Version 05.29.2013

Protein-Protein Conjugation Kit

Technical Manual

Catalog # S-9010-1

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Safety Information

WARNING – CHEMICAL HAZARD. Some chemicals used can be potentially hazardous, and can cause injury or illness.

- Read and understand the Material Safety Data Sheets (MSDS) available at Solulink.com before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with and inhalation of chemicals. Wear appropriate personal protective equipment when handling chemicals (e.g. safety glasses, gloves, or clothing). For additional safety guidelines consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's clean-up procedures as recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling and disposal.

Table of Contents

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 (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 (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^{O}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, reducing agents, or metals are required in the preparation of conjugate.

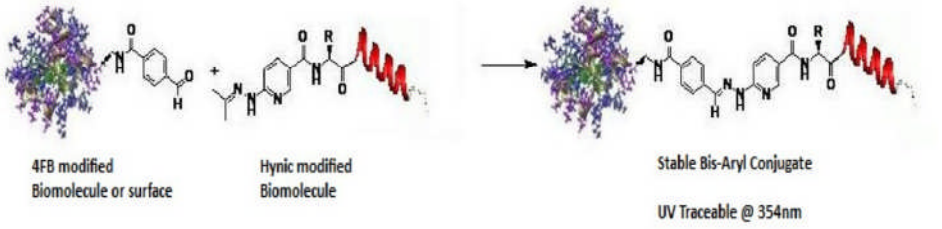


Figure 1: Linking chemistry behind HydraLink™ technology.

Fastest, most efficient:

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 aniline to the reaction mixture converts >95% of the antibody to conjugate in 2 hours 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.

Traceable conjugation:

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

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 was always unconjugated limiting protein in the final reaction. The HyNic-4FB conjugation couple catalyzed by aniline yields more than 90% conjugate.

2) **The reaction is efficient:** The reaction is very stoichiometrically efficient as input of only 1-2 mole 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^{O}C and pH 2.0-10.0.

4) **The reaction conditions are extremely mild and do not cause 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, oxidizing, 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.

6) **The modifications of both the HyNic moiety on the protein and the 4FB moiety on the protein 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 348 nm with a molar extinction coefficient of 28,500. The MSR of 4FB groups can be determined colorimetrically by its reaction with 2-hydrazinopyridine forming a hydrazone that absorbs at 354 nm with a molar extinction coefficient of 24,500. This kit contains all the reagents necessary to determine both MSRs. Procedures to guide users through this process are given in the protocol below.

The Keys to Successful Conjugation

The following are three crucial requirements that must be fulfilled for a reproducibly successful preparation of a protein/protein 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 1X Modification Buffer.
2. **Protein concentration**: The recommended concentration of protein must be adhered to in all steps.
3. **Molar substitution ratio:** The molar ratio of HyNic on the protein and 4FB on the protein must be determined and within the desired range before continuing to the next step.

Kit Components

Component	Component #	Size	Storage
S-HyNic	**S-9010-1-01**	**2 x 0.5 mg**	**Desiccated**
S-4FB	**S-9010-1-02**	**2 x 0.5 mg**	**Desiccated**
10X Modification Buffer	**S-9010-1-03**	**1.5 mL**	**4°C**
10X Conjugation Buffer	**S-9010-1-04**	**1.5 mL**	**4°C**
10X TurboLink Catalyst Buffer	**S-9010-1-05**	**1.5 mL**	**4°C**
7kDa 0.5 mL Zeba Columns	**S-9010-1-06**	**10**	**4°C**
Anhydrous DMF	**S-9010-1-07**	**2 x 1.5 mL**	**Desiccated**
0.5 mM 2-Hydrazinopyridine reagent	**S-9010-1-08**	**1 x 0.5 mL**	**4°C**
0.5 mM 2-Sulfobenzaldehyde reagent	**S-9010-1-09**	**1 x 0.5 ml**	**4°C**
2.0 mL Collection Tubes	**S-9010-1-11**	**10**	**Room temp**
7kDa 2.0 mL Zeba Columns	**S-9010-1-13**	**2**	**4°C**
10X PBS	**S-9010-1-14**	**1.5 mL**	**4°C**

