USER GUIDE

R-PE Antibody Conjugation Kit

Together we breakthrough™

Cat. No. P-9002-002

Storage 2°–8°C—Do Not Freeze.

Description The R-PE Antibody Conjugation Kit contains all the necessary reagents and components to produce two R-PE antibody conjugates. Based on SoluLINK® bioconjugation technology, it allows any purified antibody (free of carrier protein) to be conjugated and purified within 7 hours, involving less than 2 hours of hands-on time. First, the antibody is modified with S-HyNic, then the HyNic-modified antibody is conjugated to pre-activated 4FB-R-PE. After conjugation, the R-PE Antibody conjugate may be further purified by size exclusion chromatography to remove any excess R-PE, or simply buffer exchanged using the included Thermo Scientific™ Zeba™ column and used immediately. This kit has been designed to conjugate between 150 µg and 1.3 mg of each antibody to R-PE. It includes four 0.5 ml Zeba desalting columns, each with a maximum capacity of 130 µl, allowing up to 1.3 mg of a 10 mg/ml antibody solution to be desalted in preparation for modification and conjugation. Lower protein concentrations (3.0 mg/ml, minimum) and volumes (50 µl, minimum) may also be used successfully.

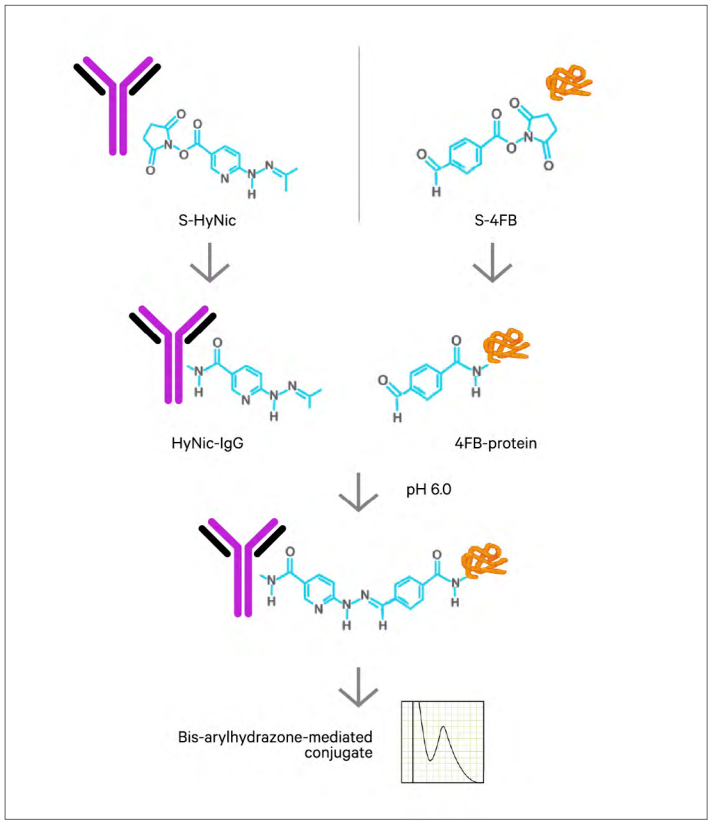

Figure 1. SoluLINK bioconjugation workflow.

Kit Components

Component	Amount
S-HyNic	2 x 1.0 mg
4FB-R-PE	2 x 3.2 mg
10X Modification Buffer	1.5 ml
10X Conjugation Buffer	1.5 ml
10X TurboLINK™ Catalyst Buffer	1.5 ml
0.5 ml Zeba Column	4
Anhydrous DMF	1.5 ml
2 ml Collection Tube	12
2 ml Zeba Column	2
2-Sulfobenzaldehyde (2-SB)	10 mg
10X MES Buffer	1.5 ml

Protocol

Before using the R-PE Antibody 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 any of the 10X buffers, dissolve the precipitate by warming in a 37°C water bath with periodic vortexing.

A. Prepare a 0.5 ml Zeba column

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

1. Prepare a 0.5 ml Zeba column (red cap) by loosening the cap one-half turn, twisting off the bottom closure, and placing it in an empty 2 ml collection 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 1,500 x g for 1 minute to remove storage buffer.

   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 spin column back into the same tube.
5. Add 300 µl of 1X Modification Buffer 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.
7. The column is now ready for antibody loading.

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B. Desalt the antibody and determine concentration

1. Place the equilibrated Zeba column in a new 1.5 ml collection tube, remove the cap, and slowly apply 50–130 µl of antibody solution to the center of the resin bed. Be careful not to let the antibody solution contact the tube wall; it must channel down through the resin itself.

