

## Protocol: NHS Ester Labeling of Amino-Biomolecules

**NHS (N-HydroxySuccinimide) esters** and other activated esters (**sulfo-NHS**, **sulfotetrafluorophenyl - STP**) are highly reactive compounds suitable for the modification of amino groups. NHS is most common type of activated esters.

Usual modifications are fluorescent labels, fluorescence quenchers, and other reporter groups. Alkyne and azido group can be attached using activated esters to adapt biomolecules to Click Chemistry. Since amino groups are nearly always contained in proteins and peptides, modification of these biopolymers is especially common. Other examples are amino-oligonucleotides, amino-modified DNA, and amino-containing sugars.

The reaction of NHS esters with amines is strongly pH-dependent: at low pH, the amino group is protonated, and no modification takes place. At higher-than-optimal pH, hydrolysis of NHS ester is quick, and modification yield diminishes. Optimal pH value for modification is 8.3-8.5.

Water is most common solvent for the labeling. If NHS ester is poorly soluble, it can be added as a solution in DMSO or DMF to a solution of protein in water, adjusted to pH 8.3-8.5. Note that DMF must not contain amines (and thus should have no odor).

We recommend using the following general protocol for the labeling of biomolecules with NHS esters produced by Lumiprobe LLC. The auxiliary reagents can be ordered at Lumiprobe LLC.

1. Calculate required amount of NHS ester:

$$\text{NHS\_ester\_weight [mg]} = 8 \times \text{amino\_compound\_weight [mg]} \times \text{NHS\_ester\_molar\_weight [Da]} / \text{amino\_compound\_molar\_weight [Da]}.$$

8 is molar excess of NHS ester. It is experimental value for mono-labeling, suitable for many common proteins and peptides. However, in some cases using less or more NHS ester is required. It depends on protein structure.

For example, to label 3 mg of insulin (molar weight 69300 Dalton) with Cy5 NHS ester (molar weight 592 Dalton), and obtain maximum yield of mono-labeled product, you should use  $10 \times 3 \text{ mg} \times 592 \text{ Da} / 69300 \text{ Da} = 0.26 \text{ mg}$  of Cy5 dye NHS ester.

The molar weights of NHS esters produced by Lumiprobe are the following:

Cy3 NHS ester	566 Da
Cy3.5 NHS ester	708 Da
Cy5 NHS ester	592 Da
Cy5.5 NHS ester	723 Da
Alkyne STP ester	352 Da
Azide NHS ester	254 Da

Please note that molar weights of reagents produced by other vendors may vary.

2. Determine volume of reaction mixture. The labeling can be performed on any scale from nanomols to dozens of grams. When the scale is low, use minimal volume (10-20 uL). Higher concentrations (1-10 mg of amino-biomolecule per mL of mixture) is optimal.
3. Dissolve NHS ester in 1/10 reaction volume of DMF or DMSO. Amine-free DMF is preferred solvent. After the reaction, NHS ester can be stored in solution for 1-2 months at -20°C.
4. Dissolve biomolecule in 9/10 reaction volume of buffer with pH 8.3-8.5. 0.1 M Sodium bicarbonate solution has appropriate pH. Other alternatives are 0.1 M Tris buffer (although Tris has amino group, it is hindered and does not react with NHS esters), or 0.1 M phosphate buffer. Note pH is most important thing. When doing large-scale labeling (hundreds of milligrams of NHS ester), note that the mixture tends to acidify with time because of hydrolysis of NHS ester. Monitor pH, or use more concentrated buffer then.
5. Add NHS ester solution to the solution of biomolecule, and vortex well. Keep on ice overnight, or at room temperature during at least 4 hours.
6. Purify the conjugate using appropriate method: gel-filtration for macromolecules is most universal. Precipitation and chromatography is another alternative. Organic impurities (such as N-hydroxysuccinimide, NHS ester, acid produced by hydrolysis) are almost always easily separated.