NOTES:

1) *For convenience all kit components can be stored at 4°C.*
   If precipitate is present in buffers upon storage at 4°C, re-dissolve by warming at 37°C before using.
2) *10X Modification Buffer: 1.0 M phosphate, 1.5 M NaCl, pH 8.0*
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*

Equipment/Regents Required But Not Provided

1) NanoDrop™ or UV-Vis Spectrophotometer
2) Protein concentration assay reagents such as BCA or Bradford assay
3) Microcentrifuge

Protein-Protein Conjugation Protocol

Over the years, Solulink's scientists have accumulated extensive protein-protein conjugation experience. Based on this experience, Solulink has developed and optimized conjugation protocols that work well. Experience has taught us that certain strict limitations need to be placed on initial buffer composition, starting mass (mg) and concentrations (mg/mL). As a consequence, before starting a conjugation project we recommend the use of the flow chart outlined in Figure 2. To use the chart, simply start at the box labeled 'your protein' and proceed to answer the questions in the flow chart. The chart eventually guides the user to the first step in the HyNic or 4FB conjugation protocol.

Conjugation kit parameters:

1. Protein molecular weight range 25,000 – 950,000 Daltons
2. Protein concentration range 1.0 – 5.0 mg/mL; mass of protein to be modified range 50 – 650 μg
3. Protein sample volume range 50 – 130 μL
4. Linker mole equivalents range 3 – 20 fold