   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.
2. Centrifuge at 1,500 x g for 2 minutes to collect the desalted antibody sample.
3. Transfer the desalted antibody into a microcentrifuge tube while measuring the volume with a pipet.
4. Discard the desalting column and collection tube after the antibody has been transferred.
5. Mix the antibody solution by gently vortexing or flicking the tube.
6. Measure the antibody concentration using a conventional UV-Vis spectrophotometer and quartz cuvette or a NanoDrop™ micro-volume spectrophotometer.

C. Modify the antibody with S-HyNic

The number of HyNic linker molecules incorporated per antibody 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. Most important among these are the antibody concentration and mole equivalents of linker added (e.g. 10X, 15X or 20X). As the antibody concentration and number of linker equivalents are increased, the MSR increases. Caution is recommended, however, since over-modification can change the isoelectric point of the antibody and result in precipitation or loss of immunoreactivity.

Antibody HyNic MSR values between 3.5–6.0 are ideal for conjugation to 4FB-modified R-PE. Therefore, an input of 20 mole equivalents of S-HyNic is recommended at an antibody concentration of 2.5 mg/ml, 15 mole equivalents of S-HyNic is recommended at 5.0 mg/ml, and 10 mole equivalents is recommended at 10 mg/ml. Optimization of conditions may be required to achieve maximal assay results.

On worksheet 1 of the Antibody / R-Phycoerythrin Conjugation Calculator, enter the antibody information in the light green input fields. Be sure to include the antibody concentration and mass of antibody to be modified with S-HyNic. The calculator will indicate the volume of antibody to be used in the modification reaction.

Next, enter the linker information in the appropriate fields. The S-HyNic supplied with this kit is a pre-weighed 1.0 mg aliquot. Enter the mole equivalents of S-HyNic desired for modification, as well as the volume of Anhydrous DMF used to dissolve the S-HyNic linker. Typically 100 µl of Anhydrous DMF is sufficient, however this volume can be increased such that the volume of linker required to modify the antibody is between 2–3 µl for greater pipetting accuracy. The volume of HyNic linker in Anhydrous DMF should not exceed 5% of the antibody volume. Ensure the actual amount of Anhydrous DMF used to dissolve the S-HyNic aliquot is entered into the calculator.

1. Dissolve a 1.0 mg vial of pre-weighed S-HyNic in 100 µl (or suitable volume) of Anhydrous DMF. Enter the volume of Anhydrous DMF used into the calculator.
2. Add the calculated volume of S-HyNic to the antibody and immediately pipet up and down, then vortex gently or flick the tube to mix.
3. Incubate the reaction at room temperature for 1.5 hours.

D. Desalt HyNic-modified antibody

Equilibrate a 0.5 ml Zeba desalting column as described in section A, except using 1X Conjugation Buffer instead of Modification Buffer. Desalt the HyNic-modified antibody into 1X Conjugation Buffer as described in section B. After modification with S-HyNic, the antibody concentration must be determined using a BCA or Bradford protein assay. Due to the UV absorption of the incorporated HyNic linkers, a spectrophotometer A_{280} reading cannot be used to accurately determine the antibody concentration during this or subsequent steps. A standard curve using bovine gamma globulins (bovine IgG) should be used rather than BSA or other protein for accurate results. Prepare the BCA or Bradford working solution and perform the assay according to the manufacturer's instructions.

E. Determine the HyNic molar substitution ratio (MSR)

Protocols to determine the MSR (number of HyNic groups per antibody) using a standard UV-Vis spectrophotometer (Method A) or a NanoDrop spectrophotometer (Method B) are described below. Both protocols begin by preparing the 2-Sulfobenzaldehyde (2-SB) assay reagent.

1. Prepare a 1X MES Buffer solution by adding 0.5 ml of 10X MES Buffer to 4.5 ml of ultrapure water in a 15 ml conical tube.
2. Weigh approximately 3–5 mg of 2-SB into a microcentrifuge tube while recording the exact mass weighed.
3. Dissolve the 2-SB in water at a concentration of 20 mg/ml. Vortex to completely dissolve.
4. Add 26.0 µl of this solution to the 15 ml conical tube containing 5.0 ml of 1X MES Buffer and mix well. Label this solution 0.5 mM 2-SB.

Protect the 2-SB solution from light and keep refrigerated. This solution remains stable for up to 90 days at 4°C.

