Standard protein labelling procedure for Fluidity One-W with fluorescent dye using Alexa Fluor™ 488 NHS Ester

Aim:

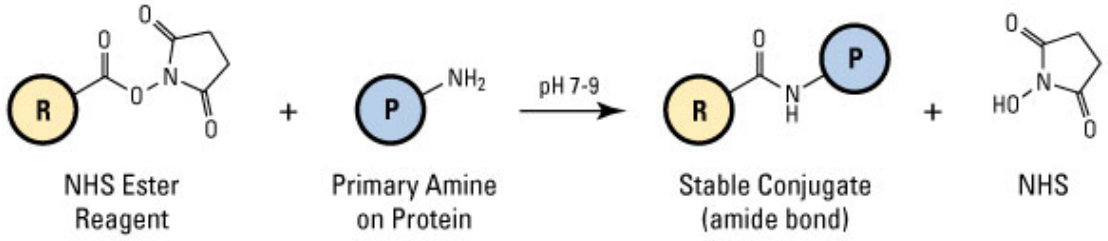
Covalently label proteins with fluorescent dyes which are optically compatible with the Fluidity One-W in a reliable and reproducible manner.

Apparatus and Materials:

- Zeba™ Desalting Chromatography Cartridges, 7K MWCO, 1.0 mL
  ThermoFisher Scientific, catalog No. 89934
- Anhydrous dimethylsulfoxide (DMSO)
- Reaction Buffer - 0.2 M $NaHCO_3$ at pH 8.2 (pH adjusted with HCl), filter sterilized (0.22 µm)
- Alexa Fluor™ 488 NHS ester fluorescent dye
  ThermoFisher Scientific, catalog No. A20100
- PBS buffer - 10 mM phosphate buffer at pH 7.4, 2.7 mM KCl and 137 mM NaCl
  Sigma-Aldrich P4417-100TAB
- UV-VIS spectrophotometer (Nanodrop™)
- Pipette and tips - 1 mL, 200 µL, and 10 µL
- Eppendorf tubes®

Reaction:

This procedure is for dyes containing NHS ester as a reactive group, which conjugate the dye to the primary amines on proteins (*i.e.*, N-terminus and lysine)



NHS ester reaction scheme for chemical conjugation to a primary amine. R represents a labeling reagent or one end of a crosslinker having the NHS ester reactive group; P represents a protein or other molecule that contains the target functional group (*i.e.*, primary amine).[1]

Method - Preparation of dye solution:

- Reconstitute 1 mg of Alexa Fluor™ 488 NHS ester dye with 155.4 µL of anhydrous DMSO creating a 10 mM solution of Alexa Fluor™ in DMSO.

  Optional: separate reconstituted dye into 20 µL aliquots and store at -20 °C.

Method - Protein labelling:

1. The recommended concentration range of protein in the labelling reaction is 0.1 mg/mL - 5.0 mg/mL.
2. Transfer the protein into the reaction buffer by buffer exchange, dilution or direct reconstitution.

1. https://www.thermofisher.com/uk/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/amine-reactive-crosslinker-chemistry.html

welcome@fluidic.com
www.fluidic.com

IMPORTANT: Any form of primary amines (e.g., free amino acids, Tris, urea, etc.) will react with the dye and decrease the protein-labelling efficiency. In case the storage buffer of the protein contains primary amines, it is essential to exchange buffers by desalting column, tangential-flow filtration, repeated dialysis or a similar method.

3. Determine the absorbance of the protein solution at 280 nm, $A_{280\ nm}$, using a UV-VIS spectrophotometer (e.g., Nanodrop).
4. Determine the amount of protein that requires labelling (by volume or mass). Based on that calculate the volume of dye solution required (3:1 ratio of dye to protein is recommended).
   Note: worked example of the calculations at the back of this guide.
   Volume of dye solution should not exceed 1.8 % of the total volume of the reaction mixture as high DMSO concentrations might interfere with the structural integrity of the protein.
6. To reduce the concentration of DMSO in the labelling mixture, dilute the required amount of protein stock solution into the reaction buffer. If addition of buffer is not required, directly add the dye solution to the protein solution in a stepwise manner (steps of 20 µL) mixing slowly by pipetting up and down after each step (do not vortex).
7. Incubate the reaction mixture at 4 °C overnight or at room temperature for 1 hour (depending on the stability of the protein).