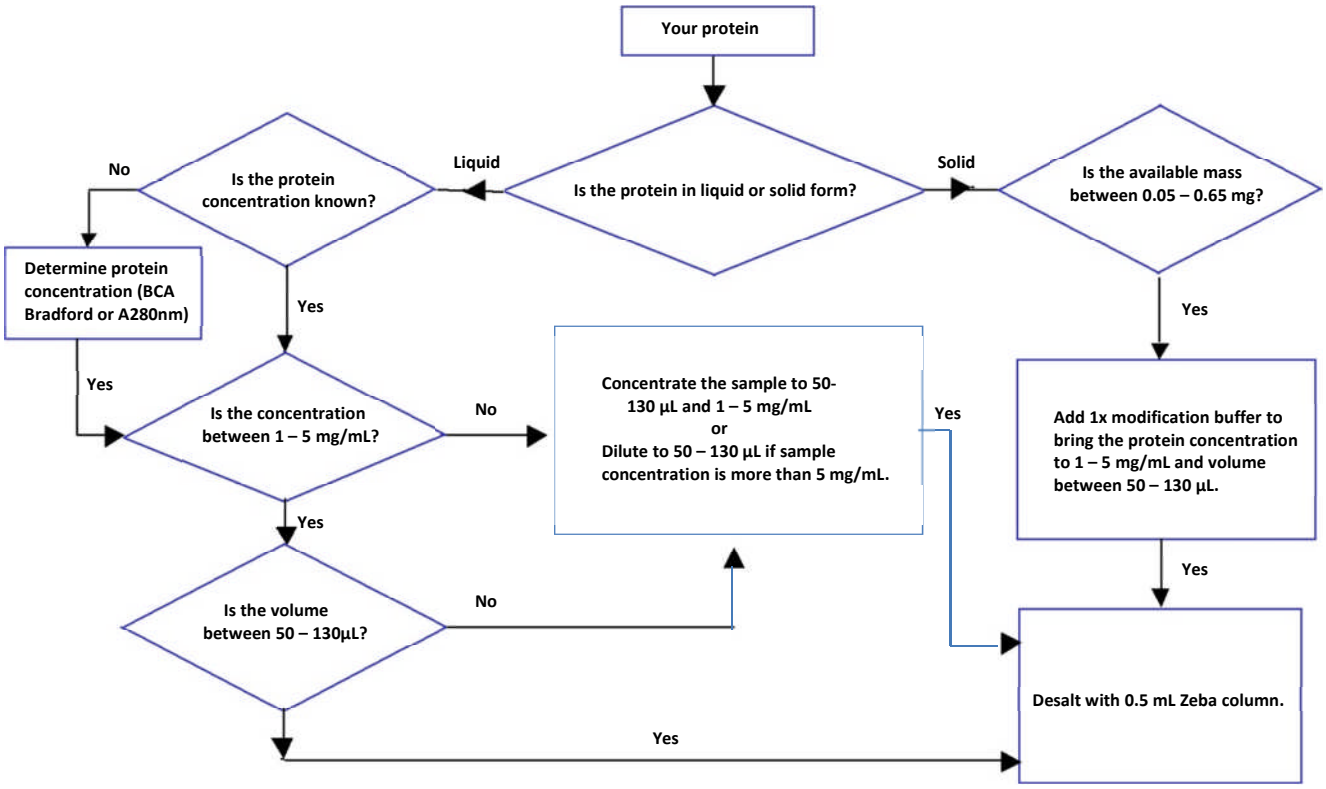


Figure 2. Flow-chart used for guiding a user to the start of the conjugation process.

Protein Desalting

Proteins must be desalted prior to modification. Proteins must be completely desalted into 1X Modification Buffer (dilute from 10X Modification Buffer) before they are modified with S-HyNic or S-4FB. This step ensures that the protein is in the correct buffer for modification, and that all traces of interfering amines such as Tris, azide, or glycine are removed.

SoluLink recommends the use of Zeba™ desalting columns (provided) to desalt proteins as required by our conjugation protocol. These rapid spin columns are recommended because they do not significantly dilute the protein during desalting and recover 85-90% of protein.

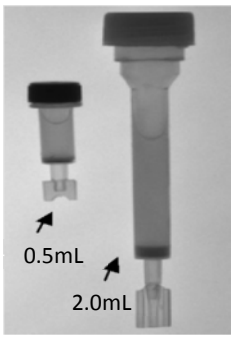


Figure 3. Zeba™ desalting spin columns (0.5 and 2 mL) used to desalt starting protein and HyNic or 4FB-modified protein.

This kit includes 0.5 mL Zeba™ desalting columns (Figure 3) that have a maximum capacity of 130 µL (and a minimum capacity of 50 µL). Therefore up to 0.65 mg of a 5 mg/mL solution of protein can be desalted. As this kit has been designed for two conjugations, included are eight columns, one to initially desalt each protein into 1X Modification Buffer and one to desalt and exchange the modified protein into 1X Conjugation Buffer. This kit also includes two 0.5 mL or 2 mL Zeba™ desalting columns that have a capacity of 50 – 150 µL or 150-700 µL, respectively, to desalt the final two protein conjugates into PBS storage buffer depending on the final reaction volume after conjugation.

Zeba™ Column Desalting Protocol

0.5mL Zeba™ Spin Column Preparation (sample volumes 50-130 µL)

1. Remove spin column's bottom closure and loosen the top cap (do not remove cap).
2. Place spin column in a 2.0 mL microcentrifuge collection tube.
3. Centrifuge at 1,500 x g for 1 minute; discard the flow through from the collection tube.
4. Place a mark on the side of the column where the compacted resin is slanted upward. Place the column in the centrifuge with the mark facing outward and away from the center of the rotor in all subsequent centrifugation steps.
5. Add 300 µL of required buffer to the top of the resin bed.
6. Centrifuge at 1,500 x g for 1 minute; discard the flow through from the collection tube.
7. Repeat steps 5 and 6 two additional times, discarding the flow through from the collection tube.
8. Column is now ready for sample loading.

Protein Sample Loading

1. Place the equilibrated spin column **in a new 2.0 mL microcentrifuge tube**. Remove cap and *slowly* apply 50-130 µL sample onto the center of the compact resin bed. Avoid contact with the tube walls — the sample must channel down through the resin itself.
2. Centrifuge at 1,500 x g for *2 minutes* to collect desalted sample.
3. Protein sample is desalted and ready for the next step.

