solulink™

RapidDirect™ Primary Antibody polyHRP Labeling Kit

Cat. No. A-9402-001

July 2011

I. Introduction

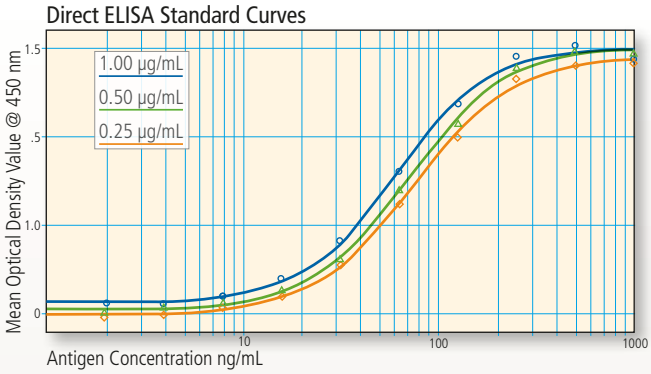
It is recognized that primary antibody-polyHRP conjugates would be superior to the classical two-step secondary antibody procedures in immunoassay protocols. Antibody-polyHRP conjugates should provide higher sensitivity as the detection conjugate would be specific for its target antigen, and would not 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.

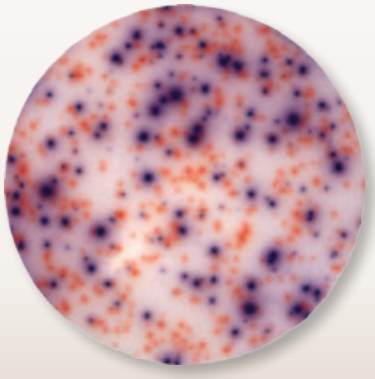
This kit converts 80–100 µg of primary antibody (in near-quantitative yield) to primary antibody-polyHRP conjugate on your benchtop, requiring only pipettes and a microcentrifuge, in <30 minutes hands-on time. An overview of Solulink's bioconjugation chemistry and a PAGE gel of the resulting antibody-polyHRP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

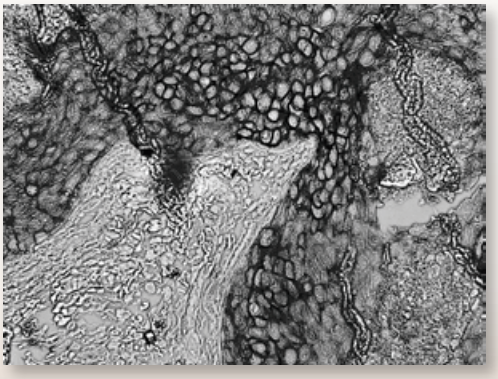
IP/Western blot



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
- UV spectrophotometer
- Microcentrifuge

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.*

Spin column setup

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 4ºC 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 4ºC.

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.*

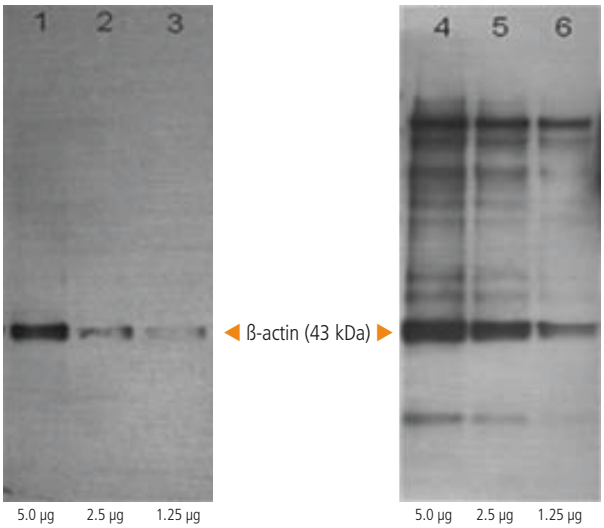
IV. Appendix A

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 ß-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 ß-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.



