Version 12.20.2018

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.

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Introduction to ChromaLink^{TM} Bioconjugation Technology

The Reaction:

ChromaLink bioconjugation 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 amine-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°C and pH 2.0-10.0. Due to 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.

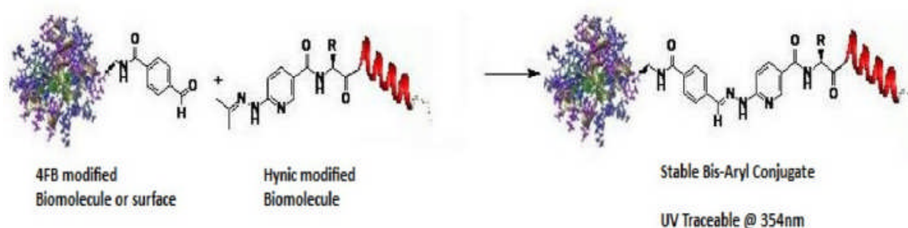


Figure 1: Linking chemistry behind ChromaLink^{TM} technology.

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 light at 354 nm and has a molar extinction coefficient of 29,000 L/(mol*cm).

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 product. The HyNic-4FB conjugation couple catalyzed by aniline yields more than 95% conjugate.

2) **The reaction is efficient:** The reaction is very stoichiometrically efficient as input of only 1-2 moles of 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 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 light at 354 nm and has a molar extinction coefficient of 29,000 L/(mol*cm).

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 groups incorporated per protein) can be quantified colorimetrically as reaction with 2-sulfobenzaldehyde yields a chromophoric product that absorbs at 350 nm with a molar extinction coefficient of 28,500 L/(mol*cm). The MSR of 4FB groups can be determined colorimetrically by its reaction with 2-hydrazinopyridine forming a hydrazone that absorbs at 348 nm with a molar extinction coefficient of 24,500 L/(mol*cm). 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 proteins must be thoroughly desalted, removing all amine contaminants and exchanging the proteins into 1X Modification Buffer.
2. **Protein concentration**: The recommended protein concentrations 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 #	Part #	Size	Storage
S-HyNic	S-9010-1-01	S-1002-1	2 x 1 mg	4°C, Desiccated
S-4FB	S-9010-1-02	S-1004-1	2 x 1 mg	4°C, Desiccated
10X Modification Buffer	S-9010-1-03	S-4000-1.5	1.5 mL	4°C
10X Conjugation Buffer	S-9010-1-04	S-4002-1.5	1.5 mL	4°C
10X TurboLink Catalyst Buffer	S-9010-1-05	S-2006-1.5	1.5 mL	4°C
7 kDa, 0.5 mL Zeba Columns	S-9010-1-06	S-4024-0.5	10	4°C
Anhydrous DMF	S-9010-1-07	S-4001-1.5	2 x 1.5 mL	Desiccated
2-Hydrazinopyridine (2-HP) reagent	S-9010-1-08	S-8082-0.5	1 x 0.5 mL	4°C
0.5 mM 2-Sulfobenzaldehyde	S-9010-1-09	S-8091-0.5	1 x 0.5 mL	4°C
2.0 mL Collection Tubes	S-9010-1-11	S-8014-2	10	Room temp
7 kDa, 2.0 mL Zeba Columns	S-9010-1-13	S-8007-2	2	4°C
10X PBS	S-9010-1-14	S-8002-1.5	1.5 mL	4°C

NOTES:

1) *For convenience all kit components may be stored at 4°C.*
   If precipitate is present in buffers upon storage at 4°C, re-dissolve by warming at 37°C and vortexing before using. Ensure all precipitate is completely dissolved before proceeding.
2) *Anhydrous DMF contains molecular sieves to absorb moisture. These appear as small beads at the bottom of the tube and are not precipitate.*
3) *10X Modification Buffer: 1.0 M sodium phosphate, 1.5 M NaCl; pH 8.0*
4) *10X Conjugation Buffer: 1.0 M sodium phosphate, 1.5 M NaCl; pH 6.0*
5) *10X TurboLink Catalyst Buffer: 100 mM aniline, 100 mM sodium phosphate, 150 mM NaCl; pH 6.0*