2.0 Protein Modification

Note- Modification of HyNic/protein-1 and 4FB/protein-2 can be done at the same time. This way the whole procedure can be finished in one day. We recommend that the HyNic-modified protein be used within 5 hours to avoid forming a dimer with itself. 4FB-modified protein is stable at 4°C for weeks.

Recommended Guidelines for Modifying Proteins with S-HyNic

The modification process is a critical element of any conjugation project. For this reason, we have included a more detailed discussion of this important step. 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 after adding 5X, 10X and 20X mole equivalents of S-HyNic at 1.0, 2.5 and 5.0 mg/mL antibody concentration.

Protein concentration	5X HyNic	10X HyNic	20X HyNic
1.0 mg/mL	1.0	1.4	3.0
2.5 mg/mL	3.2	5.3	7.9
5.0 mg/mL	4.9	6.6	7.8

Table 1: MSR of Modifying Bovine IgG with S-HyNic

Note - It is recommend that the MSR of HyNic-proteins is 4 - 8 for proteins greater than 100,000 Daltons, and 2-4 for proteins equal to or less than 100,000 Daltons.

In general, as the protein 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 molecular weight.

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

1. Desalt protein to be modified with S-HyNic into **1X Modification Buffer** (dilute 10X stock buffer 1/10 with water) following the Zeba desalting procedure on page 8. Confirm protein concentration by read at A_{280} after initial protein desalting. Between 50 - 130 µL of protein solution at a concentration between 1 - 5 mg/mL may be used.
2. On the S-HyNic Modification Calculator input the name, molecular weight, protein concentration, volume of protein-1 to be modified, and the mole equivalents of HyNic used to modify protein-1 in the green fields. Add the required volume of anhydrous DMF to a 0.5 mg vial of pre-weighed S-HyNic reagent. Pipette the solution up and down to dissolve the pellet. The volume of DMF will be output in the pink field. If the volume of DMF output is ***more than 700 µL***, two or more times dilution of the HyNic reagent may be required.

   For example if the volume of DMF output is 5857 µL, then three times dilutions are required:

   (1) Make a 58.57X stock solution by dissolving S-HyNic pellet in 100 µL of DMF (5857µL/100µL = 58.57).

   (2) Next, make a 5.857X S-HyNic solution by adding 10 µL of 58.57X S-HyNic solution into 90 µL of DMF and mix well (1:10 dilution).

   (3) Third, make 1X S-HyNic solution by adding 10 µL of 5.857X S-HyNic solution into 48.57 µL of DMF and mix well (1:5.857 dilution).

3. If protein molecular weight is *greater than* 50,000 Daltons, add 2.0 µL of S-HyNic reagent into the desalted protein. If protein molecular weight is *equal to or less than* 50,000 Daltons add 3.0 µL of S-HyNic reagent into the desalted protein as calculated using the **Protein-Protein Conjugation Calculator** on calculator worksheet 1. Immediately mix well by vortexing.
4. Incubate the reaction at room temperature for at least 2.5 hours.
5. Proceed to desalt the HyNic-modified protein using a 0.5 mL Zeba™ column equilibrated with **1X Conjugation Buffer** (dilute 10X stock buffer 1/10 with water) using the Zeba desalting protocol on page 8.

2.2 Determining the HyNic Molar Substitution Ratio (MSR)

After desalting with a Zeba™ spin column to remove excess HyNic from the modification reaction, the protein concentration is determined by using either BCA or Bradford assay (the protein concentration must *not* be determined by reading A_{280} on a spectrophotometer at this step because of the contribution at A_{280} of HyNic itself). After the HyNic-protein concentration is determined proceed to MSR assay.

