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USER GUIDE

R-PE Antibody Conjugation Kit

Catalog # P-9002-002

User Guide Introduction
This user guide provides instructions for using the R-PE Antibody Conjugation Kit.

Customer Service and Technical Support

Please contact us:
Email: technical@vectorlabs.com
Email: customerservice@vectorlabs.com
Telephone: +1 (800) 227-6666

Address:
Vector Laboratories, Inc.
30 Ingold Road
Burlingame, CA 94010

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R-PE-Antibody Conjugation Kit Introduction

Introduction

The SoluLINK R-PE-Antibody Conjugation Kit contains all reagents needed to perform two conjugation reactions between antibody and R-PE. Up to 1.3 mg of antibody may be conjugated in each reaction. This kit has been optimized to incorporate approximately one R-PE per antibody molecule for use in highly demanding applications such as flow cytometry, microarrays, and bead-based immunodiagnostics.

Advantages

SoluLINK bioconjugation technology is superior to traditional maleimide-thiol-based methods as it is:

More efficient: Greater than 95% of antibody is converted to conjugate using only a small excess (1.2 - 1.5 mole equivalents) of R-PE.

More easily purified: In most cases, the percent conversion of free antibody to conjugate is nearly quantitative. Therefore, it is only necessary to remove the small amount of excess R-PE to obtain a purified conjugate. In many applications purification is not necessary, and the conjugate can be used immediately without further purification.

Controllable: The level of R-PE conjugation, and therefore the brightness of the conjugate, can be controlled by adjusting the level of HyNic incorporation on the antibody. In flow cytometry applications a heterodimer product is preferred, and preparation of this construct can be easily optimized.

An intact antibody is incorporated: Other conjugation methods expose thiols on antibodies by DTT reduction of disulfide bonds, which cleaves the antibody into a variety of fragments. SoluLINK technology, however, gently incorporates HyNic linkers on lysine amino groups of the intact antibody, preserving its immunoreactivity.

Introduction to SoluLINK Bioconjugation Technology

SoluLINK bioconjugation technology is based on the formation of a stable hydrazone bond formed between an aromatic hydrazine and an aromatic aldehyde (Figure 1). S-HyNic is used to incorporate aromatic hydrazine linkers on biomolecules such as antibodies. S-HyNic is an amine-reactive modification reagent that directly converts amino groups (lysine amino acids) on biomolecules and surfaces to HyNic groups. S-4FB is used to convert amino groups on biomolecules and surfaces to aromatic aldehydes (4-formylbenzamide) groups. Addition of a HyNic-modified antibody to a 4FB-modified protein such as R-PE leads to the formation of conjugate via a stable bis-arylhydrazone bond.

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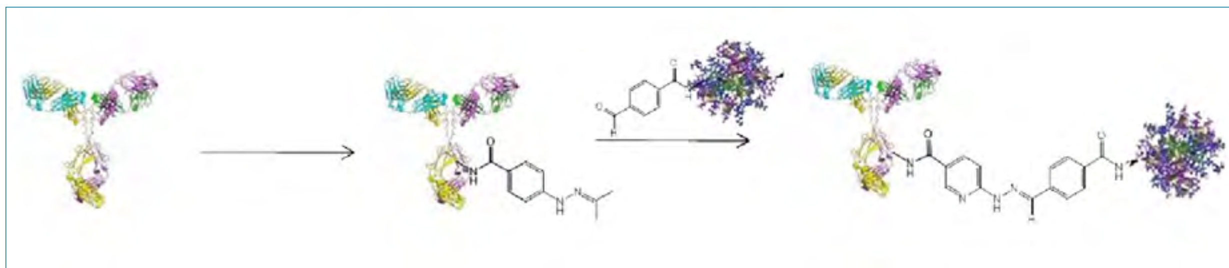

Figure 1: Reaction scheme presenting the two-step procedure to prepare R-PE-antibody conjugates using SoluLINK bioconjugation technology. Step 1 is the modification of an antibody with S-HyNic to prepare HyNic-modified antibody. Step 2 is the conjugation of HyNic-modified antibody to pre-activated 4FB-R-PE by simply mixing the two proteins.

Advantages of HyNic-4FB conjugation include:

> The conjugation reaction is high yielding. Conjugate yields of 60-80% based on starting antibody are typical.