Equipment/Regents Required But Not Provided

1) NanoDrop™ or conventional UV-Vis Spectrophotometer
2) Protein concentration assay reagents such as BCA or Bradford protein 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 limitations need to be placed on initial buffer composition, starting mass (mg) and protein 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 guides the user to the first step in the HyNic or 4FB modification 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

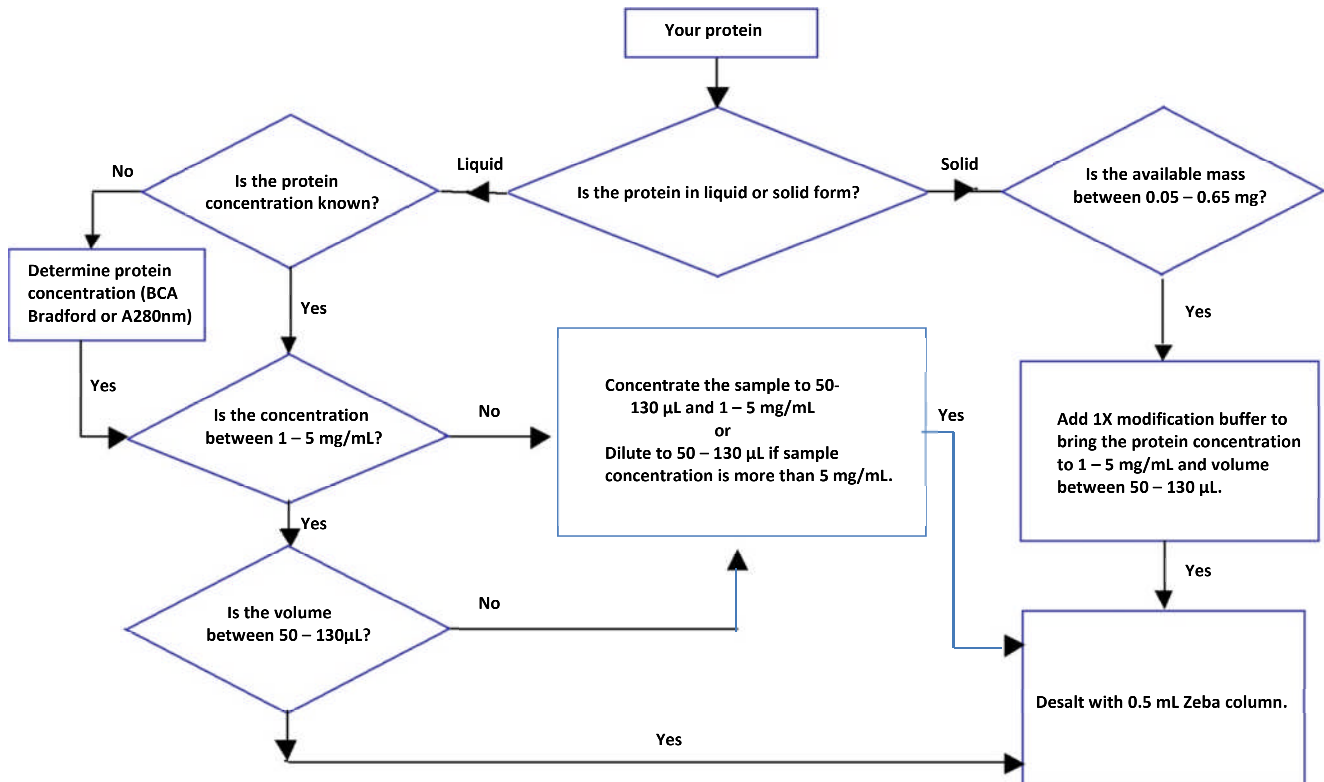


Figure 2. Flow-chart used for guiding a user to the start of the conjugation process.

