USER GUIDE

# Protein–Protein Conjugation Kit

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**Cat. No.** **S-9010-1**

**Storage** 2°–8°C — Do Not Freeze.

*The Protein-Protein Conjugation Kit requires 50 - 650 µg of each protein at a concentration of 1–5 mg/ml in a volume of 50–130 µl. The kit is suitable for conjugating proteins with a molecular weight range of 25,000–950,000 Daltons.*

## Introduction

The Protein-Protein Conjugation Kit contains all the necessary reagents and components to perform two separate protein-protein conjugations easily and efficiently. Based on SoluLINK® bioconjugation technology, the proteins are modified and conjugated within a day, involving just 90 minutes of hands-on time. First, one protein is modified with S-HyNic and the other with 4FB. Next, the molar substitution ratios are determined and the two proteins are mixed together (Figure 1). Because the HyNic-4FB conjugate bond is chromophoric, the conjugation reaction can be monitored spectrophotometrically. The conjugation reaction is stoichiometrically efficient, requiring only a slight excess (1.5–2 moles) of one of the proteins for complete conversion to conjugate.

## Kit Components

| Component | Amount |
| --- | --- |
| S-HyNic | 2 x 1.0 mg |
| S-4FB | 2 x 1.0 mg |
| 10X Modification Buffer | 1.5 ml |
| 10X Conjugation Buffer | 1.5 ml |
| 10X TurboLINK™ Catalyst Buffer | 1.5 ml |
| 7 kDa, 0.5 ml Thermo Scientific™ Zeba™ Column | 10 |
| Anhydrous DMF | 2 x 1.5 ml |
| 2-Hydrazinopyridine (2-HP) reagent | 0.5 ml |
| 0.5 mM 2-Sulfobenzaldehyde (2-SB) | 0.5 ml |
| 2 ml Collection Tube | 10 |
| 7 kDa, 2 ml Zeba Column | 2 |
| 10X PBS | 1.5 ml |

## Protocol

Before using the Protein-Protein Conjugation Kit, remove from refrigerated storage and allow components to warm up to room temperature for at least 30 minutes. If precipitate is present in the 10X buffers, redissolve by warming at 37°C and vortexing before use. Ensure all precipitate is completely dissolved before proceeding. Anhydrous DMF contains molecular sieves to absorb moisture. These appear as small beads at the bottom of the tube and are not precipitate.

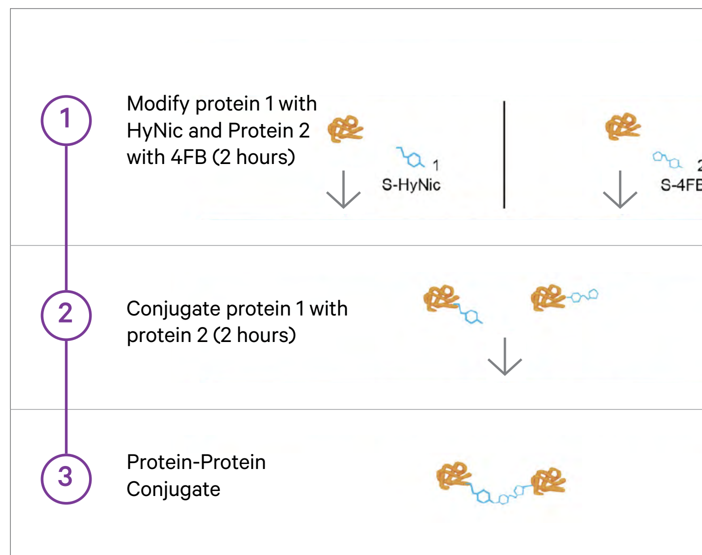


*Figure 1. Protein-Protein Conjugation Kit workflow.*

Modification of Protein 1 with S-HyNic and Protein 2 with S-4FB may be done at the same time, allowing the conjugation procedure to be finished in one day. If both proteins will be modified at the same time, prepare two spin columns (step A) and desalt both proteins simultaneously (step B). We recommend the proteins be conjugated the same day they are modified for best results.

### A. Prepare 0.5 ml Zeba column(s)

The Modification and Conjugation Buffers in this kit are supplied as 10X stock solutions. Before proceeding, create a 1X working solution of each buffer by mixing 500 µl of 10X stock with 4.5 ml of ultrapure water per conjugation reaction to be performed.

1. Prepare a 0.5 ml Zeba column(s) (red cap) by loosening the cap one-half turn, twisting off the bottom closure, and placing in an empty 2 ml collection tube.
2. Using a lab marker, place a vertical line on the outside of the column(s). Ensure that this line faces outward (away from the center of the rotor) in this and all subsequent steps.
3. Centrifuge the column(s) at 1,500 x g for 1 minute to remove storage buffer. If only one column is used, balance the centrifuge with a microcentrifuge tube containing water.

