Version 10.21.11

APC-Antibody Conjugation Kit

Technical Manual

Catalog # P-9903-001

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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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Solulink's APC-Antibody-Conjugation Kit

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APC-Antibody Conjugation Kit Using Solulink's Linking Technology

Introduction: Solulink has engineered its patented conjugation technology to more easily and efficiently prepare APC-antibody conjugates compared to maleimido/thiol-based protocols. The kit includes all the reagents and buffers needed to perform one conjugation reaction.

Advantages: Solulink's APC-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 APC 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 >95%. Therefore it is only necessary to remove the excess APC to obtain a purified conjugate. In many applications, 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 preferred and preparation of this construct can be easily optimized using this technology.

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 linkers on the intact antibody.

Introduction to Solulink Bioconjugation Technology

Solulink's core bioconjugation technology is based on the formation of a stable bond formed by the reaction of an aromatic hydrazine and an aromatic aldehyde (Figure 1). S-HyNic **1** (succinimidyl 6-hydrazinonicotinate acetone hydrazone, SANH) is used to incorporate aromatic hydrazine linkers on biomolecules. S-HyNic is an amino-reactive modification 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 APC leads to the formation of the conjugate via a bis-arylhydrazone bond. 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.

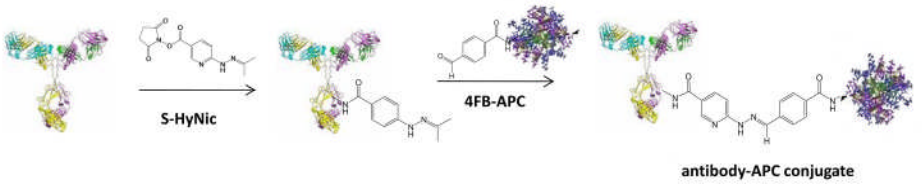

Figure 1: Scheme presenting the two-step protocol to prepare APC-antibody conjugates using Solulink's HydraLink bioconjugation technology. Step 1 is modification of Antibody with S-HyNic to prepare HyNic-Antibody. Step 2 is the conjugation of HyNic-Antibody to 4FB-APC simply by mixing in 100 mM phosphate,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

The many advantages of the HyNic-4FB conjugation couple include that:

1)The reaction is high yielding. Routinely yields of conjugate are 40-60% based on starting protein.

2)The conjugate bond is stable: The bis-arylhydrazone conjugate bond is stable to 92^{O}C and pH 2.0-10.0.

3)The reaction conditions are extremely mild and do not cause any 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, oxidation or reducing reagents are required.

4)The conjugation is traceable spectrophotometrically. The HyNic-4FB conjugate bond is chromophoric- it absorbs at 354 nm and has a molar extinction coefficient of 29000. This allows (1) real time spectrophotometric monitoring of a conjugate reaction, (2) ability to 'visualize' the conjugate during chromatographic purification using a UV or photodiode array detector and (3) quantification of conjugation.

The Keys to Success

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

1. Antibody buffer exchange: Prior to modification, the starting antibody must be completely exchanged into Modification Buffer, pH 8.0.
2. Minimum level of HyNic-modification: The HyNic-antibody molar substitution ratio (MSR) must be >3.5 as determined by a colorimetric assay.
3. The final concentration of the HyNic-antibody in the conjugation reaction must be 1-2 mg/mL.

Kit Components

Component	Size	Storage[1]
S-HyNic	1.0 mg	Desiccated
4FB-APC[2]	1.0 mg	4°C
10X Modification Buffer[3]	1.5 mL	Room temperature
10X Conjugation Buffer[4]	1.5 mL	Room temperature
0.5 mL Zeba columns	4	4°C
DMF (anhydrous)	1.0 mL	Desiccated
2-Sulfobenzaldehyde	100 mg	Room temperature
10X MES Buffer	1.5 mL	Room temperature
Collection tubes	12	Room temperature

NOTES:

1) For convenience all kit components can be stored at 4°C-
    a. If precipitates are present in buffers on storage at 4°C redissolve by warming at 37°C before using
2) **4FB-APC concentration is batch dependent; therefore see lot specific Product Data Sheet for this information**
3) 10X Modification Buffer: 1.0 M phosphate, 1.5 M NaCl, pH 8.0
4) 10X Conjugation Buffer: 1.0 M phosphate, 1.5 M 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
Spectrophotometer or Plate Reader
1.5 mL microcentrifuge tubes
Pipettors
Protein concentration assay reagents such as BCA or Bradford assays

Protocol

1.0 Desalting

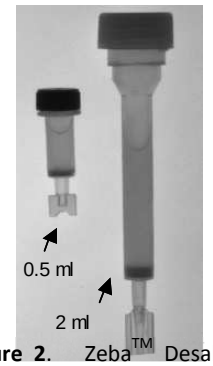

Figure 2. Zeba™ Desalt Spin Columns (0.5 and 2 ml) used to desalt starting Antibody and HyNic-modified Antibody.

Antibodies must be completely desalted into Modification Buffer (100 mM phosphate, 150 mM NaCl, pH 8.0) 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 µL. 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 antibody and one to desalt and exchange the modified antibody into conjugation buffer (see below).

