

Introduction to Solulink Bioconjugation Technology

Solulink's core technology is based on the formation of a covalent bond formed from an aromatic hydrazine and an aromatic aldehyde. S-HyNic **1** (succinimidyl 6-hydrazinonicotinate acetone hydrazone, SANH) is used to incorporate aromatic hydrazine linkers on biomolecules. S-HyNic is an amino-reactive reagent that directly converts amino groups on biomolecules and surfaces to HyNic groups. S-4FB **2** (succinimidyl 4-formylbenzoate, SFB) is used to convert amino groups to aromatic aldehydes (4-formylbenzamide (4FB) groups). Addition of a HyNic-modified biomolecule to a 4FB-modified protein 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 antibody conjugation is 6.0. Unlike thiol-based conjugation protocols where reducing reagents are required that can compromise the activity of proteins by cleaving disulfide bonds, the HyNic-4FB conjugation couple leaves disulfide bonds intact. 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.*¹ that showed that 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 (aniline) to the reaction mixture converts >95% of the antibody to conjugate in ~2 hours using 1-2 mole equivalents of second protein.

The HyNic-4FB conjugation couple is chromophoric- the conjugate bond absorbs at 354 nm and has a molar extinction coefficient of 29000. 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 (Solulink catalog# # S-2002) yields a chromophoric product that absorbs at A350 with a molar extinction coefficient of 18000 (Figure 2). Links to procedures and calculators to guide users through this process are given in the procedures below.

Sulfo-S-4FB is a water soluble analog of S-4FB. Sulfo-S-4FB is recommended for use to incorporate 4FB linkers on amino-surfaces and when modifying biomolecules when DMF or DMSO is deleterious to the biomolecule.

Methods

Additional Materials Required

Reagents

Diafiltration spin columns	S-4004
Modification Buffer	S-4003
Conjugation Buffer	S-4002
DMF (anhydrous)	S-4001

Equipment

Variable-speed bench-top microcentrifuge
Spectrophotometer or Plate Reader
1.5 mL microcentrifuge tubes
2-sulfo-benzaldehyde (S-2005)

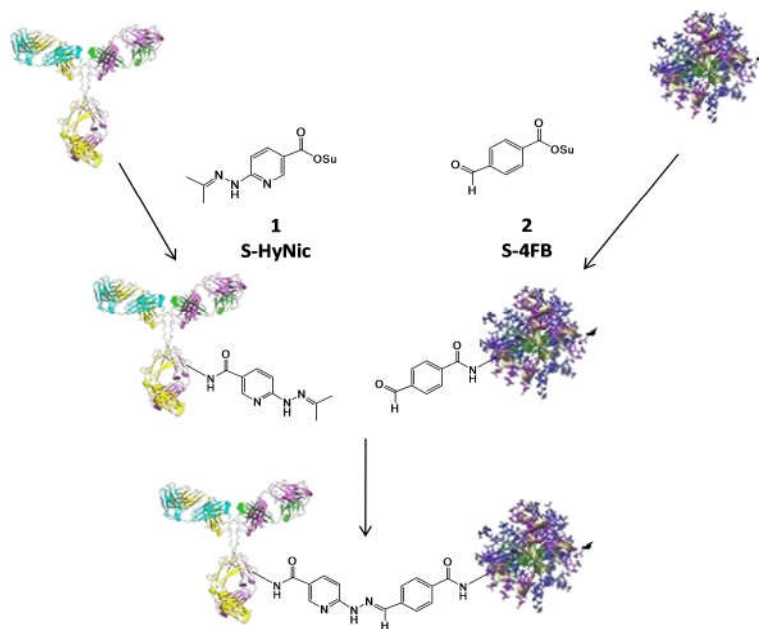


Figure 1: Schematic representation of Solulink Bioconjugation chemistry where an antibody is modified with S-HyNic to incorporate HyNic groups and a second protein is modified with S-4FB to incorporate 4FB groups. Conjugate is formed directly by simply mixing the HyNic-modified antibody with the 4FB-modified proteins.

Note: This protocol and all documents linked below can be downloaded from the appropriate category in the Solulink Library at <http://www.solulink.com/library>.

Modification Procedure

A. Desalting procedure

- Desalt/buffer exchange the protein or oligonucleotide into Modification Buffer (100 mM sodium phosphate, 150 mM sodium chloride, pH 7.4); if needed, refer to the [Protein Desalting Protocol](#) or [Oligonucleotide Desalting Protocol](#), if needed.

Notes:

- It is necessary to remove all free amine-containing contaminants, *e.g.* tris, glycine, from the protein solution before modification.
- High-level buffering capacity, *i.e.* 100 mM phosphate, is necessary for successful modification.
- For desalting proteins Solulink recommends Pierce Zeba Desalt Spin columns (# 89882); for oligonucleotides, Sartorius VivaSpin diafiltration units (#VS0112). Refer to desalting protocol for either apparatus.