> The reaction is efficient. Dirksen *et al.*[1-3] discovered that aniline catalyzes Schiff's base (hydrazone) formation. 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 approximately 2 hours using 1.2 - 1.5 mole equivalents of the second protein.

> The conjugate bond is stable. The bis-arylhydrazone conjugate bond is stable due to the aromatic nature of the linkers.

> The reaction conditions are extremely mild and do not cause antibody denaturation. Unlike thiol-based conjugation protocols where reducing agents are required to cleave disulfide bonds, the HyNic-4FB conjugation couple leaves disulfide bonds intact. No metals, oxidizing agents or reducing reagents are required to stabilize the conjugate.

> The conjugation is traceable spectrophotometrically. The HyNic-4FB conjugate bond is chromophoric, with an absorbance maximum at 354 nm and a molar extinction coefficient of 29,000 $L \cdot mol^{-1} \cdot cm^{-1}$. This allows real-time spectrophotometric monitoring of conjugate formation, and the ability to 'visualize' the conjugate during chromatographic purification using a UV or photodiode array detector.

The Keys to Success

There are three crucial requirements that must be fulfilled for a reproducibly successful preparation of an R-PE Antibody conjugate using SoluLINK's bioconjugation technology:

1. Prior to modification with S-HyNic, the starting antibody solution must be completely buffer exchanged into Modification Buffer, pH 8.0, using the included Zeba™ desalting columns.
2. The HyNic-antibody molar substitution ratio (MSR) must be ≥ 3.5 as determined by the colorimetric HyNic MSR assay.
3. The final concentration of HyNic-antibody in the conjugation reaction must be ≥ 1.5 mg/mL.

Kit Components

Component	Part No.	Units
S-HyNic	S-1002-1	2 x 1.0 mg
4FB-Modified R-PE	S-4008-003	2 x 3.2 mg
10X Modification Buffer	S-4000-1.5	1.5 mL
10X Conjugation Buffer	S-4002-1.5	1.5 mL
10X TurboLINK Catalyst Buffer	S-2006-105	1.5 mL
Anhydrous DMF	S-4001-1.5	1.5 mL
0.5 mL, 7 kDa Zeba Columns	S-4024-0.5	4
2 mL Collection Tubes	S-8014-2-12	12
2-Sulfobenzaldehyde	S-2005-10	10 mg
10X MES Buffer	S-8003-1.5	1.5 mL
2 mL, 7 kDa Zeba Columns	S-8007-2-2	2

Notes:

1. For convenience, the complete kit can be stored at 2 - 8°C. Please do not freeze the kit or any of the kit components.

   a. If precipitate forms in any of the buffers upon storage at 2 - 8°C, re-dissolve completely by warming at 37°C and vortexing before use.

2. 10X Modification Buffer: 1.0 M sodium phosphate, 1.5 M NaCl, pH 8.0
3. 10X Conjugation Buffer: 1.0 M sodium phosphate, 1.5 M NaCl, pH 6.0
4. 10X TurboLINK Catalyst Buffer: 100 mM aniline, 100 mM sodium phosphate, 150 mM NaCl, pH 6.0
5. 10X MES Buffer: 1.0 M MES, pH 6.0

Equipment/Reagents Required But Not Provided

Variable-speed bench-top microcentrifuge

Variable-speed bench-top centrifuge

Spectrophotometer and/or plate reader

1.5 mL microcentrifuge tubes

15 mL conical tubes

Handheld pipets

Protein concentration assay reagent such as BCA or Bradford

Protocols

1.0 Antibody Desalting

The R-PE-Antibody Conjugation Kit has been designed to conjugate between 300 µg and 1.3 mg of antibody to R-PE. Antibodies must be desalted into 1X Modification Buffer before they are modified with S-HyNic. Included in this kit are 0.5 mL Zeba desalting columns (Figure 2) that have a maximum capacity of 130 µL. Therefore, up to 1.3 mg of a 10 mg/mL antibody solution can be desalted in preparation of modification/conjugation. Lower protein concentrations (3.0 mg/mL, minimum) and volumes (100 µL, minimum) can also be used successfully.