   **Important:** Ensure the centrifuge is set to "g" or RCF rather than RPM in all centrifugation steps.
4. Discard the storage buffer and place the column(s) back into the same tube(s).

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5. Add 300 µl of 1X Modification Buffer to the top of the resin bed of each column and centrifuge at 1,500 x g for 1 minute.

6. Repeat steps 4 and 5 two additional times.

7. The column(s) is now ready for protein loading.

**B. Desalt the protein(s)**

1. Place the equilibrated Zeba column(s) in a new 2 ml collection tube(s).

2. Remove the 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.

3. Repeat for the second protein if both are to be desalted simultaneously.

4. Centrifuge at 1,500 x g for 2 minutes to collect desalted protein into the collection tube(s).

5. Transfer the sample(s) to new microcentrifuge tube(s) and label appropriately.

   The protein(s) are now desalted.

<u>Recommended Guidelines for Modifying Proteins with S-HyNic and S-4FB</u>

The modification process is a critical element of any conjugation project. The number of linkers 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 or S-4FB is a function of several variables that include protein concentration, number of available amino groups on the protein (often related to molecular weight), and the mole excess of linker equivalents added to the protein (e.g. 5X, 10X or 20X). Table 1 and Table 2 present the results of a study to determine the level of linker incorporation on an antibody after adding 5X, 10X and 20X mole equivalents of S-HyNic or S-4FB at 1.0, 2.5 and 5.0 mg/ml protein 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 values at various input equivalents and concentrations.*

**Note:** It is recommended that the HyNic MSR is 4–8 for proteins > 100,000 Daltons, and 2–4 for proteins ≤ 100,000 Daltons.

| 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:** The optimal 4FB MSR is 4–6 for proteins > 100,000 Daltons, and 2–4 for proteins ≤ 100,000 Daltons.

As the protein concentration and number of linker equivalents are increased, the MSR increases. Caution is recommended, however, since over-modification can change the isoelectric point of a protein and result in precipitation or loss of biological activity. This is especially important with proteins < 50 kDa molecular weight.

**C. Modify Protein 1 with S-HyNic**

Prior to modification of the proteins with S-HyNic or S-4FB, determine their concentrations using a UV-Vis spectrophotometer at 280 nm.

1. On the S-HyNic modification worksheet 1 of the Protein-Protein Conjugation Calculator input the name, molecular weight, concentration, and volume of protein 1.

2. Enter the mole equivalents of S-HyNic to modify protein 1 in the light green input field of the linker information section.

3. The volume of Anhydrous DMF required to dissolve the linker will appear in the orange field of the calculator.

4. Add the calculated volume of Anhydrous DMF to a 1.0 mg vial of S-HyNic. Pipette the solution up and down to completely dissolve the pellet.

   **Note:** If the volume of Anhydrous DMF required is > 700 µl, a dilution of the linker reagent will be necessary. For example, if the volume of Anhydrous DMF calculated is 5,857 µl, the following dilution is recommended:

   (1) Make a 58.57X stock solution by dissolving the linker in 100 µl of Anhydrous DMF (5,857 µl/100 µl = 58.57).

   (2) Next, make a 1X linker solution by adding 10 µl of 58.57X linker into 575.7 µl of Anhydrous DMF and mix well (1:58.57 dilution).

   (3) Use the volume of 1X linker solution specified by the calculator to modify the protein (step 6, below).



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5. The calculator will display the volume of S-HyNic to add to the protein.

   **Note:** If the protein molecular weight is > 50,000 Daltons, add 2.0 µl of S-HyNic reagent into the desalted protein. If the protein molecular weight is ≤ 50,000 Daltons, add 3.0 µl of S-HyNic.

6. Add the calculated volume of S-HyNic to the protein and immediately mix by pipetting up and down, then gently vortexing or flicking the tube.

7. Incubate the reaction at room temperature for 1.5–2.0 hours.

8. Desalt the HyNic-modified protein using a 0.5 ml Zeba column (red cap) equilibrated with 1X Conjugation Buffer instead of Modification Buffer, as described in steps A and B.

After desalting the sample to remove excess HyNic from the modification reaction, the protein concentration should be determined using either a BCA or Bradford protein assay. The protein concentration must not be determined by reading the A280 on a spectrophotometer at this step because the incorporated linkers inflate the absorbance at this wavelength. After the HyNic-modified protein concentration has been determined, enter this value into the HyNic MSR Calculator also on worksheet 1 of the Protein-Protein Conjugation Calculator. Proceed to the HyNic MSR determination.

D. Determine the HyNic molar substitution ratio (MSR) - optional

1. Transfer 2.0 µl of HyNic-modified and desalted protein solution to a 1.5 ml microcentrifuge tube containing 18.0 µl of 2-sulfobenzaldehyde (2-SB) solution.

