SoluLinK
The Conjugation Company™

R-Phycoerythrin- Antibody

Conjugation Kit

Technical Manual

(Catalog # P-9002-002)

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Solulink's R-PE-Antibody- Conjugation Kit

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R-PE- Antibody Conjugation Kit using SouLink's HydraLink Technology

Introduction: Solulink has engineered its HydraLink™ conjugation technology to allow you to more easily and efficiently prepare R-PE-antibody conjugates compared to past maleimido/thiol-based protocols. The kit includes all the reagents and buffers needed to perform the two step conjugation.

Advantages: SoluLink's R-PE-antibody bioconjugation technology is superior to the maleimido/thiol-based method as it is:

More efficient: Greater than 95% of antibody is converted to conjugate and only 1-1.5 molar equivalents of R-PE is required per mole of antibody to produce the conjugate.

More easily purified: In most cases, the percent conversion of free antibody to conjugate is 100%, leaving no unconjugated antibody. Therefore it is only necessary to purify out the excess R-PE to obtain a purified conjugate. In many applications even, purification is not necessary.

Controllable: The level of polymerization, and therefore the brightness of the conjugate, can be controlled by adjusting the level of HyNic-modification on the antibody. In flow cytometry applications, heterodimer product is required and preparation of this construct can be easily optimized using this technology.

and furthermore

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 species. Solulink's technology, however, gently incorporates HyNic moieties on the intact antibody.

Introduction to SoluLink Bioconjugation Technology: SoluLinK's core bioconjugation technology is based on the formation of a stable bis-arylhydrazone formed from an aromatic hydrazine and an aromatic aldehyde. S-HyNic **1** (succinimidyl 6-hydrazinonicotinate acetone hydrazone, SANH) is used to incorporate aromatic hydrazine moieties on biomolecules. S-HyNic is an amino-reactive reagent that directly converts amino groups on biomolecules and surfaces to HyNic groups. S-4FB **2** (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 R-PE leads to the formation of the conjugate (Figure 1). The bis-arylhydrazone bond is stable to 92^{O}C and pH 2.0-10.0. The recommended pH for antibody conjugation is 6.0.

Further enhancing the many advantages of the HyNic-4FB conjugation couple is the discovery by Dirksen *et al.*[1-3] that shows that 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 as strikingly demonstrated in this product.

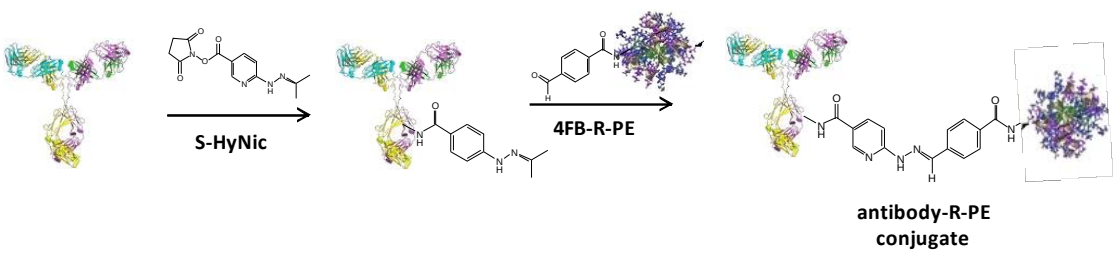


Figure 1: Scheme presenting the two-step protocol to prepare R-PE-antibody conjugates using Solulink's HydraLink bioconjugation technology. Step 1 is modification of IgG with S-HyNic to prepare HyNic-IgG. Step 2 is the conjugation of HyNic-IgG to 4FB-R-PE simply by mixing in 100 mM phosphate, 150 mM NaCl, 10 mM aniline, pH 6.0.

The Keys to Success

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

1. IgG buffer exchange: Prior to modification, the starting IgG must be completely exchanged into Modification Buffer, pH 7.4.
2. Minimum level of HyNic-modification: The HyNic/IgG molar substitution ratio (MSR) as determined by the A310/A280 ratio of the HyNic-modified antibody must be > 0.3.

