**S-SS-4FB Cleavable Linker**

C$_{17}$H$_{18}$N$_2$O$_6$S$_2$; Mol. Wt.: 410.46

Cat. No.: S-1037-010

Storage: Desiccated: -15° to -25°C

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**Introduction**

SoluLINK bioconjugation technology is based on the formation of a stable covalent bond that has a UV-traceable signal to indicate the real-time formation of conjugate. S-HyNic (succinimidyl 6-hydrazinonicotinate acetone hydrazone) is used to incorporate aromatic hydrazine linkers on biomolecules. S-HyNic is an amine-reactive linker that directly converts amino groups on biomolecules and surfaces to HyNic groups. S-4FB (succinimidyl 4-formylbenzoate) is used to convert amino groups to aromatic aldehydes (4-formyl-benzamide, or 4FB groups). S-SS-4FB incorporates the same aromatic aldehyde functionality as S-4FB and has the additional feature of being cleavable through the disulfide bond incorporated into the linker. Cleavage of the disulfide occurs spontaneously upon cellular internalization of the conjugate due to the reducing environment encountered intracellularly. This linker may also be cleaved by the addition of a reducing agent such as TCEP, DTT, or 2-mercaptoethanol to release the two conjugated biomolecules. Addition of a HyNic-modified biomolecule to a 4FB-modified biomolecule or surface directly leads to the formation of the conjugate (Figure 1). The conjugate bond is stable to 92°C and pH 2.0-10.0. The recommended pH for biomolecule modification is 8.0, and conjugation is 6.0. Unlike maleimide-thiol based conjugation protocols where reducing agents are required to liberate thiols for attachment of the linker, S-SS-4FB is coupled to the biomolecule or surface through an NHS ester. The HyNic-4FB conjugation couple therefore leaves disulfide bonds intact until cleavage is accomplished after cellular internalization or after the addition of reducing agents to deliver a payload. No oxidants, reductants or metals are required in the preparation of conjugate.

Further enhancing the many advantages of the HyNic/4FB conjugation couple is the discovery by Dirksen et al. that showed aniline catalyzes the formation of this Schiff’s base. This is especially effective for large biomolecule conjugations. In the case of antibody-protein conjugations the addition of 10 mM TurboLINK Catalyst Buffer (10X) (aniline) to the reaction converts >95% of the antibody to conjugate in ~2 hours using 1 – 2 mole equivalents of the second protein.

The HyNic-4FB conjugation couple is chromophoric - the conjugate bond absorbs at 354 nm and has a molar extinction coefficient of 29,000 L/(mol*cm). This allows (1) real time spectrophotometric monitoring of a conjugate reaction, (2) ability to ‘visualize’ the conjugate during chromatographic purification using a UV or photodiode array detector and (3) quantification of conjugation. Furthermore, the level of incorporation of 4FB groups can be quantified colorimetrically as reaction with 2-hydrazinopyridine dihydrochloride yields a chromophoric product that absorbs at 350 nm with a molar extinction coefficient of 24,500 L/(mol*cm).

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**Additional materials required**

**Reagents**

- Zeba™ Desalting Columns
- Modification Buffer (10X)
- Conjugation Buffer (10X)
- Anhydrous DMF
- 2-hydrazinopyridine dihydrochloride

**Equipment**

- Variable-speed bench-top microcentrifuge
- Spectrophotometer or Plate Reader
- 1.5 mL microcentrifuge tubes

**Modification Procedure**

A. **Desalting**

1. Desalt/buffer exchange the protein or oligonucleotide into 1X Modification Buffer (100 mM sodium phosphate, 150 mM sodium chloride, pH 8.0). If needed, refer to the Protein Desalting Protocol or Oligonucleotide Desalting Protocol.

**Notes:**

a) It is necessary to remove all free amine-containing contaminants, e.g., tris or glycine, from the protein or oligo solution before modification.

Continued on next page.
F. Quantifying modification level

1. The molar substitution ratio (MSR) can be determined using a colorimetric reaction outlined in Figure 2. Addition of 2-hydrazinopyridine to a S-SS-4FB-modified biomolecules yields a bis-aryl hydrazone that absorbs at 350 nm. Refer to the 4FB-Protein MSR Calculator or the 4FB-Oligonucleotide MSR Calculator as well as the protocol that is appropriate for your lab equipment: 4FB Protein MSR Instructions or 4FB Oligo MSR Instructions.

![Figure 2: Colorimetric reaction used to quantify the number of S-SS-4FB linkers on a biomolecule.](image)

2. The biomolecule is now modified with S-SS-4FB and ready for conjugation to HyNic-modified biomolecules and surfaces.

**Application Notes**
- Performing a Bradford assay
- Performing a BCA protein assay
- Troubleshooting Guide

b) High-level buffering capacity, i.e. 100 mM phosphate, is necessary for successful modification.

c) For desalting proteins, Zeba Desalting Columns are recommended. For oligonucleotides, Sartorius Vivaspin™ or Millipore Amicon™ diafiltration units are recommended. Refer to the desalting protein protocol.

B. Determine the concentration of the desalted protein

1. Determine the concentration of the protein to be modified using a spectrophotometer and the known E1% (280 nm). Alternatively, a Bradford assay or BCA assay can be used if the protein extinction coefficient is not known.
2. Adjust the concentration to 1.0 – 5.0 mg/mL in 1X Modification Buffer, pH 8.0, if necessary.

C. Prepare S-SS-4FB/DMF stock solution

1. Prepare a stock solution of S-SS-4FB in anhydrous DMF (or DMSO) by dissolving 2 – 4 mg of S-SS-4FB in 100 µL anhydrous DMF.

D. Modification of a protein or oligonucleotides

1. Using Table 1 as a guide, add the required volume of S-SS-4FB/DMF to the protein solution. Refer to the Protein Modification Calculator, or Amino-Oligonucleotide Modification Calculator, if needed.

<table>
<thead>
<tr>
<th>IgG Concentration (mg/mL)</th>
<th>S-SS-4FB Mole Equivalents Added</th>
<th>Determined Ratio of 4FB/Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>5</td>
<td>2.38</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
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</tr>
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</table>

Table 1: The number of 4FB groups incorporated on an antibody is dependent on the number of mole equivalents sulfo-S-SS-4FB added and the protein concentration. This table can be used as a general guide for modification of a protein with an NHS ester-based reagent.

2. Allow the reaction to incubate at room temperature for 2.0 hours.

E. Desalting procedure

1. Desalt/buffer exchange the protein or oligonucleotide into 1X Conjugation Buffer (100 mM sodium phosphate, 150 mM sodium chloride, pH 6.0). If needed, refer to the Protein Desalting Protocol or Oligonucleotide Desalting Protocol.