Labelling Antibodies with FITC

Please note – this protocol has been optimised for certain conditions and is meant as a guide only, you may need to alter some parameters to suit your own experiment

1. Dialyse purified monoclonal antibody (ideally 1 - 2 mg/ml) against carbonate/bicarbonate buffer at 4°C. Dialyse in a volume of 1-2 litres for 5 ml of antibody, with 1 change of buffer over 1 - 2 days. This will raise the pH to 9.5 and remove free $NH4^+$ ions and azide. Note: antibodies more dilute than 1mg/ml will show sub-optimal labelling.
2. Determine antibody concentration after dialysis

   (concentration antibody in mg/ml = A_{280} x 0.74 x dilution factor)
3. Remove FITC from fridge and **allow to reach room temperature prior to opening** (approx 30 mins). Note: FITC should be stored *desiccated* at 4°C
4. Dissolve FITC to 1mg/ml in anhydrous DMSO (this must be done just prior to labelling, as dissolved FITC can't be stored). For each mg of antibody, need 75 μg of FITC. As this requires weighing very small amounts of FITC, it is best to weigh an empty 5 ml glass bottle (NOT plastic), add a tiny amount of FITC to the tube, then weigh again and calculate the amount of FITC added. Then make up in the appropriate volume of DMSO.
5. Add 75 μl of FITC solution for every 1 mg of antibody. FITC solution should be added gradually, eg in 10 μl aliquots, while mixing continuously. Wrap tubes in alfoil and mix on a rotator for 2 hours at room temperature.
6. Remove unbound FITC by one of the following methods:

   (A) Size-exclusion chromatography on a Sephadex G-25 column (eg pre-prepared 'PD-10' column from Pharmacia Biotech)

   - Equilibrate column with 5 ml of PBS/1% BSA (to block non-specific protein-binding sites), and then wash with 20 ml of PBS. Do not allow to dry out

   - Allow the level of PBS to fall to the top of the gel bed, then add labelled antibody in a volume of 2 ml - 2.5 ml (if presently at less than 2 ml, first dilute in carbonate/bicarbonate buffer).

   - Begin collecting 0.5-1 ml fractions, then when the level of liquid falls to the top of the gel bed, elute with 5 ml of PBS. Note: If the column is placed against a white background, should be able to see two yellow bands, one corresponding to the labelled antibody, the other to unbound FITC. The first band to come off is the labelled antibody, and this should come off in about the 2^{nd} and 3^{rd} mls off the column.

   - Determine A_{280} of the first 8 fractions collected (assuming 0.5 ml fractions were collected) and pool those with high readings

   - The column can be stored for re-use by washing thoroughly in PBS/0.01% azide.

or...

(B) Dialyse against 2 L of PBS at 4°C with 1-2 changes over 2 days. This method takes longer, but will result in less dilution and/or loss of antibody than column

7. Dilute a sample of labelled antibody appropriately (A_{280} should be below 2.0) and determine A_{280} and A_{492}.
8. The concentration of labelled antibody is calculated by:

$$\text{mg/ml} = A_{280} - \frac{(A_{492} \times 0.35)}{1.4}$$

(where 1.4 is the reciprocal of the FITC-conjugated antibody molar coefficient)

9. To determine the FITC:antibody ratio, must first calculate the moles of antibody and FITC:

$$\text{moles antibody} = \frac{\text{mg/ml labelled antibody}}{1.5 \times 10^5}$$

$$\text{moles FITC} = \frac{A_{492}}{0.69 \times 10^5}$$

10. The FITC:antibody ratio can now be calculated by :

$$\text{FITC:antibody} = \frac{\text{moles of FITC}}{\text{moles of antibody}}$$

(for flow cytometry, a ratio of 5:1 - 6:1 is usually optimal)

11. Add sodium azide to 0.04% and store at 4°C for at least 1 year, protected from light. Ideally, antibodies should be stored at at least 1 mg/ml; if they are significantly more dilute than this, add BSA to 1%.

    Note: the formation of aggregates can increase background staining by allowing binding to Fc receptors. Aggregates can be removed by ultracentrifugation (eg 15 mins at 100 000 x *g*)

Carbonate/bicarbonate buffer:

	(to make 2 L)	*(to make 4 L)*
Na_2CO_3	17.2 g	34.4 g
$NaHCO_3$	34.4 g	68.8 g
in distilled water		

Weigh out solids, dissolve in approx ¾ of the final volume of distilled water. When dissolved, pH to 9.5 (will probably need at least 10 NaOH pellets) then make up to final volume in distilled water.