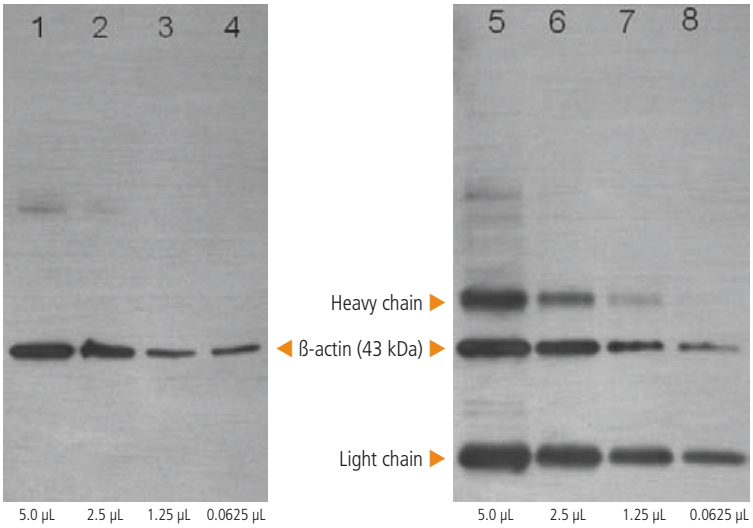
One-Step
1° antibody-BRP conjugate

Two-Step
2° antibody-BRP conjugate

Mouse Splenocyte Cell Lysate

Capture / Elution Protocol

1. 25 µg mouse splenocyte incubated with 2 µg mouse α-actin antibody 1 hour.
2. Add to 10 µL goat α-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

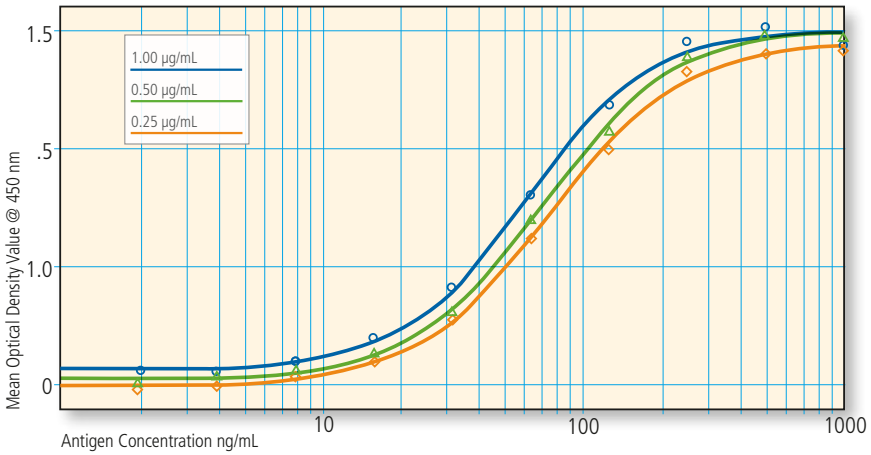
1. Load IP sample 5 µL to 0.625 µL.
2. Transfer to nitrocellulose membrane, 3% milk block, 1 hour.
3. Incubate with 0.2 µg/mL ß-actin antibody-HRP conjugate, 1 hour.
4. ECL development.

2° Antibody Western Blot Protocol

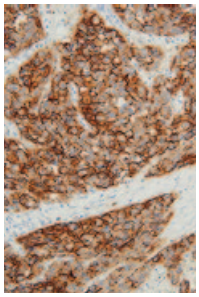
1. Load IP sample 5 µL to 0.625 µL.
2. Transfer to nitrocellulose membrane, 3% milk block, 1 hour.
3. Incubate with 0.2 µg/mL ß-actin antibody, 1 hour.
4. Incubate with 0.2 µg/mL goat anti-mouse-HRP conjugate, 1 hour.
5. ECL development.

Direct ELISA Standard Curves

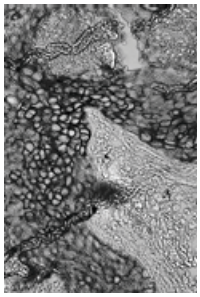
Direct ELISA curves generated using a Primary HRP conjugate prepared with the Solulink's Direct Primary Antibody Conjugation Kit. A mouse anti-FITC monoclonal antibody was conjugated to HRP as described in the manual. Antigen consisting of FITC-labeled BSA (FITC MSR = 2) was coated on plates in a 2-fold dilution series (100 µL @ 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 µg/mL, 0.5 µg/mL, and 0.25 µg/mL) using TMB substrate (20 minutes @ 450 nm) on a Molecular Devices plate reader.



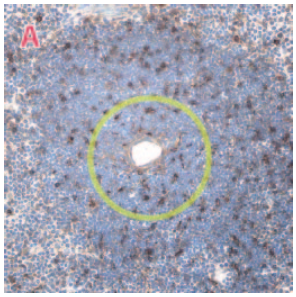
Immunohistochemistry



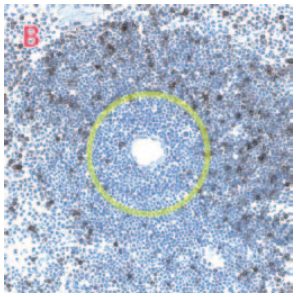
Two Steps
1. Herceptin-biotin
2. Streptavidin-polyHRP conjugate



