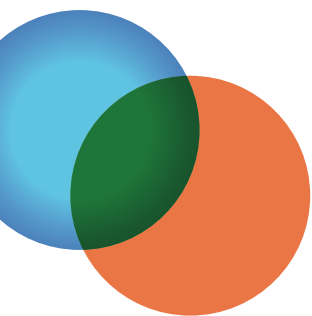


# **RapidDirect<sup>TM</sup> Primary Antibody polyHRP Labeling Kit**

Cat. No. A-9402-001

July 2011

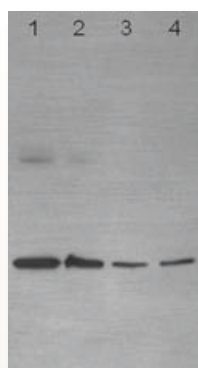
# I. Introduction

It is recognized that primary antibody-polyHRP conjugates would be superior to the classical two-step secondary antibody procedures in immunoassay protocols. Antibody-polyHRP conjugates should provide higher sensitivity as the detection conjugate would be specific for its target antigen, and would not be diluted by nonspecific binding. Furthermore a primary antibody-polyHRP conjugate would offer the researcher significant time savings since the steps of secondary antibody incubation (1 hour) and washing (15 minutes) would not be required. However, straightforward benchtop, user-friendly methods to prepare primary antibody-polyHRP conjugates have not been possible due to inefficient chemistries required to produce these complex conjugates.

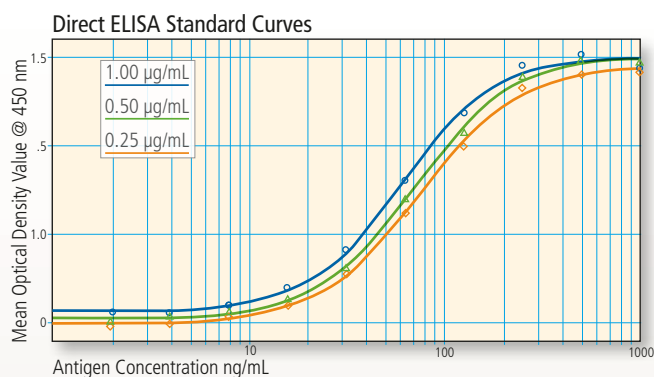
Solulink's catalyzed HyNic/4FB bioconjugation chemistry possesses the required characteristics to produce a Primary Antibody-polyHRP Labeling Kit that allows direct labeling of HRP to any primary antibody in near quantitative yield on one's benchtop without chromatography. We have demonstrated that the primary antibody-polyHRP conjugate prepared with this kit retains its immunoreactivity, yielding a highly sensitive one-step detection reagent. Uses for the primary antibody-polyHRP conjugates prepared with this kit, including IP/western blot, ELISA, IHC, and ELISpot assays, are presented in the figures below.

This kit converts 80–100 µg of primary antibody (in near-quantitative yield) to primary antibody-polyHRP conjugate on your benchtop, requiring only pipettes and a microcentrifuge, in <30 minutes hands-on time. An overview of Solulink's bioconjugation chemistry and a PAGE gel of the resulting antibody-polyHRP conjugate formed using this kit are also presented in the Appendix.

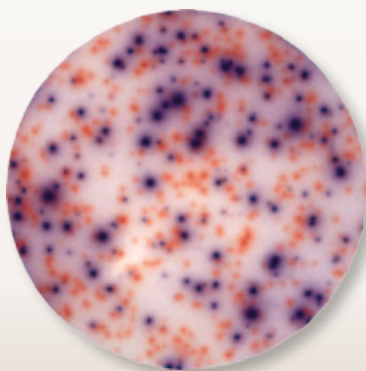
Examples of the use of primary antibody-polyHRP conjugates prepared using the Rapid Direct Primary Antibody-polyHRP Labeling Kit