Protein Desalting

Proteins must be desalted prior to modification. Proteins must be thoroughly desalted into 1X Modification Buffer (dilute 10X Modification Buffer stock 10-fold using protease-free, ultrapure water) 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 the 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 and two 2 mL Zeba™ desalting columns that have a capacity of 50 – 130 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 by twisting the tab until it snaps off, then loosen the cap one-half turn (do not completely remove the cap).
2. Place spin column in a 2 mL microcentrifuge collection tube and place it in the centrifuge. Place a balance tube opposite the column in the centrifuge rotor.
3. Centrifuge at 1,500 x *g* (ensure the centrifuge is set to *g* or RCF, and *not* RPM) 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 each time.
8. Column is now ready for sample loading.

Protein Sample Loading

1. Place the equilibrated spin column in a **new 2 mL collection tube**. Remove cap and slowly apply 50 - 130 µL of protein 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 protein sample.
3. Transfer sample to a new microcentrifuge tube (*e.g.* Eppendorf tube) and cap the sample. Protein sample is desalted and ready for the next step.

2.0 Protein Modification

> ***Note:*** *Modification of Protein 1 with S-HyNic and modification of Protein 2 with S-4FB may be done at the same time. This way the conjugation procedure can be finished in one day. We recommend that the HyNic-modified protein be conjugated within several hours of modification to avoid forming a dimer with itself (homodimer formation). 4FB-modified protein is stable at 4°C for weeks, and does not dimerize.*

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 molecular weight), 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 in Modification Buffer, pH 8.0.

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	7.8
5.0 mg/mL	4.9	6.6	7.9

Table 1: HyNic MSR values at various input equivalents and concentrations.

Note: *It is recommend that the HyNic MSR 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 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 kDa 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 protease-free ultrapure water) following the Zeba desalting procedure on page 8. Confirm protein concentration by spectrophotometer reading 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 in the modification reaction.
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 1.0 mg vial of pre-weighed S-HyNic reagent. Pipette the solution up and down to completely dissolve the pellet. The volume of DMF to dissolve the linker will be output in the pink field of the calculator. If the volume of DMF output is ***more than 700µL***, a dilution of the HyNic reagent will be required.

   For example, if the volume of DMF output is 5857µL, the following dilution is recommended:

   (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 1X S-HyNic solution by adding 10 µL of 58.57X S-HyNic solution into 575.7 µL of DMF and mix well (1:58.57 dilution).

   (3) Use the volume of 1X linker solution specified by the calculator (2 µL for proteins > 50 kDa or 3 µL for proteins ≤ 50 kDa) to modify the protein.
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 worksheet 1. **Immediately mix well by gently inverting the sample several times or by gently vortexing.**
4. Incubate the reaction at room temperature for 1.5 - 2.0 hours.
5. Desalt the HyNic-modified protein using a 0.5 mL Zeba column equilibrated with **1X Conjugation Buffer** (dilute 10X stock buffer 1/10 with protease-free water) using the Zeba desalting protocol on page 8.

2.2 Determining the HyNic Molar Substitution Ratio (MSR)

After desalting the sample to remove excess HyNic from the modification reaction, the protein concentration is determined using either a BCA or Bradford protein assay (the protein concentration must ***not*** be determined by reading the 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 the MSR assay.

MSR Assay Protocol

1. Transfer 2 µL of HyNic-modified and desalted protein solution (1-5 mg/mL in 1X Conjugation Buffer) to a new 1.5 mL microfuge tube containing 18 µL of 2-Sulfobenzaldehyde solution. Prepare another reaction tube (blank) containing 2 µL of 1X Conjugation Buffer and 18 µL of 2-Sulfobenzaldehyde solution.
2. Cap both tubes, vortex to mix well, then incubate both reactions at 37°C for 1 hour.
3. Briefly centrifuge both samples at 10,000 x *g* to collect condensate.

4. Vortex both samples to mix before reading the A_{350} 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 if required (NanoDrop ND-1000 only).
2. Blank the NanoDrop with 2 µL blank (Conjugation Buffer + 2-SBA) solution and clean the pedestal.
3. Set the λ1 wavelength to 350nm. Place 2 µL of the HyNic-protein MSR reaction on the pedestal and click the "Measure" icon. The 1.0 mm A350 absorbance will appear. **Multiply this number by 10** (to convert from 1 mm to 1 cm pathlength) and then enter this value into the HyNic MSR Calculator on worksheet 1 to obtain the HyNic MSR. Be sure to enter the other required information into the green fields as well.

