



Protein-Protein Conjugation Kit

User Manual Catalog # S-9010-1

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Introduction to the Solulink Bioconjugation Technology

The Reaction: SoluLinK's core technology is based on the formation of a stable 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. Due to the lability of the immunoreactivity of antibodies at low pH, *i.e.* < 5.0, 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.

Fastest, most efficient: 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 < 1 hour using 1-2 mole equivalents of second protein.

Traceable modification:

Reproducibility of any reaction is dependent on accurate characterization of all components. As both HyNic and 4FB are aromatic their incorporation can be readily quantified using colorimetric assays (Figures 3 and 4).

Traceable conjugation: The HyNic-4FB conjugate bond is chromophoric- it absorbs at 354 nm and has a molar extinction coefficient of 29,000. 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.

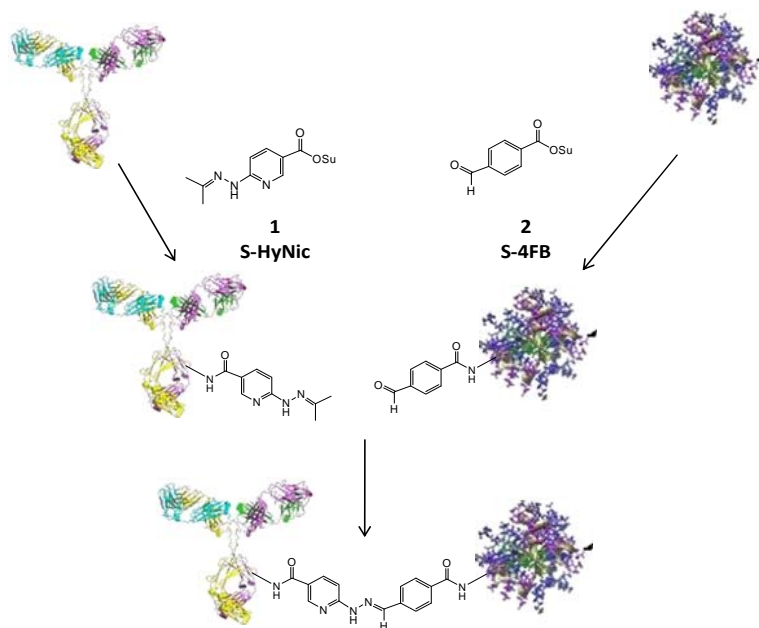


Figure 1: Schematic representation of SoluLinK Bioconjugation chemistry where an antibody is modified with S-HyNic to incorporate HyNic groups and a second protein is modified with S-4FB to incorporate 4FB groups. Conjugate is formed directly by simply mixing the HyNic-modified antibody with the 4FB-modified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

Furthermore compared to previous methods the HyNic/4FB technology offers the following practical advantages:

- 1)The reaction goes to completion: In all previous bi-functional linker based conjugations the reaction never went to completion, *i.e.* there is always unconjugated limiting protein in the final reaction. The HyNic-4FB conjugation couple catalyzed by aniline yields >95% conjugate.
- 2)The reaction is efficient: The reaction is very stoichiometrically efficient as input of only 1.0-1.5 moles second protein/mole first protein is required for complete conversion to conjugate.
- 3)The conjugate bond is extremely stable: The bis-arylhydrazone conjugate bond is stable to 92°C and pH 2.0-10.0.
- 4)The reaction conditions are extremely mild and do not cause any antibody denaturation: 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 metals, oxidation or reducing reagents are required.
- 5)The conjugation is traceable spectrophotometrically. The HyNic-4FB conjugate bond is chromophoric- it absorbs at 354 nm and has a molar extinction coefficient of 29,000. 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.
- 6)The modifications of both the HyNic moiety on the protein and the 4FB moiety on the oligonucleotide is quantifiable using a colorimetric assay. The reproducibility of any reaction is dependent on accurate characterization of all components. The Molar Substitution Ratio (MSR), *i.e.* the number of HyNic moieties/protein, of HyNic groups can be quantified colorimetrically as reaction with 2-sulfobenzaldehyde yields a chromophoric product that absorbs at 350 nm with a molar extinction coefficient of 18000 (Figure 4). The MSR of 4FB groups can be determined colorimetrically by its reaction with 2-hydrazinopyridine forming a hydrazone that absorbs at 350 nm with a molar extinction coefficient of 20000. This kit contains all the reagents necessary to determine both MSRs. Procedures to guide users through this process are given in the procedures below .