B. Determine the concentration of the protein

- Determine the concentration of the protein to be modified using a [Bradford assay](#) (BioRad, #500-0006) or the [BCA assay](#) (ThermoScientific, #23223). Alternatively the A280 can be used if the protein extinction coefficient is known (E1%).
- Adjust the concentration to 1-2.5 mg/mL in Modification Buffer pH 7.4, if necessary

C. Prepare a Sulfo-S-4FB/DMF stock solution

- Prepare a stock solution of Sulfo-S-4FB in anhydrous DMF by dissolving 2-4 mg of Sulfo-S-4FB in 100 μ L anhydrous DMF or buffer.

Note:

- The Sulfo-S-4FB/DMF stock solution is stable for 2 weeks if prepared with anhydrous DMF (Solulink catalog# S-4001) and stored desiccated.
- Stock solution prepared in buffer must be used immediately.

D. Modification of a protein or oligonucleotide

- Using Table 1 as a guide, add the requisite volume of Sulfo-S-4FB/DMF to the protein solution; refer to the [Protein Modification With An NHS Ester Calculator](#) or [Amino-Oligonucleotide Modification With An NHS Ester Calculator](#), if needed.
 - Note:** be sure to use the correct values for Sulfo-S-4FB in the **Reagent Information** section of the calculator
- Allow reaction to incubate at room temperature for 2.0 hours.

E. Desalting procedure

- Desalt/buffer exchange the protein or oligonucleotide into 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](#), if needed.

F. Quantifying modification level

- The molar substitution ratio (MSR) can be determined using a colorimetric reaction outlined in Figure 2. Addition of 2-hydrazinopyridine to a 4FB-modified biomolecules yields a compound that absorbs at 350 nm. Refer to the [4FB-Protein Colorimetric MSR Calculator](#) or the [4FB-Oligonucleotide Colorimetric MSR Calculator](#) as well as the protocol that is appropriate for your lab equipment: [4FB Colorimetric MSR Assay Protein Nanodrop Method Protocol](#), [4FB Colorimetric MSR Assay Protein Spectrophotometer Method Protocol](#), [4FB Colorimetric MSR Assay Oligonucleotide Nanodrop Method Protocol](#), or [4FB Colorimetric MSR Assay Oligonucleotide Spectrophotometer Method Protocol](#).

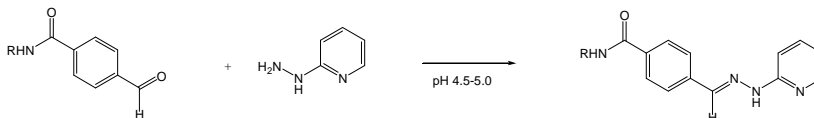


Figure 2: Colorimetric reaction used to quantify number of 4FB linkers on a biomolecule

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

Troubleshooting

Problem	Possible Cause	Solution
Protein was not modified or poorly modified.	Protein has been contaminated with amine containing compounds	Desalt the protein more thoroughly with a new Zeba Spin column or VivaSpin diafiltration apparatus
	The concentration of the protein was too low	Increase the concentration of the protein to >2.0 mg/mL
Sulfo-S-4FB was hydrolyzed	The buffer hydrolyzed the NHS ester	Modify the surface, protein or oligo with the linker immediately after dissolving.

Stability

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IgG concentration (mg/mL)	Sulfo-S-4FB molar equivalents added	Determined ratio of 4FB/protein
1.0	5	2.38
	10	4.73
	15	6.20
2.5	5	3.08
	10	6.58
5.0	5	3.74
	10	6.80

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

The 4FB linkers incorporated on biomolecules are stable at 4°C for >30 days.

Related Solulink Products

S-9002-1	S-HyNic Kit	S-4004	VivaSpin diafiltration device	S-2002	2-hydrazinopyridine
S-1037	SS-S-4FB Linker	S-4001	DMF anhydrous	S-2006	TurboLink Catalyst Buffer
S-4000	Modification Buffer	S-4002	Conjugation Buffer		

References

1. Dirksen, A., et al., Nucleophilic catalysis of hydrazone formation and transimination: implications for dynamic covalent chemistry. *J Am Chem Soc*, 2006. 128(49): p. 15602-3.
2. Ryan C. Bailey, Gabriel A. Kwong, Caius G. Radu, Owen N. Witte, and James R. Heath, DNA-Encoded Antibody Libraries: A Unified Platform for Multiplexed Cell Sorting and Detection of Genes and Proteins, *J. Amer. Chem. Soc.* 2007, **129**, 1959-1967.

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