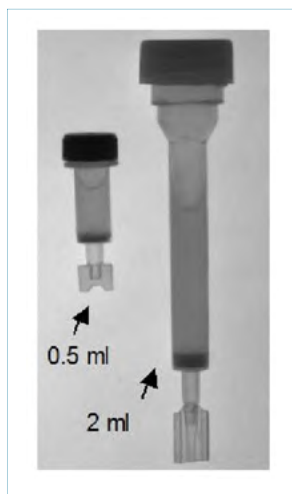

Figure 2. Zeba desalting columns (0.5 mL and 2 mL) used to desalt antibody solutions.

1.1 Zeba Desalting Protocol

<u>0.5 mL Zeba Spin Column Preparation (Sample Volumes 100 - 130 µL)</u>

1. Prepare a 1X Modification Buffer solution by adding 0.5 mL of 10X Modification Buffer to 4.5 mL of ultrapure water. Mix well.
2. Remove the column's closure by holding the body of the column and twisting off the bottom tab. Loosen the cap one-half turn, but do not remove it.
3. Place column in a 2 mL collection tube (provided). Centrifuge at 1,500 x g or RCF (not RPM) for 1 minute to remove storage solution. After centrifugation is complete, discard the liquid in the collection tube.
4. Using a lab marker, place a mark on the side of the column where the compacted resin is slanted upward. Place column in the microcentrifuge with the mark facing outward (away from the center of the rotor) in all subsequent centrifugation steps. Improper orientation may result in low sample recovery and/or incomplete desalting.
5. Add 300 µL of 1X Modification Buffer to the top of the dry resin bed and cap loosely. Properly orient the column in the rotor and centrifuge at 1,500 x g for 1 minute. Discard the flow-through.
6. Repeat the column equilibration (Step 5) with fresh 1X Modification Buffer two additional times (3 total). Discard the flow-through from each equilibration.

7. Place the equilibrated column in a new 2 mL collection tube. Slowly apply 100 - 130 µL of antibody solution to the center of the resin bed without disturbing the resin. Loosely cap the column.
8. Centrifuge at 1,500 x g for **2 minutes** to collect the desalted antibody. Transfer the antibody solution from the bottom of the collection tube into a new microcentrifuge tube, noting the volume recovered from the desalting process.
9. Discard the Zeba Spin Column after use.

After desalting the antibody into Modification Buffer, measure the concentration using a spectrophotometer and the E1% A280 mass extinction coefficient (see table below).

Antibody Source	Antibody E1% (1-cm path)
Human IgG	13.60
Human IgE	15.30
Rabbit IgG	13.50
Donkey IgG	15.00
Hourse IgG	15.00
Mouse IgG	14.00
Rat IgG	14.00
Bovine IgG	12.40
Goat IgG	13.60
Avian IgY	12.76

Table 1: E1% (280 nm) values for antibodies produced in various host species.

2.0 Antibody Modification with S-HyNic

Recommended Guidelines for Modifying Antibodies 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. For example, the number of HyNic 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, excess linker equivalents added (e.g. 10X, 15X or 20X) and reaction pH.

Table 2 presents the results of a study to determine the level of HyNic incorporation on an antibody after adding 5X, 10X and 20X equivalents of S-HyNic at 1.0, 2.5 and 5.0 mg/mL antibody concentration in Modification Buffer. These data may be used as an aid in determining the number of mole equivalents of S-HyNic to add to the antibody to achieve a given molar substitution ratio (MSR).

In general, as the antibody 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 or loss of immunoreactivity.

Antibody HyNic MSR values between 3.5 – 6.0 are ideal for conjugation of 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 an antibody concentration of 5.0 mg/mL, and 10 mole equivalents of S-HyNic is recommended at an antibody concentration of 10 mg/mL.

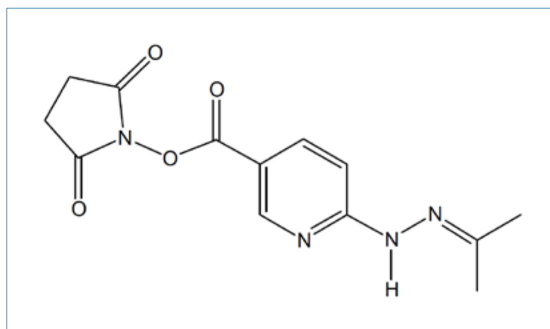

Figure 3: Structure of S-HyNic

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

Table 2: Results of a study to determine the level of HyNic incorporation on an antibody after adding 5X, 10X and 20X equivalents of S-HyNic at 1.0, 2.5 and 5.0 mg/mL antibody concentration in Modification Buffer.