Kit Components

Component	Size	Storage[1]
S-HyNic	1.0 mg vial	Desiccated
4FB-R-PE[2]	1.5 mg	**4°C**
10X Modification Buffer[3]	1.5 mL vial	Room temperature
10X Conjugation Buffer[4]	1.5 mL vial	Room temperature
100 mM Aniline Buffer[5]	1 mL vial	4°C
0.5 mL Zeba	4	4°C
DMF (anhydrous)	1 mL vial	Desiccated
Collection tubes	12	Room temperature
Flash Drive		

NOTES:

1) **For convenience all kit components can be stored at 4°C**
2) 4FB-R-PE concentration is batch dependent therefore see lot specific Product Data Sheet
3) 10X Modification Buffer: 1.0 M phosphate, 1.5 M NaCl, pH 7.4
4) 10X Conjugation Buffer: 1.0 M phosphate, 1.5 M NaCl, pH 6.0
5) 100 mM Aniline Buffer: 100 mM aniline, 100 mM phosphate, 150 mM NaCl, pH 6.0

Equipment Required

Variable-speed bench-top microcentrifuge
Spectrophotometer or Plate Reader
1.5 mL microcentrifuge tubes
Pipettors

Protocol

1. Desalting

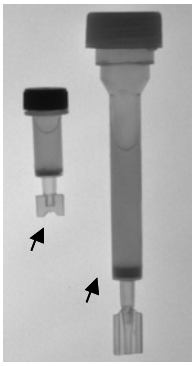


Figure 2. Zeba™ Desalt Spin Columns (0.5 and 2 ml) used to desalt starting IgG and HyNic-modified IgG.

Antibodies must be completely desalted into modification buffer (100 mM phosphate, 150 mM NaCl, pH 7.4) before they are modified with S-HyNic.

Any desalting method, such as dialysis, Sephadex desalting columns (NAP columns, GE Healthcare) or Zeba Desalt Spin Columns (Pierce Chemical, Cat. #89882 or 89889) can be used (Figure 2).

SoluLink recommends the use of Zeba™ Desalt Spin Columns to desalt proteins as required by our conjugation protocol. These rapid spin columns are recommended because they do not significantly dilute the antibody during desalting.

Included in this kit are 0.5 mL Zeba Spin Desalt columns (Figure 3) that have a maximum capacity of 130 uL. Therefore up to 1.3 mg of a 10 mg/mL solution of protein can be desalted. As this kit has been designed for two conjugations, included are four Zeba columns, one to initially desalt the protein and one to desalt and exchange the modified protein into conjugation buffer (see below).

Zeba Desalting Protocol

0.5ml Zeba™ Spin Column Preparation (Sample volumes 30-130uL)

1. Remove spin column's bottom closure and loosen the top cap (do not remove cap).
2. Place spin column in a 1.5 ml microcentrifuge collection tube.
3. Centrifuge at 1,500 x g for 1 minute to remove storage solution.
4. Place a mark on the side of the column where the compacted resin is slanted upward. Place column in the microfuge with the mark facing outward in all subsequent centrifugation steps.
5. Add 300 µl of 1x Modification buffer (pH 7.4) or Conjugation buffer (pH 6.0) as required 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, discarding buffer from the collection tube.
7. Column is now ready for sample loading.

Protein Sample Loading

1. Place the equilibrated spin column in a new 1.5 ml collection tube, remove cap and slowly apply 30-130 µl sample volume to the center of the compact resin bed.

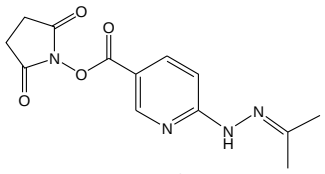
   Note- for sample volumes less than 70 µl apply a 15 µl buffer (stacker) to the top of the resin bed after the sample has fully absorbed to ensure maximal protein recovery. Avoid contact with the sides of the column when loading.

2. Centrifuge at 1,500 x g for 2 minutes to collect desalted sample.

3. Discard desalting column after use.

4. Protein sample is now desalted and ready for modification.

2. S-HyNic Antibody Modification Protocol



S-HyNic

$C_{13}H_{14}N_4O_4$ MW290.27

Figure 3: Structure of S-HyNic

1. Dissolve a vial of pre-weighed 1.0 mg S-HyNic (Figure 3) vial in 100 µL anhydrous DMF.

   Note-Always maintain the percentage of DMF (vol/vol) in the final S-HyNic modification reaction at or below 5% of the total reaction volume.

2. Prepare the antibody solution to be 2.5-4.0 mg/mL in Modification Buffer. Concentration can be determined by A280 determination or the BCA (Uptima, UP40840A) or Bradford Assays (Uptima, UPF86400).

3. Using the calculator supplied on the flash drive (or available from http://www.solulink.com/protocols.php ; Figure 4) add the required volume of S-HyNic/DMF stock solution to the antibody in modification buffer (100 mM phosphate, 150 mM NaCl, pH 7.4).