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 350nm with 100 µL of the blank solution prepared in 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 350nm 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 on worksheet 1 to obtain the HyNic MSR. Be sure to enter the other required information into the green fields as well.

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: 4FB MSR values at various input equivalents and concentrations.

Note: *It is recommend that the 4FB MSR 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 protease-free water) following the Zeba desalting procedure on page 8. Confirm protein concentration by spectrophotometer reading 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 in the modification reaction.

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 1.0 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 of the calculator. If the volume of DMF output is ***more than 700 μL***, a dilution of the S-4FB linker will be required.

   For example, if the volume of DMF required is 5857 μL, the following dilution is recommended:
   (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 1X S-4FB solution by adding 10 μL of 58.57X S-4FB solution into 575.7 μL of DMF and mix well (1:58.57 dilution).
   (3) Use the volume of 1X linker solution specified by the calculator (2 μL for proteins > 50 kDa or 3 μL for proteins ≤ 50 kDa) to modify the protein.
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 gently inverting the sample several times or by gently vortexing.**
4. Incubate the reaction at room temperature for 1.5 - 2.0 hours.
5. Desalt the 4FB-modified protein using a 0.5 mL Zeba column equilibrated with **1X Conjugation Buffer** (dilute 10X stock buffer 1/10 with protease-free water) using the Zeba desalting protocol on page 8.

2.4 Determining the 4FB Molar Substitution Ratio (MSR)

After desalting the sample to remove excess 4FB from the modification reaction, the protein concentration is determined by using either a BCA or Bradford protein 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 the MSR assay.

MSR Assay Protocol

1. Transfer 2 μL of 4FB-modified and desalted protein solution (1-5 mg/mL in 1X Conjugation Buffer) to a new 1.5 mL microfuge tube containing 18 μL of 2-HP solution. Prepare another reaction tube (blank) containing 2 μL of 1X Conjugation Buffer and 18 μL of 2-HP solution.
2. Cap both tubes, 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 condensate. Vortex both samples thoroughly before reading the A_{248} 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 if required (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 348nm. Place 2 µL of the 4FB-protein MSR reaction on the pedestal and click the "Measure" icon. The 1.0 mm A348 absorbance will appear. **Multiply this number by 10** (to convert from 1 mm to 1 cm pathlength) and enter this value into the 4FB MSR Calculator on worksheet 2 to obtain MSR. Be sure to enter the other required information in the green fields.

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 348nm 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 A348nm absorbance value of the 4FB-protein MSR sample. **Multiply this number by 10** (to account for the 10-fold dilution) and enter this value into the 4FB MSR Calculator on calculator worksheet 2 to obtain MSR. Be sure to enter the other required information in the green fields.

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 (1.5 – 2 fold) over protein-1 in order to more efficiently drive the conjugation reaction to completion.

1. On the HyNic-protein-1 / 4FB-protein-2 conjugation calculator (tab 3), input both protein names, molecular weights, protein concentrations, the mass of protein-1 to be conjugated (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, add 1/10 volume of 10X TurboLink Catalyst Buffer, and mix well. The calculator will output the required volume of 10X TurboLink Catalyst Buffer in the 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 (PBS).
4. The TurboLink™ Catalyst should be removed after the conjugation reaction is complete. The conjugation reaction may either be purified using chromatography immediately, or may be desalted into storage buffer (PBS) 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 – 130 µL; the 2 ml Zeba column has a capacity of 130 – 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 (dilute 10X stock PBS 1/10 with protease-free water). For desalting with the 2 mL Zeba™ column please see the procedure below.

3.1 2mL Zeba™ Column Desalting Protocol

2 mL Zeba™ Spin Column Preparation and Sample Loading (130 – 700 µL)

1. Remove spin column bottom closure by twisting and snapping off the bottom tab. Loosen the cap one-half turn (do not remove cap).
2. Place spin column in a 15 mL conical collection tube. Place spin column in centrifuge with an appropriate balance tube opposite the desalting column.
3. Centrifuge at 1,000 x *g* for 2 minutes (ensure the centrifuge is set to RCF or *g* and **not** RPM). 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 conjugate sample onto the center of the compacted resin bed (150–700 µL).