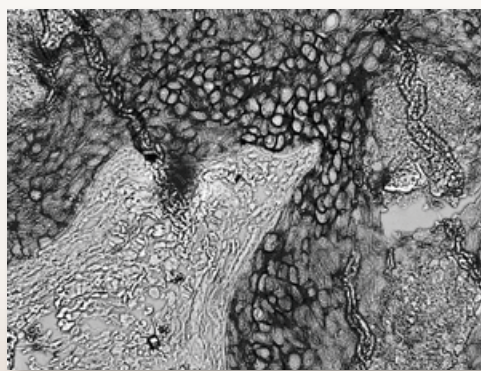
IP/Western blot



ELISA



ELISpot



IHC

## II. Kit Components and Storage

| Components          | Quantity   | Storage conditions |
|---------------------|------------|--------------------|
| S-HyNic             | 1 x 100 µg | 2–8°C or RT        |
| 4FB-modified HRP    | 1 x 50 µL  | 2–8°C              |
| Modification Buffer | 5 mL       | 2–8°C              |
| Spin Columns        | 3          | 2–8°C              |
| Collection Tubes    | 6          | 2–8°C or RT        |
| DMF                 | 0.2 mL     | 2–8°C or RT        |

## III. Experimental Protocols

Materials required, but not included

- Primary anti-antigen antibody
- Microcentrifuge
- UV spectrophotometer

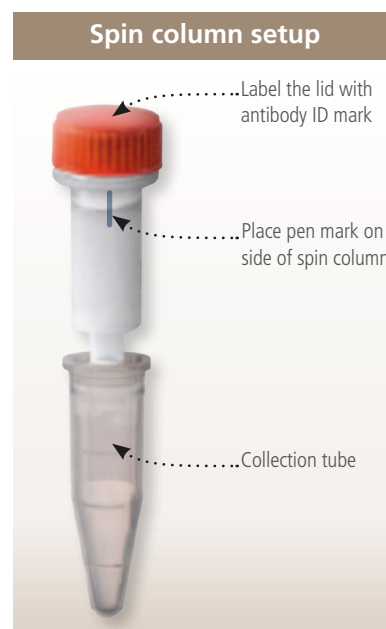
**Note:** The protocol for HRP-primary antibody conjugation requires the antibody samples to be free of protein carriers such as BSA, gelatin or high concentration of glycerol before proceeding.

**Note:** This protocol is specifically designed to conjugate 80–100 µg of antibody to HRP.

### Step 1: Antibody Preparation

Depending on the initial form of your antibody (lyophilized or solubilized), proceed as follows:

- 1.1 **Lyophilized antibody:** Reconstitute the lyophilized antibody (80–110 µg) in 50–150 µL of Modification Buffer. Mix well to obtain a solution of 0.5–2 mg/mL.  
**Solubilized antibody:** If the concentration is between 0.5–2 mg/mL, transfer 50–130 µL to a labeled microcentrifuge tube for use. If the antibody concentration is greater than 2 mg/mL, transfer a volume equivalent to 100 µg of antibody to a labeled microcentrifuge tube.
- 1.2 Prepare a **red cap spin column** by twisting off the bottom closure. Using an appropriate balance tube opposite the assembly, place the spin column into a collection tube (provided). Centrifuge at 1,500X g for 1 minute.  
**Note:** Place a pen mark on the spin column aiming outward and away from the center of the rotor.
- 1.3 Discard the flow-through from the collection tube. Place the column into a new, empty collection tube (provided).
- 1.4 Load the antibody sample from step 1.1 to the top of the dry resin bed. Orient the spin column mark outward and centrifuge at 1,500X g for 2 minutes.  
**Note:** Briefly spin down sample before loading.
- 1.5 Transfer the buffer-exchanged antibody solution (50–130 µL) from the bottom of the collection tube into a new 1.5 mL tube. Label the tube appropriately.
- 1.6 Measure the volume and confirm the antibody concentration by measuring  $A_{280}$  using an appropriate spectrophotometer. Record total microgram amount of the antibody at the beginning of the conjugation (e.g., 92 µL at 1.0 mg/mL contains a total 92 µg of antibody).