2. Prepare a reaction blank by adding 2.0 µl of 1X Conjugation Buffer to 18.0 µl of 2-SB solution.

3. Cap both tubes, vortex to mix, then incubate both reactions at 37°C for 1 hour.

4. Briefly centrifuge both samples at 10,000 x g to collect condensate at the bottom of the tube, then vortex to mix.

5. Read the absorbance at 350 nm using either a conventional UV-Vis spectrophotometer or a micro-volume spectrophotometer (e.g., NanoDrop™) by following the appropriate protocol below.

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 instrument with 2 µl of the blank solution prepared above.

3. Set the λ1 wavelength to 350 nm. Place 2 µl of the HyNic-protein MSR reaction on the pedestal and click the "Measure" icon.

4. The 1-mm 350 nm absorbance will appear. Multiply this number by 10 to convert from 1-mm to a 1-cm pathlength and enter this value into the HyNic MSR Calculator on worksheet 1 to obtain the HyNic MSR.

   **Note:** Ensure the HyNic protein concentration has been entered into the MSR calculator as well.

Method B: Conventional UV-Vis spectrophotometer (≤ 200 µl micro-cuvette)

1. Prepare a 1:10 dilution of both the blank and MSR reactions by adding 180 µl of water to each tube.

2. Mix well by vortexing.

3. Using a 1-cm pathlength quartz micro-cuvette, blank the spectrophotometer at 350 nm with the blank solution prepared above.

4. Read the 350 nm absorbance of the HyNic-protein MSR sample.

5. Multiply this number by 10 to account for the 10-fold dilution.

6. Enter this value into the HyNic MSR Calculator on worksheet 1 to obtain the HyNic MSR.

   **Note:** Ensure the HyNic protein concentration has been entered into the MSR calculator as well.

E. Modify Protein 2 with S-4FB

Prior to modification of the proteins with S-HyNic or S-4FB, determine their concentrations using a UV-Vis spectrophotometer at 280 nm.

1. On the Protein 2/S-4FB Mocificaiton section of worksheet 2 of the Protein-Protein Conjugation Calculator input the name, molecular weight, concentration, and volume of protein 2.

2. Enter the mole equivalents of S-4FB to modify protein 2 in the light green input field of the linker information section.

3. The volume of Anhydrous DMF required to dissolve the linker will appear in the orange field of the calculator.

4. Add the calculated volume of Anhydrous DMF to a 1.0 mg vial of S-4FB. Pipette the solution up and down to completely dissolve the pellet.

   **Note:** If the volume of Anhydrous DMF required is more than 700 µl, follow the dilution instructions in step C.4. above.



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5. The calculator will display the volume of S-4FB to add to the protein.
6. Add the calculated volume of S-4FB and immediately mix by pipetting up and down, then gently vortexing or flicking the tube.
7. Incubate the reaction at room temperature for 1.5–2.0 hours.
8. Desalt the 4FB-modified protein using a 0.5 ml Zeba column (red cap) equilibrated with **1X Conjugation Buffer** instead of Modification Buffer, as described in steps A and B.

After desalting the sample to remove excess 4FB from the modification reaction, the protein concentration should be 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 the incorporated linkers inflate the absorbance at this wavelength. After the 4FB-modified protein concentration has been determined enter this value into the 4FB MSR calculator on worksheet 2 of the Protein-Protein Conjugation Calculator. Proceed to the 4FB MSR determination.

### F. Determine the 4FB molar substitution ratio (MSR) - optional

1. Transfer 2.0 µl of 4FB-modified protein solution to a 1.5 ml microcentrifuge tube containing 18.0 µl of 2-hydrazinopyridine (2-HP) solution.
2. Prepare a reaction blank by adding 2.0 µl of 1X Conjugation Buffer to 18.0 µl of 2-HP solution in a separate tube.
3. Cap both tubes, vortex to mix, then incubate both reactions at 37°C for 1 hour.
4. Briefly centrifuge both samples at 10,000 x g to collect condensate at the bottom of the tubes, then vortex to mix.
5. Read the absorbance at 348 nm using either a conventional UV-Vis spectrophotometer or a micro-volume spectrophotometer (e.g., NanoDrop) by following the appropriate protocol below.

#### 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 instrument with 2 µl of the blank solution prepared above.
3. Set the λ1 wavelength to 348 nm. Place 2 µl of the 4FB-protein MSR reaction on the pedestal and click the "Measure" icon.
4. The 1-mm 348 nm absorbance will appear. Multiply this number by 10 to convert from 1-mm to a 1-cm pathlength and enter this value into the 4FB MSR Calculator on worksheet 2 to obtain the 4FB MSR.