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

1. Enter the antibody information in Worksheet 1 of the HyNic Antibody/4FB-R-Phycoerythrin Conjugation Calculator. Once the antibody concentration and mass of antibody to be modified are entered, the calculator will output the volume of antibody to be used.
2. Enter the mole equivalents of S-HyNic to use over antibody in the Reagent Information field. Typically, 15 to 20 mole equivalents of S-HyNic are used over antibody, depending on the antibody concentration.
3. Thoroughly dissolve a 1.0 mg vial of S-HyNic in 100 µL of anhydrous DMF.
4. Add the required volume of S-HyNic in DMF to the antibody solution as calculated using the HyNic Antibody/4FB-R-Phycoerythrin Conjugation Calculator. Immediately mix the solution well.

   Notes
   a. Maintain the percentage of DMF (vol/vol) in the S-HyNic modification reaction at or below 5% of the total reaction volume.

   b. The antibody concentration should be ≥ 2.5 mg/mL for efficient S-HyNic modification and subsequent conjugation.
5. Incubate the modification reaction at room temperature for 1.5 hours.
6. Proceed to desalt the HyNic-modified antibody into 1X Conjugation Buffer following the procedure below.

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2.2 Desalting of HyNic-Modified Antibody

0.5 mL Zeba Desalting Column Preparation (Sample Volumes 100 - 130 µL)

1. Prepare a 1X Conjugation Buffer solution by adding 0.5 mL of 10X Conjugation Buffer to 4.5 mL of ultrapure water. Mix well.
2. Remove the column's bottom closure by holding the body of the column and twisting off the bottom tab. Loosen the cap one-half turn, but do not remove it.
3. Place column in a 2 mL collection tube (provided). Centrifuge at 1,500 x g or RCF (not RPM) for 1 minute to remove storage solution. After centrifugation is complete, discard the liquid in the collection tube.
4. Using a lab marker, place a mark on the side of the column where the compacted resin is slanted upward. Place the column in the microcentrifuge with the mark facing outward (away from the center of the rotor) in all subsequent centrifugation steps. Improper orientation may result in low sample recovery and/or incomplete desalting.
5. Add 300 µL of 1X Conjugation Buffer to the top of the dry resin bed and loosely cap the column. Properly orient the column in the rotor and centrifuge at 1,500 x g for 1 minute. Discard the flow-through.
6. Repeat the column equilibration (Step 5) with fresh 1X Conjugation Buffer two additional times (3 total). Discard the flow-through from each equilibration.
7. Place the equilibrated column in a new 2 mL collection tube. Slowly apply 100 - 130 µL of HyNic-antibody solution to the center of the resin bed without disturbing the resin. Loosely cap the column.
8. Centrifuge at 1,500 x g for **2 minutes** to collect the desalted HyNic-antibody. Transfer the modified antibody solution from the bottom of the collection tube into a new microcentrifuge tube, noting the volume recovered from the desalting process.
9. Discard the Zeba Spin Column after use.

2.3 Determination of HyNic-Antibody Concentration

After modification with S-HyNic, the antibody concentration is determined using a BCA or Bradford protein assay. Due to the UV absorption of the incorporated HyNic linkers, an A280 concentration check cannot be used to accurately determine the antibody concentration during this or subsequent steps. A standard curve based on antibody (e.g., bovine IgG) should be used rather than BSA or other unrelated protein for accurate results. Prepare the BCA or Bradford working solution and perform the assay according to the manufacturer's instructions.

2.4 Determining the HyNic Molar Substitution Ratio (MSR)

Determination of the number of HyNic groups per antibody is accomplished using a colorimetric assay as shown in Figure 4. Incorporated HyNic groups on the antibody react with 2-sulfobenzaldehyde to form a bis-arylhydrazone, which is measured at 350 nm.