   Notes-

   a) A high buffering capacity is necessary for efficient conjugation. It is for this reason that we recommend using a 100mM phosphate buffer solution to ensure modification efficiency.
   b) It is important to have the protein concentration at 2.5-4.0 mg/ml for efficient HyNic modification.

4. Incubate the reaction at room temperature for 2 hours.

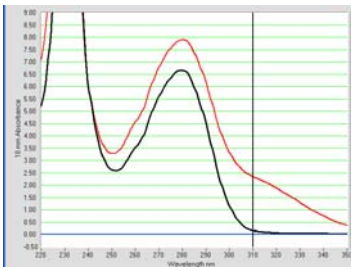


Figure 5: Overlapping UV spectra of bIgG (black) and HyNic-modified bIgG (red). In this example the IgG was modified at 4.9 mg/mL with 20X S-HyNic and the A310/A280 is 0.30.

5. Proceed to desalt the HyNic-modified IgG into Conjugation buffer (100 mM phosphate, 150 mM NaCl, pH 6.0).

6. Measure the A280 and A310 nm absorption and determine the A310/A280 ratio. **A310/A280 ratio must be > 0.3** (see Figure 5).

HyNic-antibody/4FB-R-PE Conjugation Calculator

Worksheet 1: Antibody HyNic Modification Calculator

Name of antibody to be modified	goat anti-mouse IgG	
Molecular weight of antibody	150000	*daltons*
Concentration of protein to be modified with linker	2.5	*mg/ml*
Mass of protein to be modified with linker	1.0	*mg*
Volume of protein to be modified (uL)	400.0	*uL*
S-HyNic	S-HyNic (M.W. 290.27)	
Molecular weight of linker	290.3	*daltons*
Mass of linker weighed by experimenter	1	*mg*
Volume of anhydrous DMF used to dissolve linker	100	*uL*
Equivalents of linker used to modify protein	20	
Volume of linker required to modify protein	3.9	*uL*

Legend
User Input
No Input Required
Calculator Output

Figure 4: Picture of Antibody/S-HyNic Modified Calculator supplied on flash drive and available from http://www.solulink.com/protocols.php

3. HyNic-Antibody/4FB-R-PE Conjugation Protocol

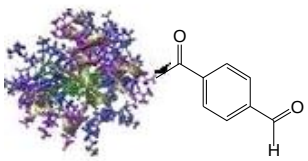


Figure 7: Representative structure of 4FB-R-PE.

1. Input amount, in milligrams, of HyNic-antibody to be conjugated into the **HyNic-antibody/4FB-R-PE into calculator** (worksheet 2; Figure 6) supplied on flash drive or available at from http://www.solulink.com/protocols.php .

2. Add calculated volume of 4FB-R-PE (Figure 7) to the Antibody solution. SoluLinK recommends a 1/1 HyNic-IgG/4FB-R-PE ratio but this may be changed as desired.

3. Add 1/10 volume Aniline Buffer (100 mM aniline, 100 mM phosphate, 150 mM NaCl, pH 6.0). Mix thoroughly.

4. Incubate at room temperature for 2 hours.

5. Desalt reaction into PBS using an appropriately sized NAP sephadex column (GE HealthCare) or Zeba spin filter (ThermoPierce) in order to remove the aniline catalyst.

6. The crude 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 below (Figure 8) compared. Gel results using DTT-cleaved IgG/maleimido-R-PE is presented in Figure 10.

7. Conjugates can be used directly without purification or may be purified to remove unconjugated R-PE by preparative size exclusion chromatography.

