1 instrument to determine the degree of free fluorophore. If the degree of free fluorophore is high, then perform another dye removal (purification).

Measure the absorbance and the labelled protein at both 280 nm (A_{280}) and 495 nm (A_{495}). Calculate the protein concentration using this equation:

$$\frac{C_{protein} mg}{mL} = \frac{[A_{280} - (A_{495} \times CF_{280})] \times dilution\ factor}{\epsilon_{protein}} \times MW_{protein}$$

- CF_{280} is the correction factor of the dye at 280 nm, $CF_{280, FL480} = 0.17$, $CF_{280, ALC480} = 0.10$, $CF_{280, ALC640} = 0.08$
- $\epsilon_{protein}$ is the molar extinction coefficient of the protein at 280 nm
- The dilution factor corresponds to how much you diluted the protein before measuring the absorbance.

Calculate the Degree of Labelling with this equation:

$$Dye\ molecules\ per\ protein = \frac{A_{495} \times dilution\ factor}{\epsilon_{dye} \times C_{protein}} \times MW_{protein}$$

- ϵ_{dye} is the extinction molar coefficient of the conjugated dye,
 $\epsilon_{dye, FL480} = 74,000\ L\ mol^{-1}\ cm^{-1}$
 $\epsilon_{dye, ALC480} = 71,800\ L\ mol^{-1}\ cm^{-1}$
 $\epsilon_{dye, ALC640} = 191,800\ L\ mol^{-1}\ cm^{-1}$

Storing the conjugates

Store the conjugates at 2°C – 8°C, protected from light. If the final concentration of purified protein conjugate is less than 1 mg/mL, add BSA or other stabilizing protein at 1–10 mg/mL. In the presence of 2 mM sodium azide, the conjugate should be stable at 2°C - 8°C for several months. For longer storage, divide the conjugate into small aliquots and freeze at $\leq -20^{\circ}C$. Avoid repeated freezing and thawing. Protect from light.

Products

Catalogue no.	Product name
410-003	FL 480 Fida 1 Protein Labelling Kit (3 reactions, 100 µg each)
420-003	ALC 480 Fida 1 Protein Labelling Kit (3 reactions, 100 µg each)
430-003	ALC 640 Fida 1 Protein Labelling Kit (3 reactions, 100 µg each)

Safety Data Sheets (SDS)

- FAM NHS ester, 6-isomer
- AF 488 NHS ester
- AF 640 NHS ester
- DMSO
- Sodium Bicarbonate
- Resin

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