Assay Protocol

1. Transfer 2 µL of HyNic-modified (desalted) protein solution (1-5 mg/mL in 1X Conjugation Buffer) to a new 2.0 mL microfuge tube containing 18 µL of SBA working solution. Prepare another reaction tube (blank) containing 2 µL of 1X Conjugation Buffer and 18 µL of 2-SBA working solution.
2. Vortex to mix well, then incubate both reaction tubes at 37°C for 1 hour.
3. Remove the reaction tubes from 37°C and briefly centrifuge at 10,000 x g to collect any condensate. Vortex

both samples thoroughly before reading the A_{348} by one of the following methods:

Method A: NanoDrop™ Method

1. Launch the NanoDrop™ software and select the UV-VIS menu option. Initialize the instrument with 2 µL water (NanoDrop™ ND-1000 only).
2. Blank the NanoDrop™ with 2 µL blank (Conjugation Buffer + SBA) solution and clean the pedestal.
3. Set the λ1 wavelength to 348nm. Place 2 µL of the HyNic-protein MSR reaction on the pedestal and click the "Measure" icon. The 1.0 mm A354 absorbance will appear. **Multiply this number by 10** (converts from 1 mm to 1 cm) and then enter this value into the HyNic MSR Calculator **Protein-Protein Conjugation Calculator** on calculator worksheet 1 to obtain MSR.

Method B: Cuvette Spectrophotometer (100 µL, 1-cm micro-cuvette)

1. Prepare a 1:10 dilution of blank (Conjugation Buffer + SBA) solution by adding 180 µL of deionized water into the blank sample tube and mix well.
2. Prepare a 1:10 dilution of HyNic-protein MSR reaction solution by adding 180 µL of deionized water into the HyNic-protein MSR reaction tube and mix well.
3. In a 1 cm, 100 µL quartz micro-cuvette, blank the spectrophotometer at 348nm with 100 µL of the blank solution prepared step 1 above.
4. Remove the blank solution and add 100 µL of the HyNic-protein MSR sample solution from step 2 above to the cuvette.
5. Record the 348nm absorbance value of the HyNic-protein MSR sample. **Multiply this number by 10** (to account for the 10-fold dilution) and then enter this value into the HyNic MSR Calculator **Protein-Protein Conjugation Calculator** on calculator worksheet 1 to obtain MSR.

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

Recommended Guidelines for Modifying Proteins with S-4FB

Protein concentration	5X S-4FB	10X S-4FB	20X S-4FB
1.0 mg/mL	3.7	4.3	9.8
2.5 mg/mL	4.8	7.3	14.0
5.0 mg/mL	5.6	8.6	14.3

Table 2: MSR of Modifying Bovine IgG with S-4FB

Note - It is recommend that the MSR of 4FB-proteins is 4 - 8 for proteins greater than 100,000 Daltons, and 2-4 for proteins equal to or less than 100,000 Daltons.

1. Desalt protein to be modified with S-4FB into **1X Modification Buffer** (dilute 10X stock buffer 1/10 with water) following the Zeba desalting procedure on page 8. Confirm protein concentration by read at A_{280} after initial protein desalting. Between 50 - 130 µL of protein solution at a concentration between 1 - 5 mg/mL

may be used.

2. On the S-4FB Modification Calculator input the name, molecular weight, protein concentration, volume of protein-2 to be modified, and the mole equivalents of S-4FB used to modify protein-2 in the green fields. Add the required volume of anhydrous DMF to a 0.5 mg vial of pre-weighed S-4FB reagent. Pipette the solution up and down to dissolve the pellet. The volume of DMF will be output in the pink field. If the volume of DMF output is ***more than 700 µL***, two or more times dilution of the S-4FB reagent may be required.

   For example if the volume of DMF output is 5857 µL, then three times dilutions are required:

   (1) Make a 58.57X stock solution by dissolving S-4FB pellet in 100 µL of DMF (5857µL/100µL = 58.57).

   (2) Next, make a 5.857X S-4FB solution by adding 10 µL of 58.57X S-4FB solution into 90 µL of DMF and mix well (1:10 dilution).

   (3) Third, make 1X S-4FB solution by adding 10 µ of 5.857X S-4FB solution into 48.57 µL of DMF and mix well (1:5.857 dilution).