### Step 2: Antibody Modification

- 2.1 Add 20 µL DMF to the vial of S-HyNic reagent. Pipette the solution up and down to re-suspend the reagent pellet.  
**Note:** A small but visible pellet can be seen at the bottom of the vial.
- 2.2 Add 2.0 µL dissolved S-HyNic reagent to the buffer exchanged antibody solution from step 1.6. Pipette the solution up and down to mix. Incubate the reaction for 2–3 hours at room temperature.
- 2.3 Prepare **yellow cap spin column** as described in step 1.2. After discarding the flow-through, place the column back into a new, empty collection tube (provided).
- 2.4 Load the completed HyNic / antibody modification reaction from step 2.2 to the top of the dry resin bed. Orient the spin column mark outward as before and centrifuge at 1,500X g for 2 minutes. Then transfer the solution from the bottom of the collection tube to a new 1.5 mL tube.

### Step 3: Antibody-HRP Conjugation

- 3.1 Briefly spin the brown vial containing modified HRP to collect the contents at the bottom of the tube. Transfer ~50 µL of the modified HRP to the Hynic-modified antibody. Incubate at room temperature for 2–3 hours or overnight at 4°C by covering the tube with aluminum foil to avoid light.
- 3.2 Prepare another **red cap spin column** as described in step 1.2, after discarding the flow-through, place the column back into a provided new empty collection tube (provided).
- 3.3 Load the completed antibody/HRP conjugate reaction from step 3.1 to the top of the dry resin bed. Orient the spin column mark outward as before and centrifuge at 1,500X g for 2 minutes. Transfer the solution from the bottom of the collection tube to an amber 0.5 mL microcentrifuge tube, measure the volume, and store at 4°C.
- 3.4 The final antibody concentration is based on the total starting amount of antibody from step 1.6 and the final conjugated volume from step 3.3 (e.g., for a starting amount of 92 µg and final volume of 150 µL, the concentration of the conjugated antibody is 0.61 mg/mL).
- 3.5 For long term storage, add the same volume of glycerol to the antibody/HRP conjugate solution and mix well from step 3.4. The final concentration of the conjugated antibody is half-diluted from step 3.4.

**Note:** The conjugated HRP-antibody will be stable in 50% glycerol at -20°C for 3 months.

## IV. Appendix A

### Western Blot results comparing One-Step 1° antibody-HRP conjugate protocol to Two-Step 2° antibody-HRP conjugate protocol

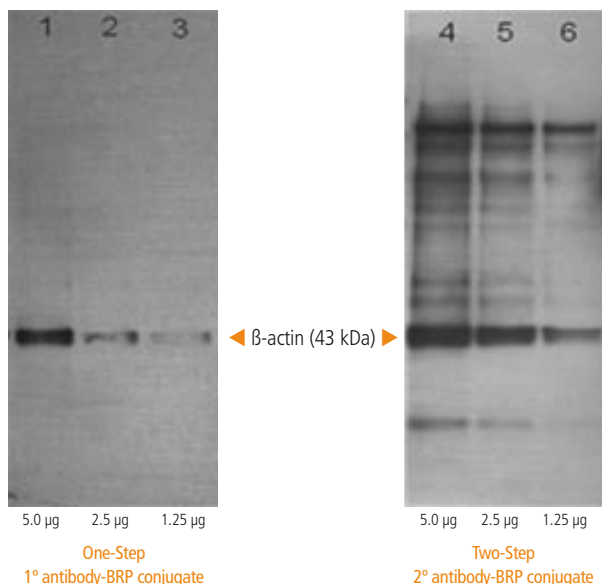
#### Mouse Spleen Whole Cell Lysate

##### Lanes 1-3

**Primary antibody-HRP One Step Western Blot:**  
Mouse spleen whole cell lysate loading from 5 µg to 1.25 µg, nitrocellulose membrane blocked with 3% milk, 1 hour at room temperature; mouse  $\beta$ -actin antibody-HRP conjugate, 0.2 µg/mL, 1 hour at room temperature; ECL development.