The Keys to Successful Conjugation

The following are the three crucial requirements that must be fulfilled for a reproducibly successful preparation of a protein/oligonucleotide conjugate using SoluLink's bioconjugation technology:

1. **Desalting:** Prior to modification, the starting protein must be thoroughly desalted, removing all amine contaminants, and exchanged into Modification Buffer, pH 7.4.
2. **Antibody concentration:** The recommended concentration of the antibody must be adhered to in all steps.
3. **Molar substitution ratio:** The Molar ratio of the Hynic on the protein and the 4FB on the oligo must be determined and within the desired range before continuing to the next step.

Kit Components

Component	Size	Storage ¹
S-HyNic	2 X 1.0 mg vial	Desiccated
S-4FB	2 X 1.0 mg vial	Desiccated
10X Modification Buffer ²	1.5 mL vial	Room temperature
10X Conjugation Buffer ³	1.5 mL vial	Room temperature
10X TurboLink Catalyst Buffer ⁴	1.0 mL vial	4°C
0.5 mL Zeba Columns	4	4°C
Collection tubes	12 X 1.5 mL	Room temperature
DMF (anhydrous)	1 mL vial	Desiccated
2-Hydrazinopyridine.2HCl	25 mg	
2-Sulfobenzaldehyde	25 mg	Room temperature
10X MES Buffer ⁵	1.5 mL vial	Room temperature
2 mL Zeba Column	2	4°C
10X PBS	1.5 mL vial	Room temperature

Flash Drive

NOTES:

- 1) For convenience all kit components can be stored at 4°C-
 - a. If precipitates are present in buffers on storage at 4°C redissolve by warming at 37°C before using
- 2) 10X Modification Buffer: 1.0 M phosphate, 1.5 M NaCl, pH 7.4
- 3) 10X Conjugation Buffer: 1.0 M phosphate, 1.5 M NaCl, pH 6.0
- 4) 10X TurboLink Catalyst Buffer: 100 mM aniline, 100 mM phosphate, 150 mM NaCl, pH 6.0
- 5) 10X MES Buffer: 1.0 M MES, pH 6.0

Equipment/Reagents Required But Not Provided

Variable-speed bench-top microcentrifuge
Spectrophotometer or Plate Reader
1.5 mL microcentrifuge tubes
Pipettors
Protein concentration assay reagents such as BCA or Bradford assays

Protein-Protein Conjugation Protocol

Protein Desalting

Proteins must be completely desalted into 1x Modification Buffer (100 mM Phosphate, 150 mM NaCl, pH 7.4) before they are modified with S-HyNic.

Any desalting method, such as dialysis, Sephadex desalting columns (NAP columns, GE Healthcare) or Zeba™ Desalt Spin Columns (Pierce Chemical, Cat. #89882 or 89889 (Figure 2)) can be used.

SoluLink recommends the use of Zeba™ Desalt Spin Columns to desalt proteins as required by our conjugation protocol. These rapid spin columns are recommended because they do not significantly dilute the antibody during desalting.

Included in this kit are 0.5 mL Zeba™ Spin Desalt columns (Figure 3) that have a maximum capacity of 130 μ L. Therefore up to 1.3 mg of a 10 mg/mL solution of protein can be desalted. As this kit has been designed for two conjugations, included are four columns, one to initially desalt the protein and one to desalt and exchange the modified protein into 1x Conjugation Buffer (100 mM Phosphate, 150 mM NaCl, pH 6.0).

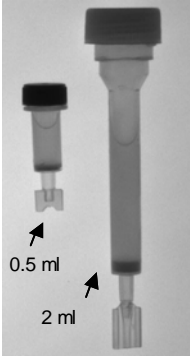


Figure 2. Zeba™ Desalt Spin Columns (0.5 and 2 ml) used to desalt starting Antibody and HyNic-modified Antibody.

Zeba Desalting Protocol

0.5ml Zeba™ Spin Column Preparation (Sample volumes 30-130 μ L)

1. Remove spin column's bottom closure and loosen the top cap (do not remove cap).
2. Place spin column in a 1.5 mL microcentrifuge collection tube.
3. Centrifuge at 1,500 x g for 1 minute to remove storage solution.
4. Place a mark on the side of the column where the compacted resin is slanted upward. Place column in the microfuge with the mark facing outward in all subsequent centrifugation steps.
5. Add 300 μ L of 1x Modification Buffer (pH 7.4) or Conjugation Buffer (pH 6.0) as required to the top of the resin bed and centrifuge at 1,500 x g for 1 minute to remove buffer.
6. Repeat steps 4 and 5 two additional times, discarding buffer from the collection tube.
7. Column is now ready for sample loading.

