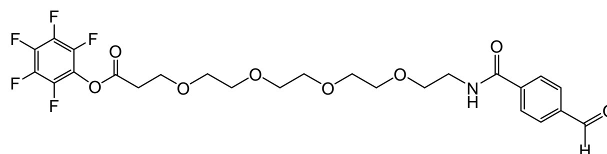
Peg4/PFB

$C_{25}H_{26}F_5NO_8$ MW: 563.47
S-1034-010



Introduction to SoluLinK Bioconjugation Technology

SoluLinK's core technology is based on the formation of a bis-arylhydrazone formed from an aromatic hydrazine and an aromatic aldehyde. S-HyNic **1** (succinimidyl 6-hydrazinonicotinate acetone hydrazone, SANH) is used to incorporate aromatic hydrazine moieties 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 biomolecule or surface directly leads to the formation of the conjugate (Figure 1). The bis-arylhydrazone 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 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 aniline to the reaction mixture converts >95% of the antibody to conjugate in ~2 hours using 1-2 mole equivalents of second protein.

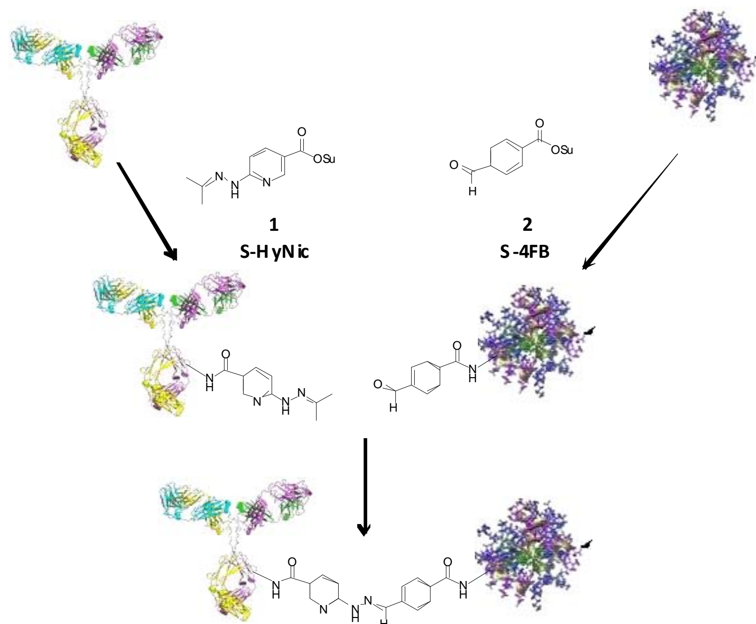


Figure 1: Schematic representation of SoluLinK Bioconjugation chemistry where an antibody is modified with S-HyNic to incorporate HyNic groups and a second protein is modified with S-4FB to incorporate 4FB groups. Conjugate is formed directly by simply mixing the HyNic-modified antibody with the 4FB-modified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

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-2002-100) yields a chromophoric product that absorbs at A360 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.

Peg4/PFP (Solulink catalog: S-1034-010 (10 mg)) is an analog of S-4FB with Peg4 extended hydrophilic linker if required to space biomolecules from surfaces or each other.

Methods

Additional Materials Required

Reagents		Equipment
Diafiltration spin columns	S-4004-025	Variable-speed bench-top microcentrifuge
Modification Buffer	S-4003-005	Spectrophotometer or Plate Reader
Conjugation Buffer	S-4002-005	1.5 mL microcentrifuge tubes
DMF (anhydrous)	S-4001-005	2-sulfo-benzlaldehyde (S-2005-100)

Note: This Protocol and all links below can be downloaded at http://www.SoluLinK.com/protocols.php

Modification Procedure

A. **Desalting procedure (More detailed protocols at LINK)**
   1. Desalt/buffer exchange the protein into Modification Buffer (100 mM sodium phosphate, 150 mM sodium chloride, pH 7.4).

Notes:

a) It is necessary to remove all free amine-containing contaminants, *e.g.* tris, glycine, from the protein solution before modification.
b) High-level buffering capacity, *i.e.* 100 mM phosphate, is necessary for successful modification.
c) 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 (More detailed protocols at LINK)

1. Determine the concentration of the protein to be modified using the BCA assay (ThermoScientific, #23223) or spectrophotometrically if the extinction coefficient of the protein is known. The extinction coefficient for antibodies is 1.4 for a 1 mg/mL solution.
2. Bring the concentration to 1-5 mg/mL in Modification Buffer pH 7.4

C. Prepare a Peg4/PFB/DMF stock solution

1. Prepare a stock solution of Peg4/PFB in anhydrous DMF (or DMSO) by dissolving 2-4 mg of Peg4/PFB in 100 µL anhydrous DMF.

Note:

a) The Peg3/PFB /DMF stock solution is stable for 2 weeks if prepared with anhydrous DMF (SoluLinK catalog# S-4001-010) and stored desiccated.

D. Modification of protein (More detailed protocols at LINK) and of oligonucleotides (LINK)

1. Using Table 1 as a guide, add the requisite volume of Peg3/PFB /DMF to the protein solution.
2. Allow reaction to incubate at room temperature for 2.0 hours.

IgG concentration (mg/mL)	Peg4/PFB 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.

E. Desalting procedure (detailed protocols at LINK)

1. Desalt/buffer exchange the protein into Conjugation Buffer (0.1M sodium phosphate, 0.15M sodium chloride, pH 6.0). For proteins SoluLinK recommends Pierce Zeba Desalt Spin columns (# 89882) or for oligonucleotides SoluLinK recommends Sartorius Vivaspin diafiltration units (#VS0112). Refer to desalting protocol for either apparatus.

F. Quantifying modification level (detailed protocols at LINK)

1. 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 bis-arylhydrazone that absorbs at 350 nm. Calculator/protocols can be downloaded from: LINK

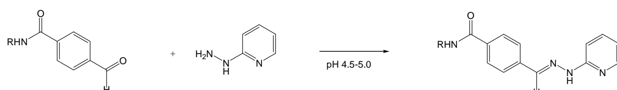


Figure 2: Colorimetric reaction used to quantify number of 4FB moieties on a biomolecule

2. 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
S-4-FB was hydrolyzed	Wet or poor quality DMF/DMSO hydrolyzed the NHS ester	Use a good quality anhydrous DMF/DMSO to solubilize the S-HyNic molecule.

Stability

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

Related SoluLinK Products

S-9002-1 S-HyNic Kit	S-4004-025 VivaSpin diafiltration device	S-2002-100 2-hydrazinopyridine
S-1004-025 S-4FB Linker	S-4001-005 DMF anhydrous	S-4023-005 Aniline
S-4003-005 Modification Buffer	S-4002-005 Conjugation Buffer	S-4024-005 Aniline 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.