   Note: For sample volumes less than 400 µL, apply a 75 µL PBS Buffer stacker to the top of the resin bed after the conjugate sample has fully absorbed to ensure maximum recovery.
8. Centrifuge column at 1,000 x *g* for 2 minutes to collect desalted sample. Transfer desalted conjugate from the 15 mL conical tube into a new storage tube, such as a sterile 1.5 mL microcentrifuge tube, and label appropriately.
9. Determine final protein concentration using a BCA or Bradford protein assay. Add bacteriostat (e.g. 0.05% sodium azide or 0.01% thimerosal) 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 in each lane depends on the type of stain and the sensitivity of the staining method used. For example, Coomassie blue stain can easily detect 2-4 micrograms of conjugate per lane and 1 microgram of individual protein. 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 (*e.g.* Superdex 200) or ion exchange (*e.g.* Q or S Sepharose) chromatography. Other media such as metal chelate affinity chromatography or protein A/G columns may be used where appropriate.

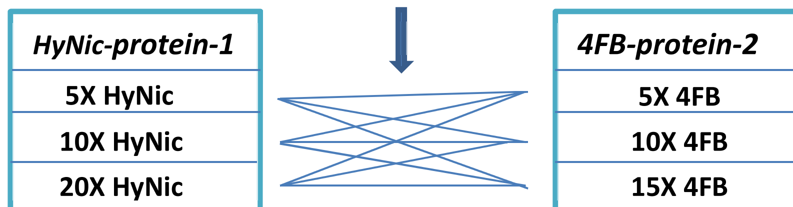
5.0 Troubleshooting

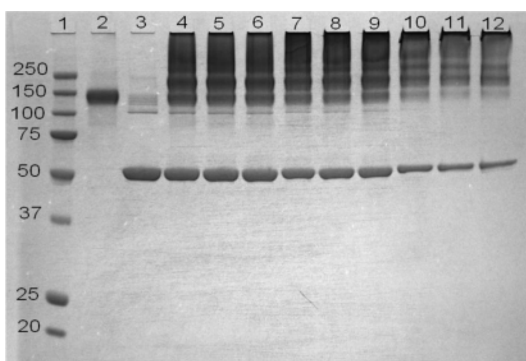
Problem	**Possible Cause**	**Recommended Action**
Poor modification of protein	Initial protein concentration is too low	Concentrate protein using a diafiltration apparatus to 1-5 mg/mL for efficient modification of protein
	Insufficient equivalents of linker added	Use higher equivalents of linker to protein. Up to 30 equivalents can sometimes be added
	Amine contaminant, *e.g.* Tris or glycine, present in starting biomolecule solution	Buffer exchange the protein by desalting, dialysis or diafiltration before modification
	The protein being modified has insufficient amino groups	Verify using the NCBI protein database, if possible
Molar substitution assay readings are out of range	Precipitation of the modified protein on treatment with quantification reagents can lead to spurious reading	Ensure MSR reactions are clear and free of precipitate
Precipitation of linker-modified protein	Over-modification with linker	Decrease equivalents of linker to protein in the modification reaction
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 optimal conjugation conditions, we recommend a titration of HyNic-protein-1 with 4FB-protein-2 of different equivalents before scaling up, as shown below:

Conjugation cross titration guide





Bovine IgG (3.0 mg/mL)	5X HyNic	10X HyNic	20X HyNic
MSR	3.3	5.4	8.0
BSA (2.5 mg/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). HyNic-bovine IgG and 4FB-BSA were conjugated by each cross titration illustrated above at mole ratio of 1:1.5 (bIgG:BSA). After final desalting, 4ug of each conjugate 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 Mainly 1:1 bIgG/BSA conjugate at low mole equivalents of HyNic/4FB modified proteins.

Lanes 7-12 1:2, 1:3 and polymer bIgG/BSA conjugate are predominantly 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 conditions to yield the desired conjugate. Please contact Solulink if you need assistance in choosing conditions for modification/conjugation of your particular proteins.