3. If the protein molecular weight is *greater than* 50,000 Daltons, add 2.0 µL of S-4FB reagent into the desalted protein. If protein molecular weight is *equal to or less than* 50,000 Daltons add 3.0 µL of S-4FB reagent into the desalted protein. Immediately mix well by vortexing.
4. Incubate the reaction at room temperature for at least 2.5 hours.
5. Proceed to desalt the 4FB-modified protein using a 0.5 mL Zeba™ column equilibrated with **1X Conjugation Buffer** (dilute 10X stock buffer 1/10 with water) using the Zeba desalting protocol on page 8.

2.4 Determining the 4FB Molar Substitution Ratio (MSR)

After desalting with a Zeba™ spin column to remove excess 4FB from the modification reaction, the protein concentration is determined by using either BCA or Bradford assay (the protein concentration must *not* be determined by reading A_{280} on a spectrophotometer at this step because of the contribution at A_{280} of 4FB itself). After the 4FB-protein concentration is determined proceed to MSR assay.

Assay Protocol

1. Transfer 2 µL of 4FB-modified (desalted) protein solution (1-5 mg/mL in 1X Conjugation Buffer) to a new 2.0 mL microfuge tube containing 18 µL of 2-HP working solution. Prepare another reaction tube (blank) containing 2 µL of 1X Conjugation Buffer and 18 µL of 2-HP working solution.
2. Vortex to mix well, then incubate both reaction tubes at 37°C for 1 hour.
3. Remove the reaction tubes from 37°C and briefly centrifuge at 10,000 x g to collect any condensate. Vortex both samples thoroughly before reading the A_{354} by one of the following methods:

Method A: NanoDrop™ Method

1. Launch the NanoDrop™ software and select the UV-VIS menu option. Initialize the instrument with 2 µL

water (NanoDrop™ ND-1000 only).

2. Blank the NanoDrop™ with 2 µL blank (Conjugation Buffer + 2-HP) solution and clean the pedestal.
3. Set the λ1 wavelength to 354nm. Place 2 µL of the 4FB-protein MSR reaction on the pedestal and click the "Measure" icon. The 1.0 mm A354 absorbance will appear. **Multiply this number by 10** (converts from 1 mm to 1 cm) and then enter this value into the 4FB MSR Calculator **Protein-Protein Conjugation Calculator** on worksheet 2 to obtain MSR.

Method B: Cuvette Spectrophotometer Protocol (100 µL, 1-cm micro-cuvette)

1. Prepare a 1:10 dilution of blank (Conjugation Buffer + 2-HP) solution by adding 180 µL of deionized water into the blank sample tube and mix well.
2. Prepare a 1:10 dilution of 4FB-protein MSR reaction solution by adding 180 µL of deionized water into the 4FB-protein MSR reaction tube and mix well.
3. In a 1 cm, 100 µL quartz micro-cuvette, blank the spectrophotometer at 354nm with 100 µL of the blank solution prepared step 1 above.
4. Remove the blank solution and add 100 µL of the 4FB-protein MSR sample solution from step 2 above to the cuvette.
5. Record the A354nm absorbance value of the 4FB-protein MSR sample. **Multiply this number by 10** (to account for the 10-fold dilution) and then enter this value into the 4FB MSR Calculator **Protein-Protein Conjugation Calculator** on calculator worksheet 2 to obtain MSR.

3.0 Protein-Protein Conjugation (Calculator Worksheet 3)

Conjugate formation is initiated by mixing the desired equivalents of each modified protein together with TurboLink Catalyst Buffer. Often protein-2 is added in molar excess (1 – 2 fold) over protein-1 in order to more efficiently drive the conjugation reaction to completion.