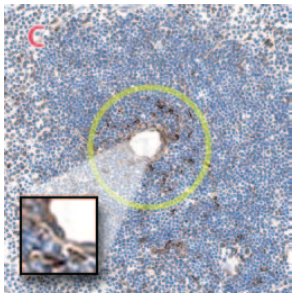
One Step
1. Herceptin-polyHRP conjugate



Panel A: *Typical IHC staining using un-conjugated rat α-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 α-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)*

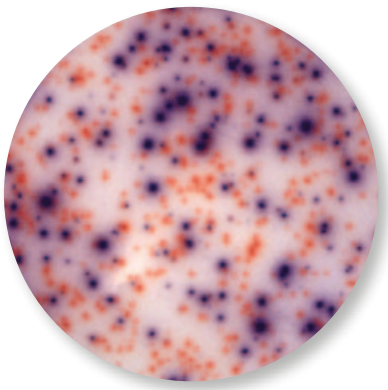


Panel C: *IHC staining using direct rat-α-mouse CCL21-poly-HRP conjugate prepared using Solulink's Direct Primary Antibody-poly-HRP Conjugation Kit followed by DAB chromagen staining (brown). Note there is specific staining (within the yellow circle) and a lack of non-specific staining (outside the yellow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

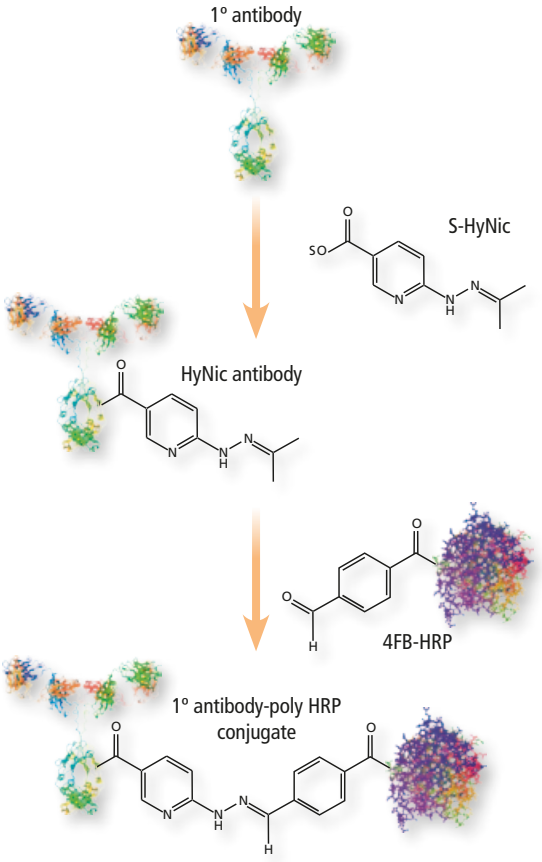
Two-color ELISpot

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

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

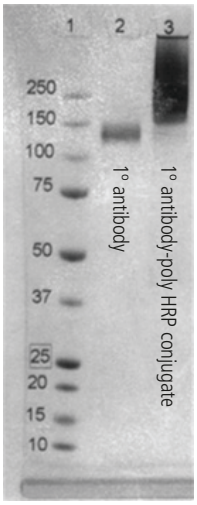


Solulink's Bioconjugation Technology Used to Prepare 1° Antibody-HRP Conjugates and PAGE Results



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

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



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- Read and understand the Material Safety Data Sheets (MSDS) available at www.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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Cat. No. A-9402-001

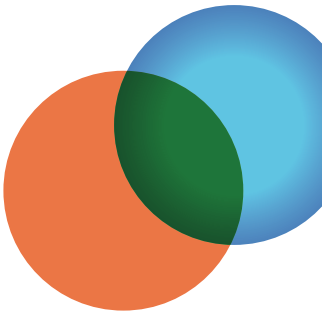
100% Satisfaction Guarantee

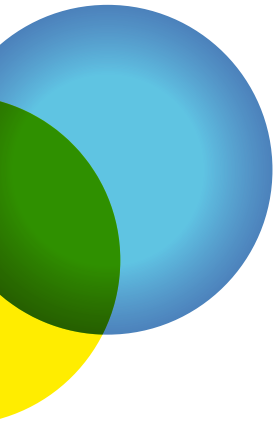
Exceptional quality and batch-to-batch consistency are of paramount importance for all of us at Solulink. Consequently, Solulink has established strict quality control guidelines for each format of our products and each batch must pass these stringent biochemical and biological/immunological testing requirements.

However, if any of our products do not meet these specifications in your hands, please contact us; your concerns will be addressed quickly, and after investigation, the product will either be immediately replaced or credited for the original purchase price.

How to Order

Our Customer Service Department is available 8 AM–6 PM (Pacific Time), Monday through Friday to help you with your orders. Orders can be placed via email, telephone, fax, mail, or on our website.





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