Fluorescent Dye 647-I Maleimide

Catalog ID: U0249

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This protocol only provides a guideline, and should be modified according to your specific needs.

1. Prepare protein stock solution (Solution A):
    1. (Optional) if your protein does not contain a free cysteine, you must treat your protein with DTT or TCEP to generate a thiol group. 10 molar equivalents of DTT or TCEP are sufficient for converting a disulfide bond to two free thiol groups. If DTT is used you must remove free DTT by dialysis or gel filtration before conjugating a dye maleimide to your protein. Following is a sample protocol for generating a free thiol group:

        a. Prepare a fresh solution of 1 M DTT (15.4 mg/100 µl) in distilled water.
        b. Make IgG solution in 20 mM DTT: add 20 µl of DTT stock per ml of IgG solution while mixing. Let stand at room temp for 30 minutes without additional mixing (to minimize reoxidation of cysteines to cystines).
        c. Pass the reduced IgG over a filtration column pre-equilibrated with "Exchange Buffer". Collect 0.25 ml fractions off the column.
        d. Determine the protein concentrations and pool the fractions with the majority of the IgG. This can be done either spectrophotometrically or colorimetrically.
        e. Carry out the conjugation as soon as possible after this step (see below).

        Note 1: IgG solutions should be >4 mg/ml for the best results. The antibody should be concentrated if less than 2 mg/ml. Include an extra 10% for losses on the buffer exchange column.
        Note 2: The reduction can be carried out in almost any buffers from pH 6 to 7, e.g., MES, phosphate or TRIS buffers.
        Note 3: Steps c and d can be replaced by dialysis.

    2. Mix 100 µL of a reaction buffer (e.g., 100 mM MES buffer with pH ~6.0) with 900 µL of the target protein solution (e.g. antibody, protein concentration >2 mg/ml if possible) to give 1 mL protein labeling stock solution.
        Note 1: The pH of the protein solution (Solution A) should be 6.5 ± 0.5.

Note 2: Impure antibodies or antibodies stabilized with bovine serum albumin (BSA) or other proteins will not be labeled well.

Note 3: The conjugation efficiency is significantly reduced if the protein concentration is less than 2 mg/mL. For optimal labeling efficiency the final protein concentration range of 2-10 mg/mL is recommended.

2. Prepare dye stock solution (Solution B):

   Add anhydrous DMSO into the vial of dye to make a 10-20 mM stock solution. Mix well by pipetting or vortex under subdued light (if possible).

   Note: Prepare the dye stock solution (Solution B) before starting the conjugation. Use promptly. Extended storage of the dye stock solution may reduce the dye activity. Solution B can be stored in freezer for up to 4 weeks when kept from light and moisture. Avoid freeze-thaw cycles.

3. Determine the optimal dye/protein ratio (optional):

   Note: Each protein requires distinct dye/protein ratio, which also depends on the properties of dyes. Over labeling of a protein could detrimentally affects its binding affinity while the protein conjugates of low dye/protein ratio gives reduced sensitivity. We recommend you experimentally determine the best dye/protein ratio by repeating Steps 4 and 5 using a serial different amount of labeling dye solutions. In general 4-6 dyes/protein are recommended for most of dye-protein conjugates.

   1. Use 10:1 molar ratio of Solution B (dye)/Solution A (protein) as the starting point: Add 5 µl of the dye stock solution (Solution B, assuming the dye stock solution is 10 mM) into the vial of the protein solution (95 µl of Solution A) with effective shaking. The concentration of the protein is ~0.05 mM assuming the protein concentration is 10 mg/mL and the molecular weight of the protein is ~200KD.

      Note: The concentration of the DMSO in the protein solution should be <10%.
   2. Run conjugation reaction (see Step 4 below).
   3. Repeat #3.2 with the molar ratios of Solution B/Solution A at 5:1; 15:1 and 20:1 respectively.
   4. Purify the desired conjugates using premade spin columns.
   5. Calculate the dye/protein ratio (DOS) for the above 4 conjugates (see next page).
   6. Run your functional tests of the above 4 conjugates to determine the best dye/protein ratio to scale up your labeling reaction.

4. Run conjugation reaction:

1. Add the appropriate amount of dye stock solution (Solution B) into the vial of the protein solution (Solution A) with effective shaking.

   Note: The best molar ratio of Solution B/Solution is determined from Step 3.6. If Step 3 is skipped, we recommend to use 10:1 molar ratio of Solution B (dye)/Solution A (protein).
2. Continue to rotate or shake the reaction mixture at room temperature for 30-60 minutes.

5. Purify the conjugation:

   The following protocol is an example of dye-protein conjugate purification by using a Sephadex G-25 column.

   1. Prepare Sephadex G-25 column according to the manufacture instruction.
   2. Load the reaction mixture (directly from Step 4) to the top of the Sephadex G-25 column.
   3. Add PBS (pH 7.2-7.4) as soon as the sample runs just below the top resin surface.
   4. Add more PBS (pH 7.2-7.4) to the desired sample to complete the column purification. Combine the fractions that contain the desired dye-protein conjugate.

      Note 1: For immediate use, the dye-protein conjugate need be diluted with staining buffer, and aliquoted for multiple uses. Note 2: For longer term storage, dye-protein conjugate solution need be concentrated or freeze dried.