1. Using the **Protein-Protein Conjugation Calculator.** On the HyNic-protein-1/4FB-protein-2 conjugation calculator, input both protein names, molecular weights, protein concentrations, the mass of protein-1 to be conjugated in mg, and the mole equivalents of 4FB-protein-2 to conjugate with protein-1 in the green fields. The calculator will determine the volumes of HyNic-modified protein-1 to combine with 4FB-modified protein-2. Mix the two indicated volumes of proteins, and add 1/10 volume of 10X TurboLink Catalyst Buffer. The calculator will output the required volume of 10X TurboLink Catalyst Buffer at blue field.
2. Incubate the reaction at room temperature for 2-3 hours or overnight at 4°C.
3. The reaction is now ready for purification by column chromatography, if required (see section 4 below), or for desalting into storage buffer.
4. The TurboLink™ Catalyst should be removed soon after the conjugation reaction is complete. The reaction solution may either be purified by using chromatography immediately, or may be desalted into storage buffer using either a 0.5 or 2 mL Zeba™ column based on the conjugation reaction volume (0.5 ml Zeba™

column has a capacity of 50 – 150 µL; 2 ml Zeba™ column has a capacity of 150 – 700 µL).

5. If using a 0.5 mL Zeba, proceed to buffer exchange the conjugated proteins into **1X PBS Buffer** as described on page 8. For using the 2 mL Zeba™ column, please see the protocol below.

3.1 Zeba™ Column Desalting Protocol

2 mL Zeba™ Spin Column Preparation and Sample Loading (150 – 700 µL)

1. Remove spin column bottom closure and loosen the top cap (do not remove cap).
2. Place spin column in a 15 mL conical collection tube.
3. Centrifuge at 1,000 x g for 2 minutes. Discard the flow through from the collection tube.
4. Place a mark on the side of the column where the compacted resin is slanted upward. Place column in the 15 mL conical collection tube with the mark facing outward and away from the center of the rotor in all subsequent centrifugation steps.
5. Add 1 mL of **1X PBS Buffer** to the top of the resin bed and centrifuge at 1,000 x g for 2 minutes. Discard the flow through from the collection tube.
6. Repeat step 5 an additional two times, discarding the flow through from the collection tube.
7. Place the Zeba™ column in a new 15 mL conical collection tube, remove cap, and slowly apply sample onto the center of the compacted resin bed (150–700 µL).
   Note- For sample volumes less than 350 µL, apply a 40 µL 1X PBS Buffer stacker to top of the resin bed after the sample has fully absorbed to ensure maximal protein recovery. Avoid contact with the sides of the column when loading.
8. Centrifuge column at 1,000 x g for 2 minutes to collect desalted sample.
9. Determine final protein concentration using BCA or Bradford assay. Add bacteriostat (e.g. 0.05% sodium azide) and/or a protein stabilizer (e.g. 0.5% BSA) if necessary, then store at 4°C.

3.2 Analysis of the Conjugated Proteins

After completion of the conjugation reaction, a small aliquot of the crude reaction mixture is often analyzed using a 4-12% Bis-Tris gel in an SDS (non-reducing) running buffer system. The amount of crude sample loaded on these gels depends on the type and sensitivity of staining method being used. For example, Coomassie blue stain can easily detect 2-4 micrograms of crude conjugate per lane. An appropriate protein marker is loaded side by side, as well as both HyNic- and 4FB-modified proteins as references. The protein conjugate will migrate more slowly in the gel and is visualized as higher molecular weight species (see appendix for an example).

4.0 Purification

All conjugation reactions will be a mixture of reaction products consisting of the desired conjugate along with some un-conjugated HyNic- and 4FB-modified proteins. For this reason, some conjugation reactions are purified using chromatographic methods. Various FPLC or HPLC chromatography workstations are available for this purpose. Solulink routinely purifies conjugates using size exclusion or ion exchange chromatography media. Other media such as metal chelate affinity chromatography or protein A/G columns may be used where

appropriate.

