Labeling Proteins (Antibodies) with Amine-Reactive Dyes (NHS Esters)

NHS-esters readily react with amino groups of proteins. The optimum pH range for NHS-ester coupling is pH 8.0 – 9.0. At this pH amino groups of proteins, i.e. the e-amino groups of lysines are to a high degree unprotonated and highly reactive towards dye-NHS-ester.

Required Materials

• Solution A: PBS buffer (Phosphate-Buffered Saline, pH 7.4): Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2H_2O$, and 0.24 g KH_2PO_4, in 1 liter distilled water.

• Solution B : 0.2 M sodium bicarbonate solution, adjusted to pH 9.0 with 2 M sodium hydroxide.

• Solution C : To 20 parts of Solution A add 1 part of Solution B to obtain a labeling buffer of pH 8.3. Kept in an air-tight bottle, this solution will be stable for a long period of time.

• Solution D: Dissolve 1.0 mg of dye NHS-ester in 50 – 200 µl of anhydrous, amine-free DMSO or DMF. Due to the high quality of NHS-esters such solutions are stable for a long period of time (freeze and protect from light when not in use). However, it may be difficult to avoid humidity entering a solution in continuous use. In the presence of water NHS-esters readily hydrolyze and become non-reactive. Hence we advise to freshly prepare, whenever possible, the dye NHS-ester solution immediately before starting the labeling reaction.

• Gel filtration column filled with Sephadex G-25 or equivalent.

Conjugate Preparation

• Dissolve 1 – 5 mg of protein in 1 ml of Solution C. Protein solutions must be free of any amine-containing substances such as tris-(hydroxymethyl)-aminomethane (TRIS), free amino acids or ammonium ions. Antibodies that are dissolved in amine containing buffers should be dialyzed against Solution A, and the desired coupling pH of 8.3 will be obtained by the procedure given above for Solution C. The presence of sodium azide in low concentration (< 3 mM) will not interfere with the labeling reaction.

• To obtain a degree of labeling (DOL, dye-to-protein ratio) of 2 – 3 add, while gently shaking, a threefold molar excess of reactive dye (Solution D) to the protein solution. Variations due to different reactivities of both the protein and the labeling reagent may occur. This may necessitate optimization of the dye-to-protein ratio used in the reaction

in order to obtain the desired DOL. To increase the degree of labeling a higher ratio of NHS-ester to protein has to be used and vice versa.

• Incubate the reaction mixture protected from light for up to 1 hour at room temperature. In most cases the labeling reaction will be complete within 5 – 10 minutes.

Conjugate Purification – Removal of Unbound Dye

• Due to an unavoidable side reaction part of the applied dye NHS-ester will hydrolyze during the labeling reaction and must be removed from the protein conjugate. We recommend using a Sephadex G-25 (or equivalent) gel filtration column (1 – 2 cm diameter and 10 – 20 cm length; for very hydrophilic dyes a 30 cm column is preferable) for separation of dye-protein conjugate from free dye.

• Preequilibrate the column with Solution A.

• Elute the dye-protein conjugate using Solution A.

• The first colored and fluorescent zone to elute will be the desired dye-protein conjugate. A second colored and fluorescent, but slower moving zone contains the unbound free dye (hydrolyzed NHS-ester).

• To prevent denaturation of the conjugate after elution, bovine serum albumin (BSA) or another stabilizer may be added.