1.1 Zeba Desalting Protocol

0.5 mL Zeba™ Spin Column Preparation (Sample volumes 30-130 µL)

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 8.0) 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.

1.2 Antibody Sample Loading for Desalting

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 antibody 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. Antibody sample is now desalted and ready for modification.

2.0 Protein Modification

Figure 3: Structure of S-HyNic (CAS# 362522-50-7)

Recommended Guidelines for Modifying Proteins/Antibodies with S-HyNic (Figure 3)**:** The modification process is the 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 functional groups incorporated per antibody 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 M.W.), excess linker equivalents added (e.g., 5X, 10X or 20X) and reaction pH. Table 3 presents the results of a study to determine the level of HyNic incorporation on an antibody adding 5X, 10X and 20X equivalents of S-HyNic at 1.0, 2.5 and 5.0 mg/mL antibody concentration in modification buffer (100 mM phosphate, 150 mM NaCl, pH 8.0.

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 1: results of a study to determine the level of HyNic incorporation on an antibody adding 5X, 10X and 20X equivalents of S-HyNic at 1.0, 2.5 and 5.0 mg/mL antibody concentration in modification buffer (100 mM phosphate, 150 mM NaCl, pH 8.0).

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 dramatically change the isoelectric point of the protein and result in precipitation of the antibody or loss of biological activity.

Data has been compiled in Table 1 as an aid in determining the number of equivalents of S-HyNic to be added to a protein solution required to achieve a given molar substitution ratio (MSR).

2.1 S-HyNic-Antibody Modification Protocol

1. Dissolve a vial of pre-weighed 1.0 mg S-HyNic vial in 100 µL anhydrous DMF
2. Add the required volume of S-HyNic to the antibody in modification buffer (**100 mM** phosphate, 150 mM NaCl, pH 8.0) as calculated using the **APC**

Antibody Conjugation Calculator. A volume that typically represents 15 mole equivalents/mole antibody is added and mixed thoroughly.

Notes

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

b) PBS (**10 mM** phosphate, 150 mM sodium chloride, pH 7.2) is **NOT** recommended as a modification buffer due to its poor buffering capacity. Use the Modification Buffer included in the kit.

c) It is important to have a final protein concentration at 2.0-3.0 mg/mL for efficient HyNic modification.

3. Incubate the reaction at room temperature for 1.5 hours.

4. Proceed to desalt the HyNic-modified IgG into 1X Conjugation Buffer (100 mM phosphate, 150 mM NaCl, pH 6.0). See Zeba Desalting Protocol on p. 7.

2.2 Determining the HyNic Molar Substitution Ratio (MSR)

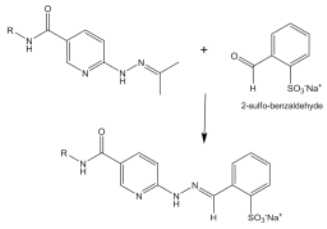

Figure 4: Scheme presenting the colorimetric assay used to quantify the number of HyNic groups on a biomolecule. The bis-arylhydrazone product absorbs at 350 nm has a molar extinction coefficient of 18000 M^{-1}.

The determination of the number of HyNic groups/antibody is accomplished by the colorimetric assay shown in Figure 4 and described below.

HyNic MSR Quantification

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-sBA) Assay Reagent described in Step 1.

1. **Assay Reagent Preparation:** Prepare a 0.5 mM working solution of 2-sBA solution in 0.1 M MES buffer, pH 5.0 as follows:

   a. Prepare a 20 mg/mL solution of 2-sulfobenzaldehyde in water.
   b. Add 52 µL of this solution to a 15 mL conical tube containing 9.948 mL 100 mM MES Buffer (pH 5.0). Label this solution 0.5 mM 2-sBA solution.
   c. Protect the solution from light and keep refrigerated. This solution remains stable for up to 30 days at 4°C.

Method A: Cuvette Spectrophotometer Protocol

1. Transfer 10 µL of HyNic-modified (desalted) antibody solution (~2-5mg/mL in 1X Conjugation Buffer) to a new 1.5 mL microcentrifuge tube containing 490 µL 2-sBA reagent. Prepare another reaction tube (negative control) containing 490 µL 2-sBA reagent and 10 µL of 1X Conjugation Buffer.

2. Incubate all reaction tubes at 37°C for 30 minutes or at room temperature for 2 hours.

3. Remove the reaction tubes from the 37°C incubator and measure the A_{350} of both reactions using a quartz cuvette as follows:

    a. blank the spectrophotometer at 350 nm using 500 µL 0.5 mM 2-sBA solution in MES (pH 5.0) in a 1 mL quartz cuvette.
    b. record the A_{350} of the sample and no antibody controls.

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

4. Using the values obtained, calculate the HyNic/ antibody MSR with the aid of the **APC Antibody Conjugation Calculator** or calculate the MSR by determining the hydrazone concentration using the known molar extinction coefficient (i.e., 18,000 at 350 nm) and dividing by the known molar antibody concentration.