Protein Sample Loading

1. Place the equilibrated spin column in a new 1.5 mL collection tube, remove cap and slowly apply 30-130 μ L sample volume to the center of the compact resin bed.

Note- For sample volumes less than 70 µl apply a 15 µl buffer (stacker) to the top of the resin bed after the sample has fully absorbed to ensure maximal antibody recovery. Avoid contact with the sides of the column when loading.

2. Centrifuge at 1,500 x g for 2 minutes to collect desalted sample.
3. Discard desalting column after use.
4. Protein sample is now desalted and ready for modification.

2.0 Protein Modification

Recommended Guidelines for Modifying Proteins/Antibodies with S-HyNic (Figure 3): The modification process is the critical element of any conjugation project. For this reason, we have included a more detailed discussion of this important step. For example, the number of functional groups incorporated per protein molecule is commonly referred to as the molar substitution ratio (MSR). The final MSR obtained after a modification reaction with S-HyNic is a function of several variables that include protein concentration, number of available amino-groups on the protein (often related to M.W.), excess linker equivalents added (e.g. 5X, 10X or 20X) and reaction pH. Table 1 presents the results of a study to determine the level of HyNic incorporation on an antibody adding 5X, 10X and 20X equivalents of S-HyNic at 1.0, 2.5 and 5.0 mg/mL antibody concentration in modification buffer (100 mM phosphate, 150 mM NaCl, pH 7.2-7.4).

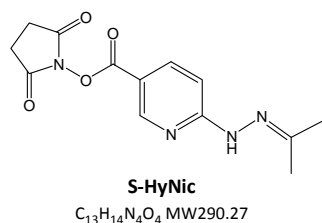


Figure 3: Structure of S-HyNic (CAS# 362522-50-7)

In general, as the protein/antibody concentration and number of linker equivalents are increased the molar substitution ratio increases. Caution is recommended since over-modification can dramatically change the isoelectric point of the protein and result in precipitation of the protein or loss of biological activity. This is especially critical with proteins <50 kD MW.

2.1 S-HyNic-Protein Modification Protocol (Calculator Worksheet 1)

- a) Dissolve a vial of pre-weighed 1.0 mg S-HyNic vial in 100 µL anhydrous DMF
- b) Add the required volume of S-HyNic to the protein in 1x Modification Buffer (100 mM Phosphate, 150 mM NaCl, pH 7.4) as calculated using the Protein-Protein Conjugation Calculator. A volume that typically represents 20 mole equivalents/mole of protein is added and mixed thoroughly.

	5X	10X	20X
MSR			
1 mg/ml	1.4	1.0	3.0
2.5 mg/ml	3.2	6.6	7.9
5 mg/ml	4.9	5.9	7.8

Table 1: results of a study to determine the level of HyNic incorporation on an antibody adding 5X, 10X and 20X equivalents of S-HyNic at 1.0, 2.5 and 5.0 mg/mL antibody concentration in modification buffer (100 mM phosphate, 150 mM NaCl, pH 7.2-7.4).

Notes

- I. Always maintain the percentage of DMF (vol/vol) in the final S-HyNic modification reaction at or below 5% of the total reaction volume.
- II. PBS (10 mM phosphate, 150mM sodium chloride, pH 7.2) is **NOT** recommended as a modification buffer due to its poor buffering capacity.
- III. It is important to have a final protein concentration at >1.0 mg/mL for efficient HyNic modification.

- c) Incubate the reaction at room temperature for 1.5 hours.
- d) Proceed to desalt the HyNic-modified protein into 1x Conjugation Buffer (100 mM Phosphate, 150 mM NaCl, pH 6.0).

2.2 Determining the HyNic Molar Substitution Ratio (MSR)

The determination of the number of HyNic groups/protein is accomplished by the colorimetric assay shown in Figure 4 and described below.

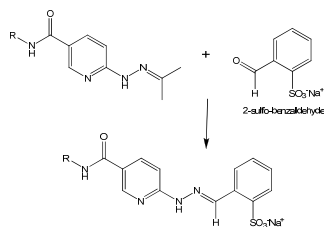


Figure 4: Scheme presenting the colorimetric assay used to quantify the number of HyNic groups on a biomolecule. The bis-arylhydrazone product absorbs at 350 nm has a molar extinction coefficient of 18000 M^{-1} .

