# Instructions for the use of Mal-$(PEG)_n$-NHS Ester

**Introduction**

The BroadPharm Mal-$(PEG)_n$-NHS Ester is a water-soluble heterobifunctionalcrosslinkers that contains a*N*-hydroxysuccinimide (NHS) ester and a maleimide group that allows covalent conjugation of amine- and sulfhydryl-containing molecules. NHS esters react with primary amines at pH 7-9 to form amide bonds, while maleimides react with sulfhydryl groups at pH 6.5-7.5 to form stable thioether bonds. In aqueous solutions, NHS ester hydrolytic degradation is a competing reaction whose rate increases with pH. The maleimide group is more stable than the NHS-ester group but will slowly hydrolyze and loses its reaction specificity for sulfhydryls at pH values > 7.5. For these reasons, conjugations with these crosslinkers are usually performed at pH 7.2-7.5, with the NHS-ester (amine-targeted) reacted before or simultaneous with the maleimide (sulfhydryl-targeted) reaction. Mal-$(PEG)_n$-NHS Esters are often used to prepare antibody-enzyme and hapten-carrier protein conjugates in a two-step reaction scheme. First, the amine-containing protein is reacted with a several-fold molar excess of the crosslinker, followed by removal of excess non-reacted reagent by desalting or dialysis; finally, the sulfhydryl-containing molecule is added to react with the maleimide groups already attached to the first protein. Mal-(PEG)n-NHS Ester is soluble in water and many other aqueous buffers to approximately 10mM, although solubility decreases with increasing salt concentration. Mal-(PEG)n-NHS Ester is not directly water-soluble and must be dissolved in an organic solvent such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF); subsequent dilution into aqueous reaction buffer is generally possible, and most protein reactants will remain soluble if the final concentration of organic solvent is less than 10%.

**Important Product Information**

• Mal-$(PEG)_n$-NHS esters are moisture-sensitive. Store the vial of reagent at -20°C with desiccant. . Equilibrate vial to room temperature before opening to avoid moisture condensation inside the container. Dissolve needed amount of reagent and use it immediately before hydrolysis occurs. Discard any unused reconstituted reagent. Do not store reagent in solution.

**Note:** Do not use phosphate-buffered saline (PBS) for initial dissolution of Mal-$(PEG)_n$-NHS esters; the reagent does not dissolve well in buffers exceeding 50mM total salts. However, once dissolved, the solution can be further diluted in PBS or other non-amine buffers.

• Avoid buffers containing primary amines (e.g., Tris or glycine) and sulfhydryls during conjugation, because they will compete with the intended reaction. If necessary, dialyze or desalt samples into an appropriate buffer such as phosphate-buffered saline (PBS).

• Molecules to be reacted with the maleimide moiety must have free (reduced) sulfhydryls. Reduce peptide disulfide bonds with **Procedure for Two-step Protein Crosslinking**

Generally, a 10- to 50-fold molar excess of crosslinker over the amount of amine-containing protein results in sufficient maleimide activation to enable several sulfhydryl-containing proteins to be conjugated to each amine-containing protein. More dilute protein solutions require greater fold molar excess of

reagent to achieve the same activation level. Empirical testing is necessary to determine optimal activation levels and final conjugation ratios for the intended application.

**A. Material Preparation**

• Conjugation Buffer (see Important Product Information) – adding EDTA to 1-5mM helps to chelate divalent metals, thereby reducing disulfide formation in the sulfhydryl-containing protein
• Desalting column to separate modified protein from excess crosslinker and reaction byproducts (e.g., Zeba® Spin Desalting Columns)
• Amine-containing (Protein-$NH_2$) and sulfhydryl-containing proteins (Protein-SH) to be conjugated

**B. Protocol**

**Note:** For best results, ensure that Protein-SH is prepared and ready to combine with Protein-$NH_2$ in step 5.

1. Prepare Protein-$NH_2$ in Conjugation Buffer.
2. Add the appropriate amount of crosslinker to the protein solution. The concentration of the Protein-$NH_2$ determines the crosslinker molar excess to use. Suggested crosslinker molar excesses are as follows:

• Protein samples < 1 mg/mL use 40-80-fold molar excess.

• Protein samples of 1-4 mg/mL use 20-fold molar excess.

• Protein samples of 5-10 mg/mL use 5- to 10-fold molar excess

3. Incubate reaction mixture for 30 minutes at room temperature or 2 hours at 4°C.
4. Remove excess crosslinker using a desalting column equilibrated with Conjugation Buffer.
5. Combine and mix Protein-SH and desalted Protein-$NH_2$ in a molar ratio corresponding to that desired for the final conjugate and consistent with the relative number of sulfhydryl and activated amines that exist on the two proteins.
6. Incubate the reaction mixture at room temperature for 30 minutes or 2 hours at 4°C.

**Note:** Generally, there is no harm in allowing the reaction to proceed for several hours or overnight, although usually the reaction will be complete in the specified time. To stop the conjugation reaction before completion, add buffer containing reduced cysteine at a concentration several times greater than the sulfhydryls of Protein-SH.

**Note:** Conjugation efficiency can be estimated by electrophoresis separation and subsequent protein staining.