Method - Purification:

1. Remove the dye using Zeba™ Desalting Chromatography Cartridges, 7K MWCO, 1 mL or similar desalting columns.

1a. Equilibrate the desalting columns with 5 column volumes (CV) of reaction buffer.

1b. Load 200 µL of labelling mixture on the column.

1c. Elute sample with 1 mL of reaction buffer.

1d. For elution, collect 10 x 100 µL fractions into clean 1.5 mL Eppendorf tubes®.

2. To determine labelling efficiency and purity; use a UV-VIS spectrophotometer and measure the absorbance of each fraction at 280 nm for the protein and 495 nm for Alexa Fluor™ 488 to determine which fractions contain the labelled protein without any free dye. For example, a Nanodrop™ can be used to determine labelling efficiency using the “Proteins & Labels” dialog.
3. Alternatively use the Fluidity One-W to test the purity of the sample where the approximate molecular weight of the protein is known. If the results given for the molecular weight 30% lower than expected, there is likely an excess of free dye remaining in the sample. Repeat purification until the results given by the Fluidity One-W do not change between purifications.

Troubleshooting:

When experimental molecular weight does not agree with nominal value:

A) size is too small

- contamination by free label
  solution: repeat purification
- contamination by lower molecular weight protein contaminants or protein degradation
  solution: run SDS-PAGE to assess protein purity

B) size is too large

- contamination by higher molecular weight protein contaminants
  solution: run SDS-PAGE to assess protein purity.
- contamination by soluble higher molecular weight oligomers/aggregates
  solution: analyse size-distribution of sample components by DLS or size-exclusion chromatography.

Additional Information:

- Ideally reconstitute dye before use.
- Do not use reconstituted dye older than 1 year.
- Make sure reconstituted dye that was stored at -20 °C is allowed to equilibirate to room temperature before opening to avoid exposure to condensation water.
- Resolution of desalting spin-columns is not high enough to properly separate protein from free dye.
- When checking the purification fractions by Nanodrop™: it will give a value for protein mass concentration as well as a molar concentration for the dye. Divide the mass concentration of the protein by its molecular weight to give you the molar concentration of the protein. Compare the absorbance values between the dye and the protein. A dye-to-protein ratio of 3:1 in the reaction mixture will usually result in dye-to-protein ratios close to 1:1 after purification.
- Higher ratio of dye to protein than expected might be caused by unbound dye which requires additional sample purification.
- Lower ratio of dye to protein than expected might indicate the presence of unlabelled protein, this could have been caused by protein aggregation, for example.

Calculation to determine mass concentration using absorbance from UV-VIS spectrophotometer:

$$c_m = \frac{A_{280\ nm}}{\varepsilon_{0.1\ \%}}$$

c_m ; mass concentration
$A_{280\ nm}$; absorbance
$\varepsilon_{0.1\ \%}$; mass-concentration extinction coefficient

Worked example:

This is an example calculation, please insert your own values when working out for your protein.

- concentration of protein in solution - 1000 µg/mL
- molecular weight of protein being labelled - 40 kDa
- molar concentration of dye in DMSO - 10 mM
- mass of protein to be labelled - 1000 µg
- molecular weight of Alexa Fluor™ 488 NHS ester - 643.4 g/mol

Calculating volume of protein solution:

$$V = \frac{m}{c_m} \qquad 1\ mL = \frac{1000\ \mu g}{1000\ \mu g/mL}$$

Calculating amount of protein:

$$n = \frac{m}{MW} \qquad 25\ nmol = \frac{1000\ \mu g}{40\ kDa}$$

Use a ratio of 3 parts dye to 1 part protein to determine amount of dye required:

$$n_{dye} = n_{pro} \times 3 \qquad 75\ nmol = 25\ nmol \times 3$$

Calculate volume of dye (Alexa Fluor™ 488 NHS ester) required:

$$V = \frac{n}{c_{mol}} \qquad 7.5\ \mu L = \frac{75\ nmol}{10\ mM}$$

Calculate percent of DMSO in reaction mixture:

$$\% = \frac{V_{DMSO}}{V_{tot}} \times 100 \qquad 0.74\ \% = \frac{7.5\ \mu L}{1000\ \mu L} \times 100$$