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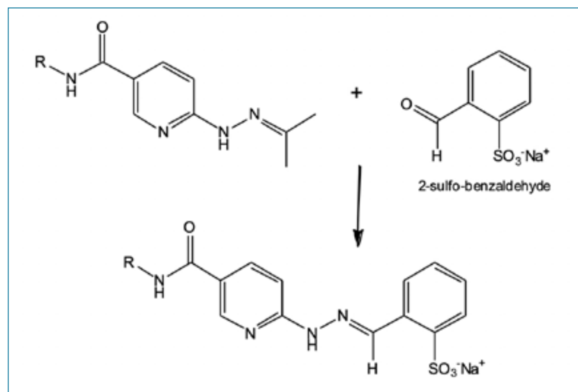

Figure 4: Scheme presenting the reaction of HyNic groups on an antibody with 2-sulfobenzaldehyde. The bis-arylhydrazone product absorbs at 350 nm and has a molar extinction coefficient of 28,500 $L \cdot mol^{-1} \cdot cm^{-1}$.

HyNic MSR Determination
Protocols to determine the MSR using a standard spectrophotometer (Method A) or a NanoDrop spectrophotometer (Method B) are described below. Both protocols begin by preparing the 2-sulfobenzaldehyde (2-SB) assay reagent described in Step 1 below.

1. 2-Sulfobenzaldehyde (2-SB) Reagent Preparation

   Prepare a 0.5 mM working solution of 2-sulfobenzaldehyde in 1X (100 mM) MES buffer, pH 5.0, as follows:

   a. 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.

   b. Weigh approximately 3 – 5 mg of 2-sulfobenzaldehyde into a microcentrifuge tube while recording the exact mass weighed.

   c. Prepare a 20 mg/mL solution of 2-sulfobenzaldehyde in water by adding a sufficient volume of ultrapure water to the weighed 2-sulfobenzaldehyde. Vortex to completely dissolve.

   d. Add 26 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-sulfobenzaldehyde.

   e. Protect the solution from light and keep refrigerated. This solution remains stable for up to 30 days at 4°C.

MSR Method A: Spectrophotometer/Cuvette Protocol

1. Transfer 10 µL of HyNic-modified antibody solution (at 2-10 mg/mL in Conjugation Buffer) to a new 1.5 mL microcentrifuge tube containing 490 µL of 0.5 mM 2-sulfobenzaldehyde solution.
2. Prepare a reaction blank by adding 10 µL of 1X Conjugation Buffer to 490 µL of 0.5 mM 2-sulfobenzaldehyde 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, measure the A_{350} of the MSR reaction using a quartz cuvette as follows:

a. Blank the spectrophotometer at 350 nm using 500 µL of the blank sample prepared above. Empty the cuvette.

b. Record the 350 nm absorbance value of the HyNic MSR sample.

Note: In instances where low HyNic incorporation occurs or when the antibody concentration is < 2 mg/mL the assay may require > 10 µL to achieve a reliable A_{350} reading.

5. Using the A_{350} value obtained, calculate the HyNic-antibody MSR with the aid of the HyNic Antibody/4FB-R-Phycoerythrin Conjugation Calculator.

Method B: NanoDrop Spectrophotometer Protocol

1. Transfer 2 µL of HyNic-modified antibody solution (at 2-10 mg/mL in Conjugation Buffer) to a new 1.5 mL microcentrifuge tube containing 18 µL of 0.5 mM 2-sulfobenzaldehyde solution.
2. Prepare a reaction blank by adding 2 µL of 1X Conjugation Buffer to 18 µL of 0.5 mM 2-sulfobenzaldehyde solution in a separate microcentrifuge tube. Mix both reactions well by vortexing, followed by a quick centrifuge spin to collect all liquid at the bottom of the tubes.
3. Incubate both reactions at 37°C for 60 minutes or at room temperature for 90 minutes.
4. Centrifuge both reactions at 10,000 x g for 15 seconds to collect condensation to the bottom of the tubes if a 37°C water bath was used during the incubation. Vortex both samples to mix.
5. Open the NanoDrop UV-Vis module and blank the instrument at 350 nm using the blank sample prepared above.
6. Record the 350 nm absorbance value of the HyNic MSR sample.
7. Using the A350 value obtained, calculate the HyNic-antibody MSR with the aid of the HyNic Antibody/4FB-R-Phycoerythrin Conjugation Calculator.