HyNic-antibody/4FB-RPE Conjugation Calculator

Worksheet 2: HyNic-antibody/4FB-RPE Conjugation Calculator

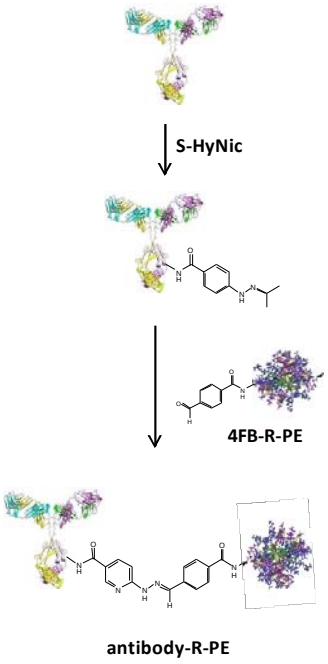
HyNic-IgG Name	IgG-HyNic	
HyNic-IgG lot#	IgG-HyNic	
4FB-RPE lot#	S434433	
Ab-HyNic (MW)	150000	*daltons*
4FB-RPE (MW)	240000	*daltons*
HyNic-IgG concentration	2.2	*mg/ml*
4FB-RPE concentration	7.25	*mg/ml*
HyNic-IgG # equivalents	1.0	
4FB-RPE # equivalents	1.0	
mg HyNic-IgG to be conjugated	1.5	*mg*
Volume of HyNic-IgG to be conjugated	681.8	*uL*
Volume of 4FB-RPE to be added	331.0	*uL*
Volume of Aniline Buffer to be added	101.3	*uL*

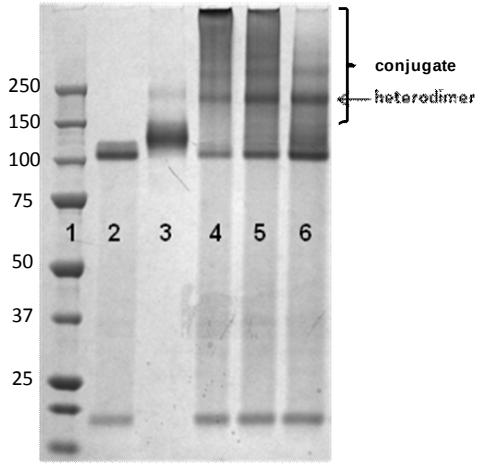
Legend
User Input
No Input Required
Calculator Output

Figure 6: Picture of HyNic-antibody/4FB-R-PE Calculator supplied on flash drive and available from http://www.solulink.com/protocols.php

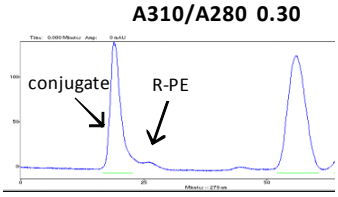
Appendix

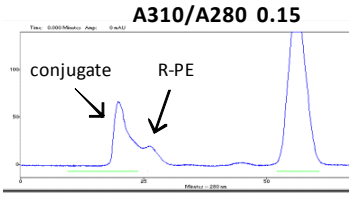
HyNic-Ab-R-PE Conjugation Results

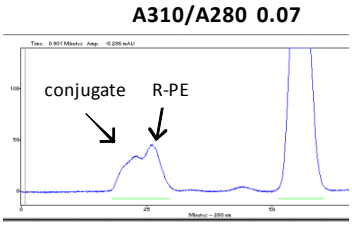




Lane	Component	catalyst	A310/A280
1	protein markers		
2	4FB-R-PE		
3	HyNic-Ab		
4	HyNic-Ab + 4FB-R-PE	+ 10 mM aniline	0.30
5	HyNic-Ab + 4FB-R-PE	+ 10 mM aniline	0.15
6	HyNic-Ab + 4FB-R-PE	+ 10 mM aniline	0.07







- **antibody/R-PE ratio 1:1**
- **conjugation buffer**
  - **100 mM phosphate/150 mM NaCl, pH 6.0**
- **2 h incubation**
- **no quenching**

Figure 8: Results of the conjugation of HyNic-bIgG conjugation to 4FB-R-PE. In 3 separate conjugation reactions the antibody modification was performed at 5.0, 2.5 and 1.0 mg/mL using 20 equivalents S-HyNic, the reaction mixture was desalted and added to 4FB-R-PE (4.4 mg/mL). The final concentration of HyNic-IgG in the aniline-accelerated reaction was 1.5, 1.1 and 0.62 mg/mL. PAGE (left) and size exclusion chromatography (right) results are presented. The results show extremely efficient conversion of antibody to conjugate and the type of conjugate formed, *i.e.* antibody/R-PE conjugate ratio, can be controlled by controlling the level of HyNic modification on the antibody.