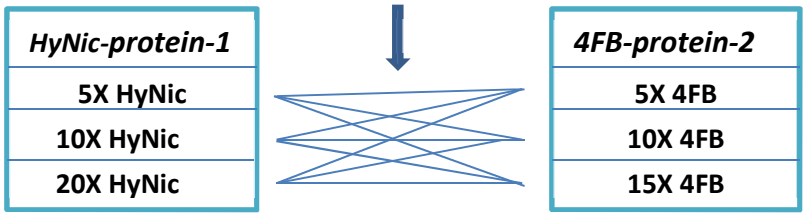
5.0 Troubleshooting

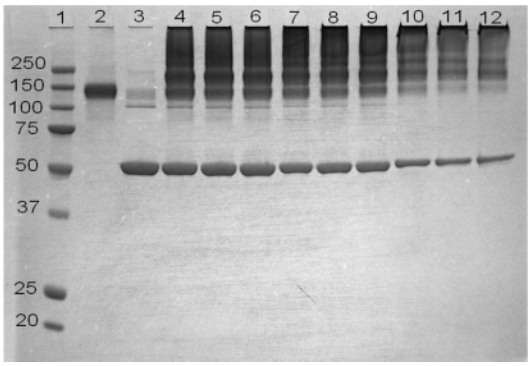
Problem	Possible Cause	Recommended Action
Poor modification of protein	Initial protein concentration is too low	Concentrate protein using a diafiltration filter to 1-5 mg/mL for efficient modification of protein
	Insufficient equivalents of modification reagent added	Add more modification reagent. Up to 40 equivalents can sometimes be added
	Amine contaminant, e.g. Tris or glycine buffer present in starting biomolecule solution	Exchange the protein buffer by desalting, dialysis or diafiltration before modification
	The protein being modified has insufficient amino groups	Verify using the NCBI protein database
Molar substitution assay readings are out of range	Precipitation of the modified protein on treatment with quantification reagents can lead to spurious reading	Make sure all solutions being used are clear
Precipitation of modified protein	Over-modification	Decrease equivalents of modification reagent
Protein precipitates during conjugation reaction	Conjugation reaction pH may be close to the isoelectric point of the conjugate being formed	Conjugate at a different pH, higher or lower than PI (but below pH 6.5)

6.0 Appendix

To determine the best conjugation condition, we recommend that the customer perform a titration of HyNic-protein-1 with 4FB-protein-2 of different equivalents before scaling up, as show below:

Conjugation cross titration guide





Bovine IgG (3.0mg/ml)	5X HyNic	10X HyNic	20X HyNic
MSR	3.2	4.8	8.0
BSA (2.5mg/ml)	5X 4FB	10X 4FB	15X 4FB
MSR	3.5	5.4	6.9

Table 3: MSR of Modified Bovine IgG and BSA

Protein-protein conjugation:

Bovine IgG was modified with 5, 10 or 20 mole equivalents of S-HyNic. BSA was modified with 5, 10 or 15 mole equivalents of S-4FB (Table 3). Then HyNic-bovine IgG and 4FB-BSA were conjugated by each cross titration illustrated above at ratio 1:1.5 of bIgG : BSA. After final desalting, 4ug each of crude conjugated was loaded on a 12% Bis-Tris SDS-PAGE gel, followed by Coomassie staining:

Lane 1. Protein Marker
2. HyNic-Bovine IgG (bIgG)
3. 4FB-BSA
4. 5xHyNic bIgG/5x4FB BSA
5. 5xHyNic bIgG/10x4FB BSA
6. 5xHyNic bIgG/15x4FB BSA
7. 10xHyNic bIgG/5x4FB BSA
8. 10xHyNic bIgG/10x4FB BSA
9. 10xHyNic bIgG/15x4FB BSA
10. 20xHyNic bIgG/5x4FB BSA
11. 20xHyNic bIgG/10x4FB BSA
12. 20xHyNic bIgG/15x4FB BSA

Result:

Lanes 4-6 Most of 1:1 bIgG/BSA conjugated at low mole equivalents of Hynic/4FB modified proteins.
Lanes 7-12 1:2, 1:3 and polymer of bIgG/BSA conjugated are gradually formed based on increasing mole equivalents of Hynic/4FB modified proteins.

Note – This example conjugation is meant as an illustration using model proteins-your sample may require modified condition to yield the desired conjugate. Please contact Solulink if you need more reagents or assistance in choosing condition for modification/conjugation of your particular proteins.