   Note: Ensure the 350 nm absorbance value is normalized to a 10-mm (1-cm) pathlength.

   It is critical that the HyNic-antibody MSR is ≥ 3.5 for efficient conjugation.

3.0 4FB-R-PE/HyNic-Antibody Conjugation Protocol

1. On Worksheet 2 of the HyNic Antibody/4FB-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.
2. Enter the 4FB-R-PE concentration and the mole equivalents of 4FB-R-PE to be used in the conjugation reaction. Use 1.2 equivalents to prepare a 1:1 mole ratio PE:antibody conjugate, or 1.5 equivalents for a conjugate with a larger mole ratio of PE to antibody.
3. Add the calculated volume of 4FB-R-PE to the HyNic-modified antibody solution and mix well.

4. Add the indicated volume of 10X TurboLINK Catalyst Buffer to the conjugation reaction and mix thoroughly.

   Note: Add the indicated volume of supplied 10X TurboLINK Catalyst Buffer directly to the conjugation reaction (no prior dilution is necessary).

5. Incubate the conjugation reaction at room temperature for 2.0 hours.

The completed reaction can be analyzed by gel electrophoresis using low voltage (≤ 80 volts), or by analytical size exclusion chromatography. An example of the results is presented in the Appendix (Figure 5).

3.1 Conjugate Purification/Desalting

After the 2-hour incubation period is complete, the conjugate may be directly purified via size exclusion chromatography, or desalted into 1X PBS (or other suitable buffer) for long-term 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. Remove the column's bottom closure by holding the body of the column and twisting off the bottom tab. Loosen the cap one-half turn, but do not remove it.

2. Place the column in a 15 mL conical tube. Centrifuge at **1,000 x g** or RCF (not RPM) for **2 minutes** to remove storage solution. After centrifugation is complete, discard the liquid in the collection tube.

3. Place a mark on the side of the column where the compacted resin is slanted upward. Place column in the centrifuge with the mark facing outward (away from the center of the rotor) in all subsequent centrifugation steps. Improper orientation may result in low sample recovery and/or incomplete desalting.

4. Add 1 mL of PBS, pH 7.2, or other desired storage buffer to the top of the resin bed and loosely cap the column. Properly orient the column in the rotor and centrifuge at 1,000 x g for 2 minutes. Discard the flow-through.

5. Repeat the column equilibration (step 4) with fresh buffer two additional times (3 total).

6. Place the column in a new 15 mL conical tube. Slowly apply up to 700 µL of conjugate to the center of the resin bed without disturbing the resin. **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.** Loosely cap the column.

7. Centrifuge at 1,000 x g for 2 minutes to collect the desalted conjugate. Transfer the desalted material into a microcentrifuge tube, noting the volume recovered from the desalting process.

8. Determine the conjugate concentration using a BCA or Bradford protein assay.

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.