Protocols to determine the MSR using a standard spectrophotometer (Method A) or a NanoDrop spectrophotometer (Method B) are described below. Both protocols begin by preparing the 2-sulfobenzaldehyde (2-sBA) Assay Reagent described in Step 1.

Assay Reagent Preparation: Prepare a 0.5 mM working solution of 2-sBA solution in 0.1 M MES buffer, pH 5.0 as follows:

- a. Prepare a 20 mg/mL solution of 2-Sulfobenzaldehyde in water.
- b. Add 52 μL of this solution to a 15 mL conical tube containing 9.948 mL 100 mM MES Buffer (pH 5.0). Label this solution 0.5 mM 2-sBA solution.
- c. Protect the solution from light and keep refrigerated. This solution remains stable for up to 30 days at 4°C .

Method A: Cuvette Spectrophotometer Protocol

1. Transfer 10 μL of HyNic-modified (desalted) protein/antibody solution ($\sim 2\text{-}5\text{mg/ml}$ in 1x Conjugation Buffer) to a new 1.5 mL microfuge tube containing 490 μL 2-sBA reagent. Prepare another reaction tube (negative control) containing 490 μL 2-sBA reagent and 10 μL of 1x Conjugation Buffer.
2. Incubate all reaction tubes at 37°C for 30 minutes or at room temperature for 2 hours.
3. Remove the reaction tubes from the 37°C incubator and measure the A_{350} of both reactions using a quartz cuvette as follows:
 - a. Blank the spectrophotometer at 350 nm using 500 μL 0.5mM 2-sBA solution in MES (pH 5.0) in a 1 mL quartz cuvette.
 - b. Record the A_{350} of the sample and no antibody controls.

Note- In instances where low HyNic incorporation occurs or when antibody concentration is $< 2 \text{ mg/mL}$ the assay may require $>10 \mu\text{L}$ to achieve a detectable A_{350} reading.

- Using the values obtained, calculate the HyNic / protein MSR with the aid of our Modification Calculator on the flash drive included with the kit or calculate the MSR by determining the hydrazone concentration using the known molar extinction coefficient (i.e. 18,000 at 350 nm) and dividing by the known molar antibody concentration.

Method B: NanoDrop Method

- Transfer 2 μ L of HyNic-modified (desalted) antibody solution (~2-5 mg/mL in 1x Conjugation Buffer) to a new 1.5 mL microfuge tube containing 18 μ L 2-sulfo benzaldehyde reagent. Prepare another reaction tube (negative control) containing 2 μ L 1x Conjugation Buffer reagent and 18 μ L of 2-sulfo benzaldehyde.
- Incubate all reaction tubes at 37°C for 30 minutes or at room temperature for 2 hours.
- Remove the reaction tubes from the 37°C incubator and measure the A_{350} of
Determining the Molar Substitution Ratio (MSR)

Using the values obtained, calculate the HyNic / protein MSR with the aid of our Modification Calculator on the flash drive included with the kit or calculate the MSR by determining the hydrazone concentration using the known molar extinction coefficient (i.e. 18,000 at 350 nm) and dividing by the known molar antibody concentration.

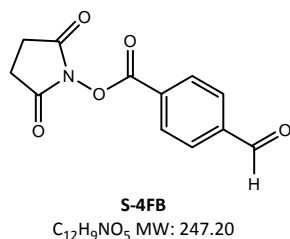


Figure 4: Structure of S-4FB

	[S-4FB] 5X	[S-4FB] 10X	[S-4FB] 20X	[S-4FB] 40X
eq added				
MSR				
1.0 mg/ml	3.7	4.3	9.84	18.6
2.5 mg/ml	4.8	8.6	14.0	23.0
5.0 mg/ml	4.6	7.3	14.3	24.8

Table 2: Results of a study to determine the level of 4FB incorporation on an antibody adding 5X, 10X and 20X equivalents of S-4FB at 1.0, 2.5 and 5.0 mg/mL antibody concentration in modification buffer (100 mM phosphate, 150 mM NaCl, pH 7.2-7.4).

