solulink™

RapidDirect™ Primary Antibody polyHRP Western Blot Kit

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I. Introduction

Western Blot Detection

Development of western blots with secondary antibody/HRP conjugates while simple in execution has historically produced results with significant background banding that in many cases obscures visualization of the target antigen. Other recent approaches to precluding interfering/irrelevant bands have distinct limitations of sensitivity. Moreover, such approaches depend on relative affinity of a secondary WB antibody for native IgG over denatured; this is relative, rather than absolute, so heavy and light chain band interference does still occur to some degree even with these indirect methods.

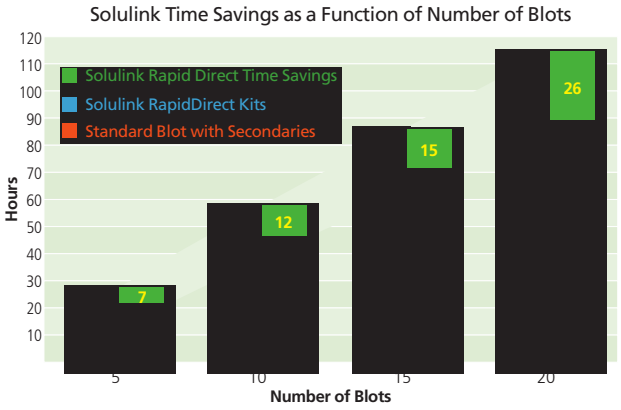
It has long been recognized that it is superior to use a primary anti-antigen/HRP conjugate in a western blot assay as it would:

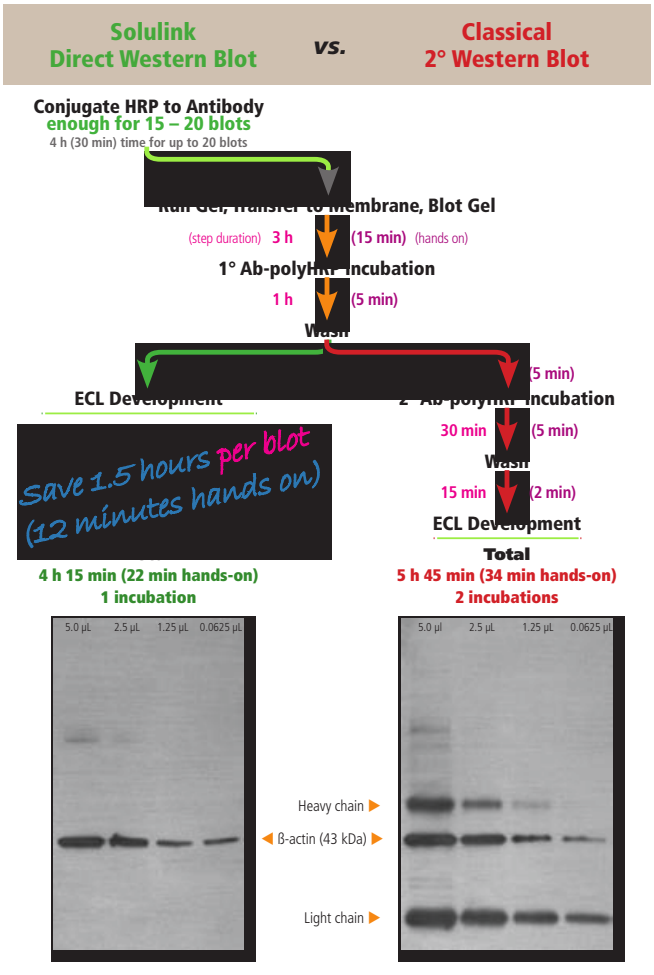
- produce a gel result with minimal background
- eliminate the second secondary antibody incubation step thereby reducing the time required to complete the protocol
- obviate the need for secondary antibodies and therefore eliminate any cross-species contamination
- allow multiplexing

Solulink's 1° Antibody-polyHRP Western Blot Labeling Kit allows direct-labeling of HRP to the anti-antigen antibody in near quantitative yield producing a highly sensitive one step detecting reagent which eliminates the root cause of the interfering bands resulting from binding of detecting reagents to heavy and light chains of antibodies and other proteins on the blot membrane.

This kit converts 75–100 µg of primary antibody to primary antibody-poly-HRP conjugate on their benchtop requiring only pipettes and a microcentrifuge in <30 minutes hands-on time. The final conjugate allows at minimum development of 10–20 western blots dependent on the inherent affinity of the primary antibody. Examples of the western blots from both mouse LLC and cell lysates are presented in Appendix A.