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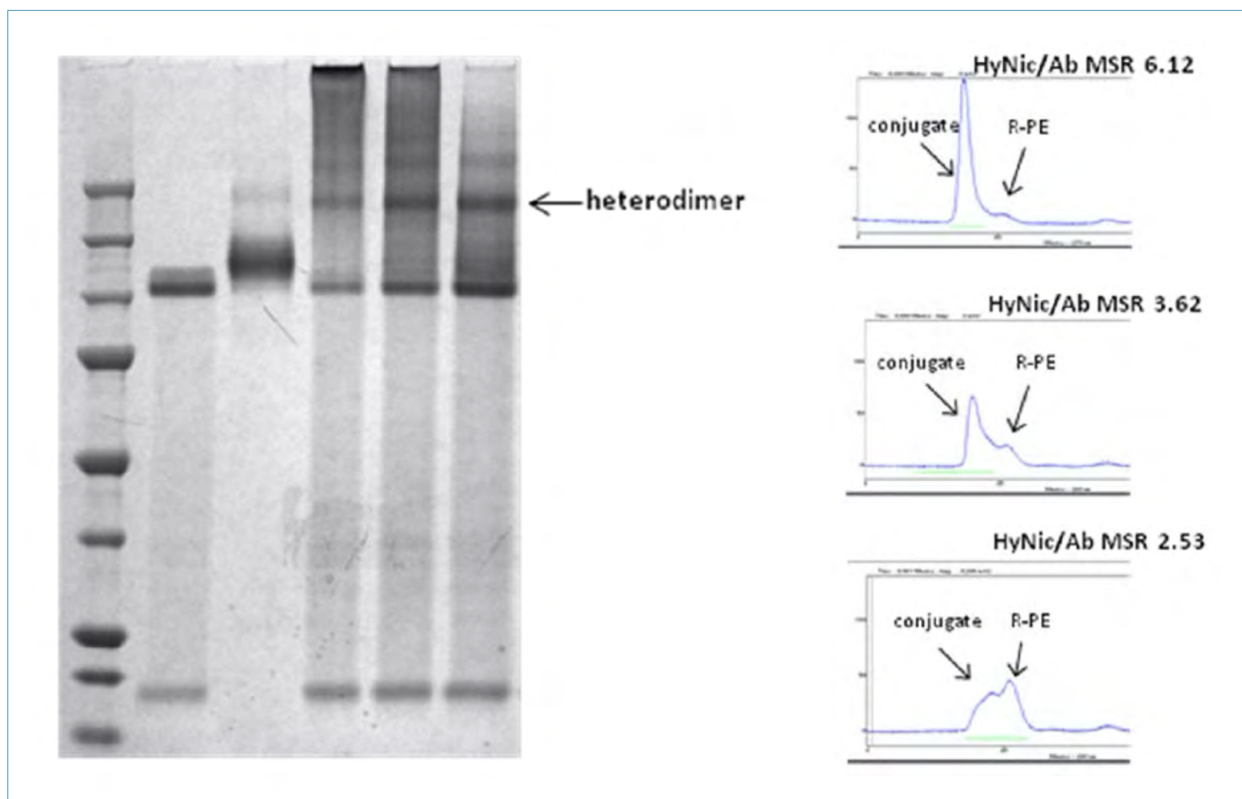
Appendix

R-PE-Antibody Conjugation Results



Lane			MSR (HyNic/Ab)
1	MW markers		
2	R-PE		
3	bIgG		
4	4FB-PE/HyNic-bIgG	10 mM Aniline	6.12
5	4FB-PE/HyNic-bIgG	10 mM Aniline	3.62
6	4FB-PE/HyNic-bIgG	10 mM Aniline	2.53

- antibody/R-PE ratio 1:1
- 2 h incubation

Figure 5: Results of the conjugation of HyNic-modified antibody to 4FB-R-PE at different HyNic MSR values. In three separate reactions, the antibody was modified with 20 mole equivalents of S-HyNic at 5.0, 2.5, and 1.0 mg/mL antibody concentration. The modification reaction was desalted and added to 4FB-R-PE (at 4.4 mg/mL). The final concentration of HyNic-antibody in the conjugation reaction was 1.5, 1.1 and 0.62 mg/mL, respectively. Non-reducing SDS-PAGE (left) and size exclusion chromatography (Superdex 200, right) results are presented. The results show efficient conversion of antibody to conjugate and the type of conjugate formed, i.e. the antibody/R-PE conjugate ratio. The R-PE to antibody ratio and resulting molecular weight distribution can be controlled by varying the HyNic MSR of the antibody.