##### Lanes 4-6

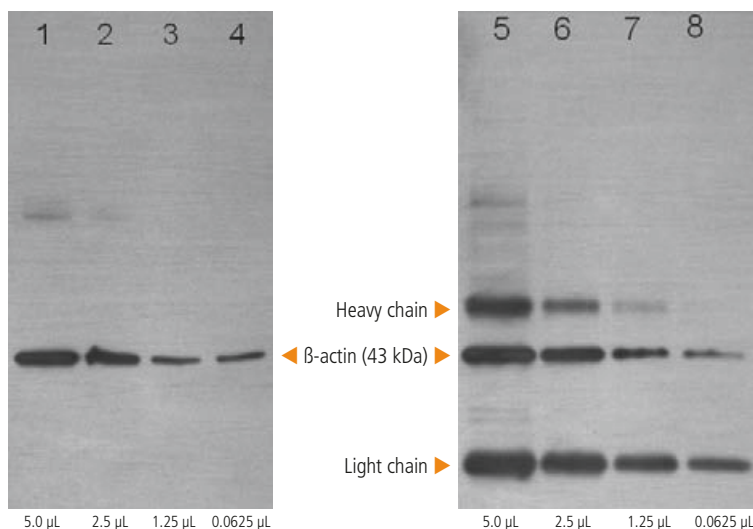
**Secondary antibody-HRP Two Step Western Blot:**  
Mouse spleen cell lysate loading from 5 µg to 1.25 µg, nitrocellulose membrane blocked with 3% milk, 1 hour at room temperature; mouse  $\beta$ -actin, 0.25 µg/mL, 1 hour at room temperature; goat anti-mouse-HRP conjugate, 0.1 µg/mL, 1 hour at room temperature; ECL development.



#### Mouse Splenocyte Cell Lysate

##### Capture / Elution Protocol

1. 25 µg mouse splenocyte incubated with 2 µg mouse  $\alpha$ -actin antibody 1 hour.
2. Add to 10 µL goat  $\alpha$ -mouse antibody immobilized NanoLink magnetic beads, incubate 1 hour.
3. Elute actin from beads by incubation with 0.1 M DTT Loading Buffer, 90°C, 10 minutes.



##### Solulink Direct IP/Western Blot

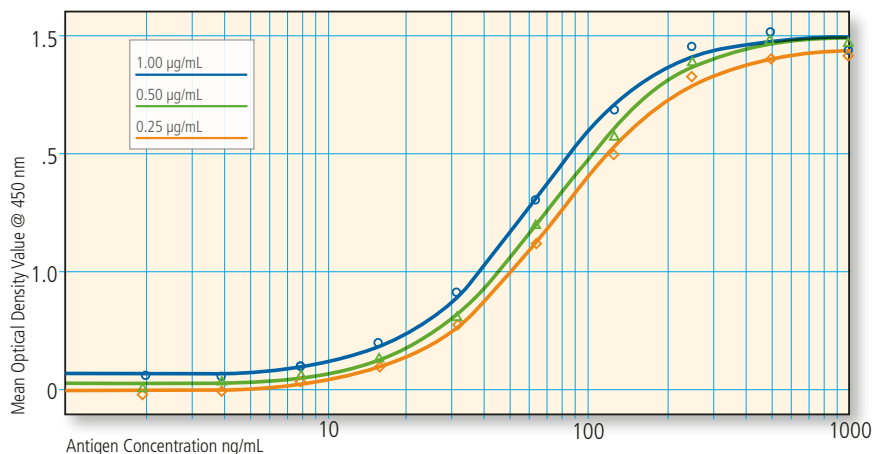
1. Load IP sample 5 µL to 0.625 µL.
2. Transfer to nitrocellulose membrane, 3% milk block, 1 hour.
3. Incubate with 0.2 µg/mL  $\beta$ -actin antibody-HRP conjugate, 1 hour.
4. ECL development.