2.3 S-4FB Antibody Modification Protocol (Calculator Worksheet 2)

1. Dissolve a vial of pre-weighed 1.0 mg S-4FB vial in 100 μ L anhydrous DMF
2. Add the required volume of S-4FB to the second protein in modification buffer (**100 mM** phosphate, 150 mM NaCl, pH 7.4), a volume that typically represents 10-15 mole equivalents/mole protein dependent on protein concentration (for antibodies see Table 2) and mix thoroughly.

Notes

- a) Always maintain the percentage of DMF (vol/vol) in the final S-HyNic modification reaction at or below 5% of the total reaction volume.
 - b) PBS (**10 mM** phosphate, 150mM sodium chloride, pH 7.2) is **not** recommended as a modification buffer due to its poor buffering capacity.
 - c) It is important to have a final protein concentration @ 2.5-4.0 mg/ml for efficient HyNic modification.
3. Incubate the reaction at room temperature for 2 hours.
 4. Proceed to desalt the 4FB-modified protein into 1x Conjugation Buffer (100 mM phosphate, 150 mM NaCl, pH 6.0).

Determining the Molar Substitution Ratio (MSR)

After desalting on Zeba™ Spin columns to remove excess linker from the modification reaction, protein concentrations are determined using the BCA assay. The modified protein samples are then ready for their respective MSR assay.

4FB MSR Quantification

1. Prepare a 0.5mM working solution of 2-hydrazinopyridine (**2-HP**) solution in 0.1 M MES buffer, pH 5.0 as follows:
 - a. Dissolve 5 mg 2HP in 100 μ L DMF.
 - b. Add 76 μ L of this solution to a 50ml conical tube containing 50ml 100mM MES Buffer (pH 5.0). Label this solution 0.5mM 2-HP solution.
 - c. Protect the solution from light and keep refrigerated. This solution remains stable for up to 30 days at 4°C.
2. Transfer 10 μ L of 4FB-modified (desalted) protein solution (~2-5mg/ml in 1x conjugation buffer) to a new 1.5 ml microfuge tube containing 490 μ L 2HP reagent. Prepare another reaction tube (negative control) containing 490 μ L 2-HP reagent and 10 μ L of 1x conjugation buffer.
3. Incubate all reaction tubes at 37°C for 30 minutes or at room temperature for 2 hours.
4. Remove the reaction tubes from the 37°C incubator and measure the A₃₉₀ of both reactions using a quartz cuvette as follows:

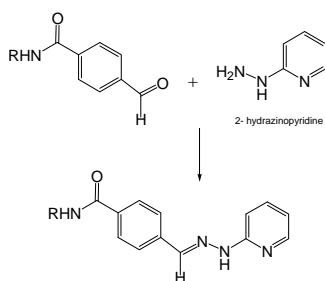


Figure 4: Scheme presenting the colorimetric assay used to quantify the number of 4FB groups on a biomolecule. The bis-arylhydrazone product absorbs at 350 nm has a molar extinction coefficient of 18000 M⁻¹.

- a. Blank the spectrophotometer at 350 nm using 500 μ l 0.5mM 2-HP solution in MES (pH 5.0) in a 1 ml quartz cuvette.
- b. Record the A_{350} of each sample and no protein controls.

Note- In rare instances that depend on the protein concentration of the desalted protein being measured and the final degree of 4FB-modification, it may require a volume greater or lesser than 10 μ l of protein to get a detectable A_{350} reading on the spectrophotometer.

5. Using the values obtained, calculate the 4FB/protein MSR with the aid of our on-line Modification Calculator found at ([LINK](#)) or calculate the MSR by determining the hydrazone concentration using the known molar extinction coefficient (i.e. 18,000 at 350 nm) and dividing by the known molar protein concentration.

3.0 Protein-Protein Conjugation (Calculator Worksheet 3)

1. Using Calculator Worksheet 3 to calculate volumes of proteins to combine mix HyNic-modified **protein 1** with 4FB-modified **protein 2** and add 1/10 volume TurboLink Buffer. Following addition of the stock TurboLink Buffer the final [aniline] = 10 mM.
 - a. **Recommendation: Add 1 mole equivalent of limiting protein, i.e. antibody, to 1.5-2.0 mol equivalents of highest abundance protein, i.e. enzyme or bioflour.**
2. Incubate at room temperature for 1.5-2.0 h
3. The conjugation reaction can be 'visualized' by removing an aliquot and analyzing by gel electrophoresis.
4. The reaction is now ready for purification by size exclusion chromatography if required.