Ab/DTT reduction method/mal-R-PE Results

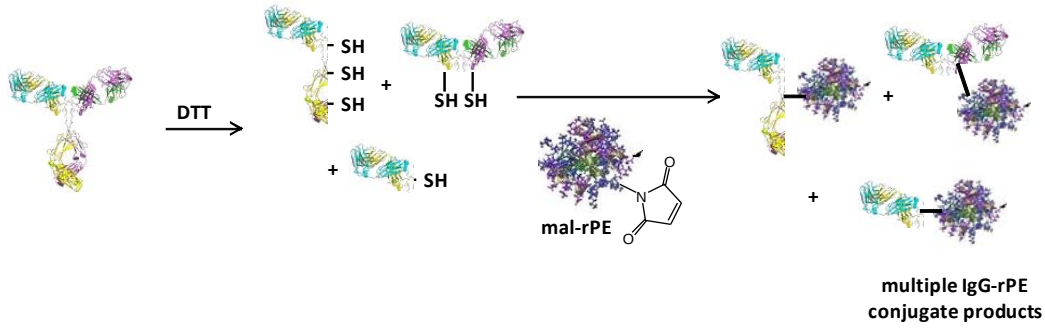
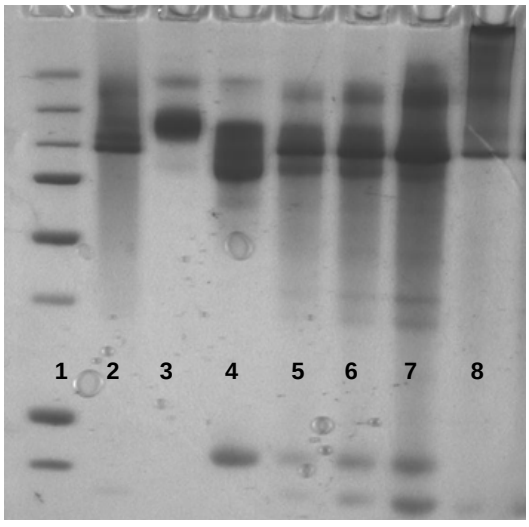


Figure 9: Schematic representation of the conjugation of IgG to R-PE using the maleido/thiol conjugation couple. Thiols are generated by cleaving antibody disulfide bonds with DTT leading to multiple fragments followed by conjugation to maleimido-R-PE. Figure 10 presents the results of a conjugation using this method.



Lane	
1	MW markers
2	R-PE
3	bIgG
4	IgG/DTT
5	'IgG-SH'/maleimido-R-PE (**1/1**)
6	'IgG-SH'/maleimido-R-PE (**1/2**)
7	'IgG-SH'/maleimido-R-PE (**1/3**)
8	HyNic-IgG/4FB-R-PE (**1/1**)

Figure 10: PAGE results of crude reaction mixtures of conjugation of maleimido-R-PE (Prozyme) to DTT-treated bIgG at 1/1, 1/2 and 1/3 'IgG-SH'/maleimido-R-PE mole ratios (lanes 5, 6 and 7 respectively). Lane 8 is crude reaction mixture of R-PE-antibody prepared with SoluLink's HyNic/4FB bioconjugation technology.

Stability

Component	Storage	Stability
Unopened Kit	4°C	3 months after receipt
S-HyNic/DMF	4°C	One week
4FB-R-PE2	4°C	3 months
HyNic-modified IgG		use immediately
R-PE-IgG conjugate following desalting without bacteriostat	4°C	4 weeks
R-PE-IgG conjugate following purification with bacteriostat- 0.05% azide	4°C	6 months

Troubleshooting

Problem	Possible Cause	Recommended Action
Poor HyNic modification of IgG	- initial IgG concentration is too low	- concentrate IgG using a diafiltration filter, - use an initial concentration of 2.5- 4 mg/ml IgG with 20-30 molar equivalents of S-HyNic for required labeling of IgG
	- amine contaminant, e.g. Tris or glycine buffer, present in starting IgG solution	- thoroughly exchange the IgG buffer by diafiltration, dialysis or desalting column before modification
R-PE-antibody conjugate has a molecular weight that is much larger than predicted	- due to high modification levels on each IgG a large molecular weight product may be formed	- decrease modification levels by using lower equivalents of S-HyNic, lower the IgG concentration during the modification reaction

References

1. Dirksen, A., et al., Nucleophilic catalysis of hydrazone formation and transimination: implications for dynamic covalent chemistry. J Am Chem Soc, **2006**, 128, 15602-3.
2. Dirksen, A., Hackeng, T.M. and Dawson, P.E., Nucleophilic Catalysis of Oxime Ligation, Angew. Chem. Int. Ed., **2006**, 45, 7581 –7584
3. Dirksen, A. and Dawson, P.E., Rapid Oxime and Hydrazone Ligations with Aromatic Aldehydes for Biomolecular Labeling, Bioconjugate Chem., **2008**, 19 , 2543-2548