##### 2° Antibody Western Blot Protocol

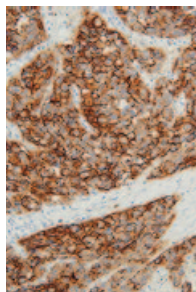
1. Load IP sample 5 µL to 0.625 µL.
2. Transfer to nitrocellulose membrane, 3% milk block, 1 hour.
3. Incubate with 0.2 µg/mL  $\beta$ -actin antibody, 1 hour.
4. Incubate with 0.2 µg/mL goat anti-mouse-HRP conjugate, 1 hour.
5. ECL development.

## Direct ELISA Standard Curves

Direct ELISA curves generated using a Primary HRP conjugate prepared with the Solulink's Direct Primary Antibody Conjugation Kit. A mouse anti-FITC monoclonal antibody was conjugated to HRP as described in the manual. Antigen consisting of FITC-labeled BSA (FITC MSR = 2) was coated on plates in a 2-fold dilution series (100  $\mu$ L @ 500, 250, 125, 62.5, 31.25, 15.625, 7.8, 3.90, and 1.95 ng/mL) using standard methods. Immobilized antigen was then detected at 3 different conjugate concentrations (1  $\mu$ g/mL, 0.5  $\mu$ g/mL, and 0.25  $\mu$ g/mL) using TMB substrate (20 minutes @ 450 nm) on a Molecular Devices plate reader.

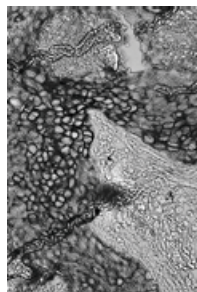


## Immunohistochemistry



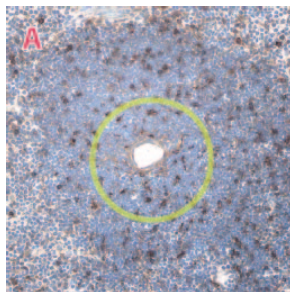
### Two Steps

1. Herceptin-biotin
2. Streptavidin-polyHRP conjugate

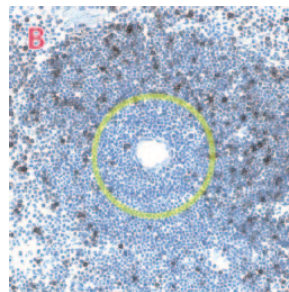


### One Step

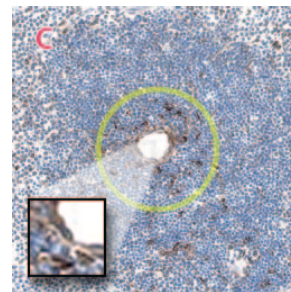
1. Herceptin-polyHRP conjugate



**Panel A:** Typical IHC staining using un-conjugated rat  $\alpha$ -mouse CCL-21 biotinylated secondary antibodies followed by Streptavidin-HRP/DAB chromagen staining (brown). In addition to specific labeling (within the yellow circle) there is also profound non-specific staining due to cross-reactivity of anti-mouse secondary antibodies on antibody expressing mouse tissue.



**Panel B:** Control IHC staining using un-conjugated rat  $\alpha$ -IgG2B (same IHC detection protocol as Panel A) (brown). There is strong non-specific labeling (outside the yellow circle) resembling the non-specific staining observed with un-conjugated primary antibodies (see Panel A)

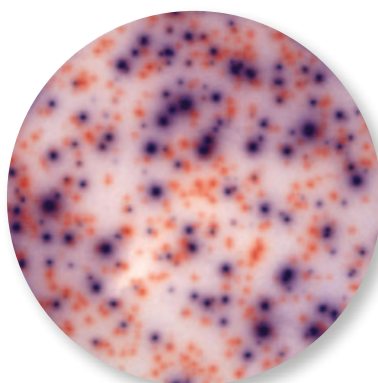


**Panel C:** IHC staining using direct rat- $\alpha$ -mouse CCL21-poly-HRP conjugate prepared using Solulink's Direct Primary Antibody-poly-HRP Conjugation Kit followed by DAB chromagen staining (brown). Note there is specific staining (within the yellow circle) and a lack of non-specific staining (outside the yellow circle) as seen in Panels A and B.