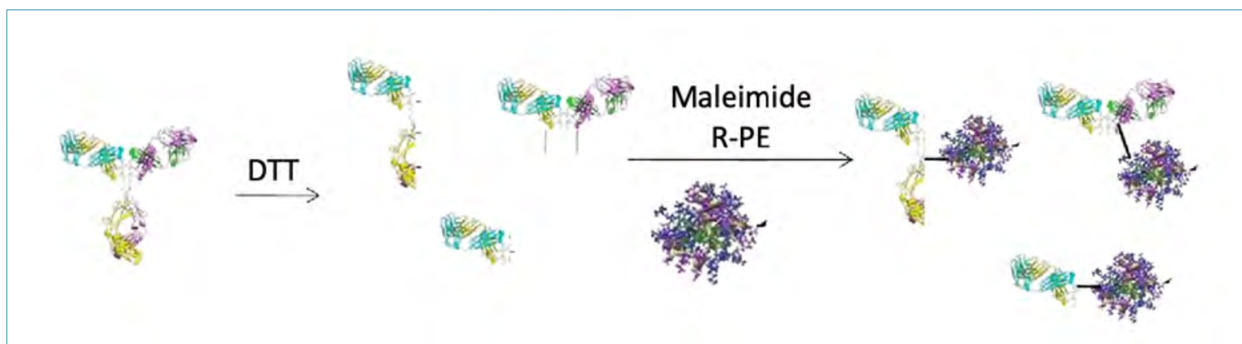
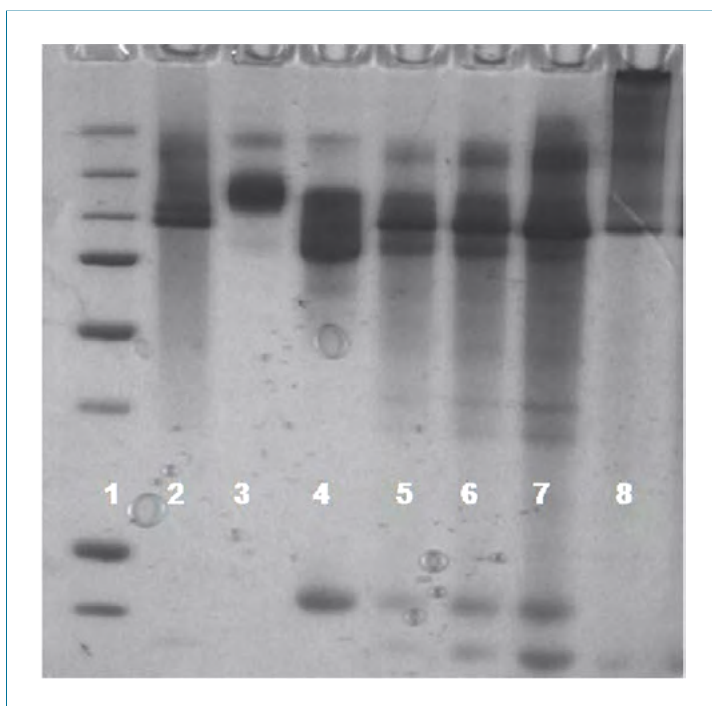


Figure 6: Schematic representation of the conjugation of antibody to R-PE using the maleimide/thiol conjugation couple. Thiols are generated by cleaving antibody disulfide bonds with DTT, leading to formation of multiple fragments, followed by conjugation to maleimide-R-PE. Figure 7 presents the non-reducing SDS-PAGE results of a conjugation using this method.



Lane	Contents
1	Precision Plus Protein™ All Blue standards
2	4FB-modified R-PE
3	Bovine IgG
4	Maleimide R-PE
5	DTT-treated Ab:Maliemide R-PE (1:1 mole ratio)
6	DTT-treated Ab:Malemide R-PE (1:2 mole ratio)
7	DTT-treated Ab:Maleimide R-PE (1:3 mole ratio)
8	R-PE-Ab conjugate prepared with the R-PE-Antibody Conjugation Kit

Figure 7: Non-reducing SDS-PAGE results showing conjugation of maleimide-R-PE (Prozyme™) to DTT-treated antibody at 1:1, 1:2, and 1:3 antibody:maleimide-R-PE mole ratios (lanes 5, 6 and 7, respectively). Lane 8 is a conjugate prepared using the R-PE-Antibody Conjugation Kit.

Troubleshooting

Problem	Possible Cause	Recommended Action
Poor HyNic modification of Antibody	Initial Antibody concentration is too low	Concentrate antibody using a diafiltration device Use an initial antibody concentration of 3-10 mg/ml with 20 mole equivalents of S-HyNic
	Amine contaminant, e.g. Tris or glycine buffer, present in starting antibody solution	Thoroughly exchange the antibody buffer by diafiltration, dialysis or desalting column before modification
R-PE-antibody conjugate has a molecular weight that is much larger than predicted	Antibody HyNic MSR is too high	Decrease modification levels by using lower equivalents of S-HyNic, or lower the antibody concentration during the modification reaction

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Vector Laboratories | 30 Ingold Road, Burlingame, CA 94010 | 650.697.3600 p | 650.697.0339 f | vectorlabs.com