Method B: NanoDrop Method

1. Transfer 2 µL of HyNic-modified (desalted) antibody solution (~2-5 mg/mL in 1X Conjugation Buffer) to a new 1.5 mL microcentrifuge tube containing 18 µL 2-sulfobenzaldehyde reagent. Prepare another reaction tube (negative control) containing 2 µL 1x Conjugation Buffer reagent and 18 µL of 2-sulfobenzaldehyde.

2. Incubate all reaction tubes at 37°C for 30 minutes or at room temperature for 2 hours.

3. Remove the reaction tubes from the 37°C incubator and measure the A_{350} of Determining the Molar Substitution Ratio (MSR)

    Using the values obtained, calculate the HyNic/ antibody MSR with the aid of the **APC Antibody Conjugation Calculator** or calculate the MSR by determining the hydrazone concentration using the known molar extinction coefficient (i.e., 18,000 at 350 nm) and dividing by the known molar antibody concentration.

    It is critical that the MSR of HyNic/antibody is >3.5.

3.0 4FB-APC/HyNic-Antibody Conjugation Protocol

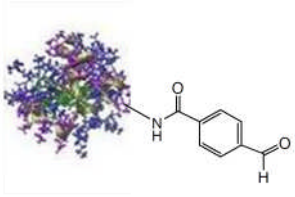

Figure 5: Representative structure of 4FB-APC.

1. Dilute the HyNic-antibody sample to 1-2 mg/mL.
2. Input amount, in milligrams, of HyNic-antibody to be conjugated into the 4FB-APC/HyNic-antibody into the **APC Antibody Conjugation Calculator**.
3. Add calculated volume of 4FB-APC (Figure 6) to the HyNic-antibody solution. Solulink recommends a 1.25/1.0 4FB-APC/HyNic-Antibody ratio but this may be changed as desired.
4. Incubate at room temperature overnight.
5. Desalt reaction into PBS, pH 7.2 using an appropriately sized NAP Sephadex column (GE HealthCare) or Zeba spin filter (ThermoPierce) in order to buffer exchange the conjugate into PBS.
6. The crude reaction can be analyzed by gel electrophoresis using low voltage, <80 volts, or by analytical size exclusion chromatography.
7. Conjugates can be used directly without purification or may be purified to remove unconjugated APC by preparative size exclusion chromatography.

Appendix

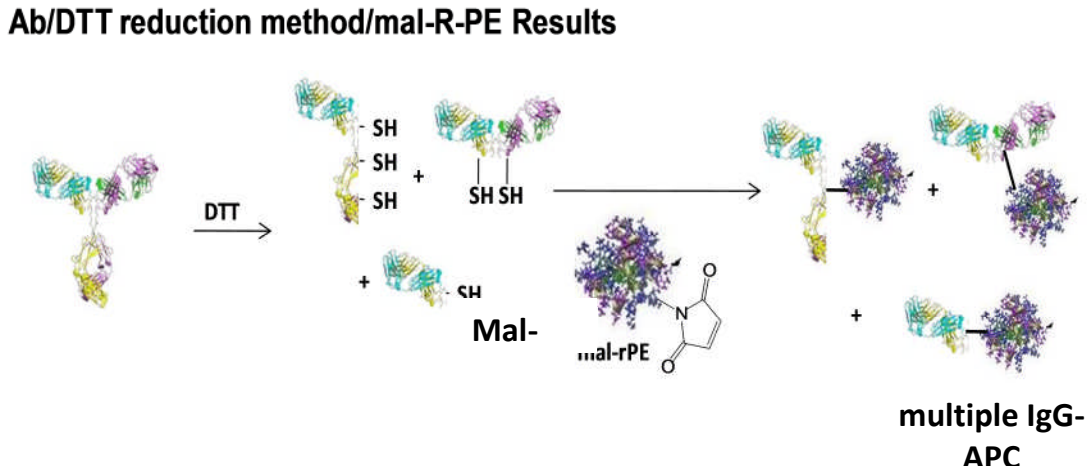

Figure 7: Schematic representation of the conjugation of Antibody to APC using the maleimido/thiol conjugation couple. Thiols are generated by cleaving antibody disulfide bonds with DTT leading to multiple fragments followed by conjugation to Maleimido-APC.

Stability

Component	Storage	Stability
Unopened Kit	4°C	Up to 18 months (see expiration date on kit)
S-HyNic/DMF	4°C	One week
4FB-APC	4°C	6 months
HyNic-modified Antibody		use immediately
APC-Antibody conjugate following desalting without bacteriostat	4°C	4 weeks
APC-Antibody conjugate following purification with bacteriostat- 0.05% sodium azide	4°C	6 months

Troubleshooting

Problem	Possible Cause	Recommended Action
Poor HyNic modification of Antibody	- initial Antibody concentration is too low	- concentrate Antibody using a diafiltration filter, - use an initial concentration of 2.5- 4 mg/mL antibody with 20-30 molar 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
APC-antibody conjugate has a molecular weight that is much larger than predicted	- due to high modification levels on each Antibody a large molecular weight product may be formed	- decrease modification levels by using lower equivalents of S-HyNic, lower the Antibody concentration during the modification reaction