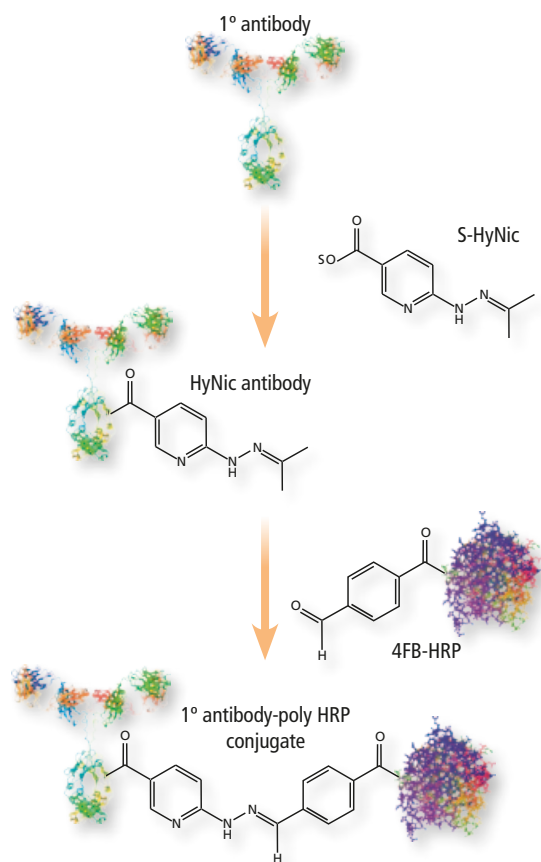
## Two-color ELISpot

**Red:** Direct 1° antibody-polyHRP conjugate developed with AEC

**Blue:** Biotinylated antibody developed with StAv-AlkPhos conjugate and BCIP/NBT

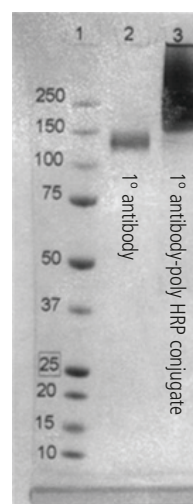


## Solulink's Bioconjugation Technology Used to Prepare 1° Antibody-HRP Conjugates and PAGE Results



**Left:** Solulink's HyNic/4FB conjugation couple as applied to primary antibody-HRP conjugates.

**Below:** PAGE results of conjugation of HyNic-modified antibody to 4FB-HRP as produced using the 1° antibody-poly-HRP couple.



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- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling and disposal.

# RapidDirect™ Primary Antibody polyHRP Labeling Kit

Cat. No. A-9402-001

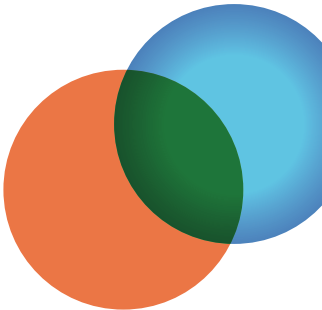
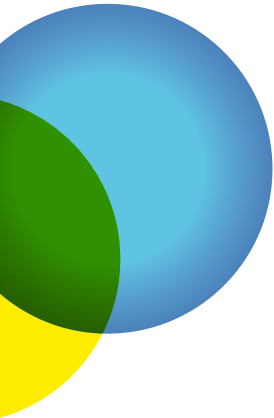
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