

Fluorophore Conjugation of Antibodies (Lamond Lab)

(here Alexa conjugation of IgG antibodies)

Materials:

Fluorophores: Alexa Fluor 488 / Alexa Fluor 546 (succinimidyl esters), Molecular Probes, Cat.No. A-20000 / A-20002, dissolved in anhydrous DMSO;

Phosphate-buffered saline (PBS);
Sodium bicarbonate, 1M;

PD-10 column, Sephadex G-25M, AmershamPharmacia, Cat.No. 17-0851-01;
Bio-Spin 6 columns, BioRad, Cat.No. 732-6002

Protein Preparation:

Antibodies in crude serum will not label well. IgG can be affinity purified by using Protein A or Protein G. Purified IgG should be in a buffer free of ammonium ions or primary amines. Replace unsuitable buffer (e.g. Tris or glycine) with PBS by dialysis or another method. The presence of low concentrations ($\leq 3\text{mM}$) of sodium azide will not interfere with the conjugation reaction.

Labeling Reaction:

To 1 ml of a 2 mg/ml IgG solution (in 2 ml Eppendorf tube), add 100 μl of 1M sodium bicarbonate (bicarbonate is added to raise the pH of the reaction mixture, since succinimidyl esters react efficiently at pH 7.5-8.5). Add appropriate amount (\rightarrow test reactions) of reactive Alexa dye, mix the reaction by inverting the tube a few times and incubate the reaction mixture on a rotating wheel for 1 hour at room temperature followed by an overnight incubation at 4 °C.

Test Reactions:

When first conjugating an antibody to Alexa Fluor 488 or Alexa Fluor 546, a range of Alexa dye to antibody concentrations should be compared. For a first-time titration of Alexa dye we recommend a range of 10 to 200 μg Alexa dye per mg antibody. Compare each conjugate by cell staining. Select the conjugate with the brightest “positive” cells which still has low background on “negative” cells.

Purification of the conjugated Antibody:

Non-conjugated Alexa dye can be removed by gel filtration using a PD-10 column (Sephadex G-25M). Elute the conjugated antibody with PBS (optionally with 0.01% sodium azide). For the test reactions Bio-Spin 6 columns can be used.

Characterizing the Conjugate:

Alexa 488:

Concentration of antibody-Alexa dye conjugate (M):

$$c = \frac{[A_{280} - (A_{494} \times 0.11)] \times dil}{203,000}$$

dil = dilution factor

0.11 = correction factor to account for absorption of the dye at 280 nm

203,000 = molar extinction coefficient of IgG

Degree of labeling (moles dye per mole protein):

$$d = \frac{A_{494} \times dil}{71,000 \times c}$$

71,000 = approximate molar extinction coefficient of the dye at 494nm

Alexa 456:

Concentration of antibody-Alexa dye conjugate (M):

$$c = \frac{[A_{280} - (A_{558} \times 0.12)] \times dil}{203,000}$$

dil = dilution factor

0.12 = correction factor to account for absorption of the dye at 280 nm

203,000 = molar extinction coefficient of IgG

Degree of labeling (moles dye per mole protein):

$$d = \frac{A_{558} \times dil}{104,000 \times c}$$

104,000 = approximate molar extinction coefficient of the dye at 558nm

For IgGs optimal labeling is achieved with 4-9 moles of Alexa Fluor 488 dye per mole of antibody or 2-7 moles of Alexa 546 per mole of antibody, respectively.

Storage:

Store the labeled antibody at 4 °C, protected from light.