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MSR Method A: Standard UV-Vis Spectrophotometer

1. Transfer 10 µl of HyNic-modified antibody solution to a 1.5 ml microcentrifuge tube containing 490 µl of 0.5 mM 2-SB solution.
2. Prepare a reaction blank by adding 10 µl of 1X Conjugation Buffer to 490 µl of 0.5 mM 2-SB solution in a separate microcentrifuge tube. Mix both reactions well by vortexing.
3. Incubate both reactions at 37°C for 60 minutes or at room temperature for 90 minutes.
4. After the incubation period is complete, centrifuge both tubes at 10,000 x g for 15 seconds to collect condensation at the bottom of the tubes if a 37°C water bath was used. Vortex both samples to mix.
5. Blank the spectrophotometer at 350 nm using 500 µl of the blank sample.
6. Record the 350 nm absorbance value of the HyNic MSR sample.

MSR Method B: NanoDrop Spectrophotometer Protocol

1. Transfer 2.0 µl of HyNic-modified antibody solution to a 1.5 ml microcentrifuge tube containing 18.0 µl of 0.5 mM 2-SB solution.
2. Prepare a reaction blank by adding 2.0 µl of 1X Conjugation Buffer to 18.0 µl of 0.5 mM 2-SB solution in a separate microcentrifuge tube.
3. Mix both reactions well by vortexing, followed by a quick centrifuge spin to collect all liquid at the bottom of the tubes.
4. Incubate both reactions at 37°C for 60 minutes or at room temperature for 90 minutes.
5. Centrifuge both reactions at 10,000 x g for 15 seconds to collect condensation at the bottom of the tubes if a 37°C water bath was used. Vortex both samples to mix.
6. Open the NanoDrop UV-Vis module and blank the instrument at 350 nm using the blank sample.
7. Record the 350 nm absorbance of the HyNic MSR sample. Ensure the 350 nm absorbance value is normalized to a 10-mm (1-cm) pathlength.

After the 350 nm absorbance value is determined by either method above, enter the required information into the light green input fields of the Antibody / R-Phycoerythrin Conjugation Calculator under the HyNic MSR Calculator section. A HyNic-antibody MSR value of ≥ 3.5 is required for efficient conjugation to 4FB-R-PE.

F. Conjugate HyNic-modified antibody to 4FB-R-PE

1. On Worksheet 2 of the Antibody/R-Phycoerythrin Conjugation Calculator, input the concentration of the HyNic-modified antibody and the mass of HyNic-modified antibody to be conjugated to 4FB-R-PE. Also enter the antibody name and molecular weight.
2. 4FB-R-PE is supplied at 7.5 mg/ml. Enter this concentration value into the 4FB-R-Phycoerythrin section of the calculator (if necessary).
3. Enter the desired mole equivalents of 4FB-R-PE to be used per antibody. 1.2 equivalents is recommended to prepare a 1:1 mole ratio PE:antibody conjugate, or up to 2.0 equivalents may be used to create a conjugate with a larger mole ratio of R-PE to antibody.
4. Add the calculated volume of 4FB-R-PE to the HyNic-modified antibody solution and mix well.
5. Add the calculated volume of 10X TurboLINK Catalyst Buffer to the conjugation reaction and mix well.

   Note: Add the indicated volume of 10X TurboLINK Catalyst Buffer directly to the conjugation reaction (no prior dilution to 1X is necessary).
6. Incubate the conjugation reaction at room temperature for 2 hours.

G. Conjugate Purification/Desalting

After the 2-hour incubation period is complete, the conjugate may be purified by size exclusion chromatography, or desalted into 1X PBS (or another suitable buffer) for immediate use and storage. A 2 ml Zeba desalting column is included with the kit for desalting the conjugate into the desired storage buffer.

Desalting using a 2 ml Zeba column

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 column back into the tube.
5. Add 1.0 ml of PBS, pH 7.2, or other desired storage buffer to the top of the resin bed and loosely cap the column.
6. Centrifuge the column at 1,000 x g for 2 minutes.
7. Repeat steps 4–6 two additional times.
8. Place the column in a new 15 ml conical tube.
9. Slowly apply up to 700 µl of conjugate to the top of the resin bed without disturbing the resin.

   Important: For sample volumes less than 500 µl, apply a 200 µl buffer stacker to the top of the resin after the conjugate has been fully absorbed to maximize conjugate recovery.

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10. Loosely cap the column and centrifuge at 1,000 x g for 2 minutes to collect the desalted conjugate.
11. Transfer the desalted conjugate into a labeled storage tube.

Determine the conjugate concentration using a Bradford or BCA Assay with bovine gamma globulin protein standards. The R-PE antibody conjugate is now ready to use. Store the conjugate at 4°C, protected from light. A bacteriostatic agent such as sodium azide may be added to extend the shelf life of the product, if desired.

Application Notes

Bradford Assay Protocol

BCA Assay Protocol

Troubleshooting guide

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