   **Note:** Ensure the 4FB protein concentration has been entered into the MSR calculator as well.

#### Method B: Conventional UV-Vis spectrophotometer (≤ 200 µl micro-cuvette)

1. Prepare a 1:10 dilution of both the blank and MSR reactions by adding 180 µl of water to each tube.
2. Mix well by vortexing.
3. Using a 1-cm pathlength quartz micro-cuvette, blank the spectrophotometer at 348 nm with the blank solution prepared above.
4. Read the 348 nm absorbance of the 4FB-protein MSR sample.
5. Multiply this number by 10 to account for the 10-fold dilution.
6. Enter this value into the 4FB MSR Calculator on worksheet 2 to obtain the 4FB MSR.

   **Note:** Ensure the 4FB protein concentration has been entered into the MSR calculator as well.

### G. Conjugate the proteins

Conjugate formation is initiated by mixing the desired mole equivalents of each modified protein together with TurboLINK Catalyst Buffer. Often protein 2 is added in slight mole excess (1.5–2-fold) over protein 1 to more efficiently drive the conjugation reaction to completion.

1. On worksheet 3 of the Protein-Protein Conjugation Calculator, input both protein names, molecular weights, protein concentrations, the mass of HyNic-protein 1 to be conjugated, and the mole equivalents of 4FB-protein 2 to conjugate with HyNic-protein 1.
2. The calculator will display the volume of HyNic-modified protein 1 to combine with 4FB-modified protein 2.
3. Mix the calculated volume of each protein together in a microcentrifuge tube.
4. Add the calculated volume of 10X TurboLINK Catalyst Buffer and mix well.

   **Note:** add the 10X TurboLINK Catalyst Buffer directly — it is not necessary to dilute it to 1X concentration first.

5. Incubate the reaction for 2–3 hours at room temperature, or overnight at 4°C.



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H. Conjugate buffer exchange or purification

After conjugation, the protein-protein conjugate may be purified if desired, or buffer exchanged into storage buffer. Size exclusion chromatography, ion exchange chromatography, hydrophobic interaction chromatography, and other methods have been used successfully to purify conjugates prepared using the Protein-Protein Conjugation Kit.

If further purification is not required, buffer exchange the conjugate into PBS or another suitable storage buffer. 10X PBS, pH 7.2, is provided with the kit but any buffer suitable for maintaining protein stability may be used. If using PBS, dilute the 10X stock by adding 0.5 ml into 4.5 ml of ultrapure water and mixing well.

Both a 0.5 ml and a 2 ml Zeba column are provided for buffer exchange, based on the volume of conjugate. The 0.5 ml Zeba column has a capacity of up to 130 µl, while the 2 ml Zeba column has a capacity of up to 700 µl. If the conjugate volume is 130 µl or less, equilibrate a 0.5 ml Zeba column as described in steps A and B using the desired buffer. Follow the procedure below if the conjugate volume is greater than 130 µl.

I. 2 ml Zeba column buffer exchange protocol

1. Prepare a 2 ml Zeba column by loosening the cap one-half turn, twisting off the bottom closure, and placing it in an empty 15 ml conical tube.
2. Using a lab marker, place a vertical line on the outside of the column. Ensure that this line faces outward (away from the center of the rotor) in this and all subsequent steps.
3. Centrifuge the column at 1,000 x g for 2 minutes to remove storage buffer.

   **Important:** Ensure the centrifuge is set to "g" or RCF rather than RPM in all centrifugation steps.
4. Discard the buffer and place the spin column back into the tube.
5. Add 1 ml of 1X PBS (or other suitable buffer) to the top of the resin bed and centrifuge at 1,000 x g for 2 minutes.
6. Repeat steps 4 and 5 an additional two times.
7. Place the 2 ml Zeba column in a new 15 ml conical tube.
8. Remove the cap and slowly apply the conjugate sample onto the center of the resin bed.

   **Important:** For sample volumes less than 500 µl, apply a 200 µl buffer stacker to the top of the resin bed after the conjugate sample has been fully absorbed to ensure maximum recovery.
9. Centrifuge the column at 1,000 x g for 2 minutes to collect the conjugate.
10. Transfer the buffer exchanged conjugate to a storage tube and label appropriately.

The conjugate concentration may be determined using a Bradford or BCA assay. Absorbance at 280 nm will not give an accurate protein concentration due to the absorbance of the linkers at this wavelength. Follow the manufacturer's instructions for the protein concentration determination method used. A bacteriostatic agent such as sodium azide or thimerosal may be added to prolong the shelf life of the conjugate. For labile proteins, a protein stabilizer such as BSA or gelatin may be added after the concentration has been determined.

## Application Notes

Bradford Assay Protocol

BCA Assay Protocol

Troubleshooting Guide



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