In addition to getting publication quality gels the first time, if you run 10–20 western blots, Solulink's technology can save you 10–25 hours compared to conventional secondary western blot, by first conjugating antibody directly to HRP.





With Solulink's technology, you first conjugate the antibody to HRP, which makes enough for 10 to 20 blots, which in turn saves you 10 to 25 hours (with just 4.5 hours hands-on time) compared to secondary antibody methods.

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
Antibody Working Buffer	120 mL	2–8°C
20X Wash Buffer	75 mL	2–8°C

III. Experimental Protocols

Materials required, but not included:

- Primary anti-antigen antibody
- UV spectrophotometer
- PVDF or nitrocellulose membrane
- Microcentrifuge
- SDS-PAGE gel & immunoblotting equipment & reagents
- ECL film
- Rocking platform or rotator
- Chemiluminescent substrate

Note: *The protocol of 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

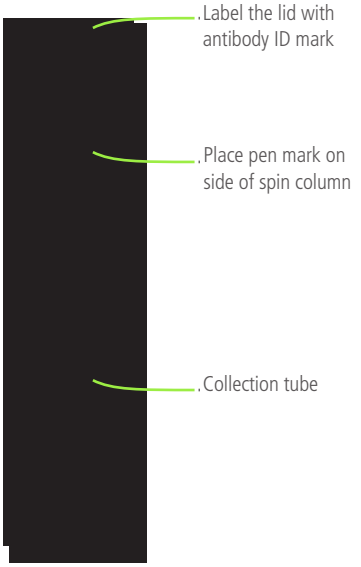
Depending on the initial form of your antibody (lyophilized or solubilized), proceed as follows:

1.1 **Lyophilized Antibody:** Reconstitute the lyophilized antibody (80–110 µg) in 50–150 µL Modification Buffer and 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 microcentrifuge 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 resuspend 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 microcentrifuge 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.

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 and 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.*

Step 4: Procedure for Western Blotting

4.1 Prepare Block Buffer by adding 3–5% BSA or dry milk (not provided) in 1X Wash Buffer (diluted from 20X Wash Buffer).

4.2 Remove the membrane from the transfer apparatus and place it in an incubated tray containing 12 mL of Blocking Buffer. Incubate for 1 hour at room temperature on shaker.

4.3 Decant Blocking Buffer and wash the membrane for 10 minutes with 22 mL of 1X Wash Buffer, then decant. Repeat this wash step 2 more times.

4.4 Prepare 11 mL of the diluted conjugated HRP-primary antibody in an Antibody Working Buffer at an appropriate concentration (typically 0.2–2 µg/mL).

4.5 Add the Antibody Working Buffer from step 4.4 to the membrane and incubate for 1 hour at room temperature or overnight at 4°C on shaker.

4.6 Decant antibody working buffer, wash the membrane for 10 minutes with 22 mL of 1X Wash Buffer, then decant. Repeat this wash step 2 more times.

4.7 Incubate the membrane in chemiluminescent HRP substrate ECL working solution for 1–5 minutes (please refer to the instructions of the ECL working solution).

4.8 Expose the membrane to ECL film for an appropriate time period.

IV. Troubleshooting Guide

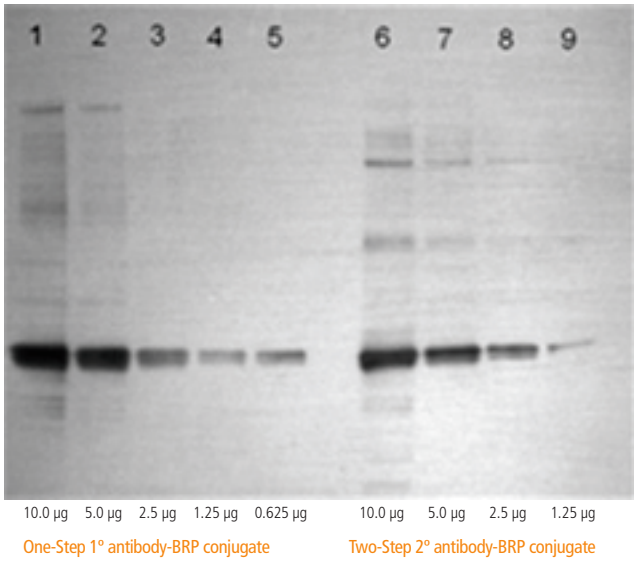
Problem	Possible Cause	Recommended Action
Poor HyNic modification	Quality and/or purity of starting antibody is poor	If antibody quality or source is undetermined, perform suitable test such as BCA or Bradford protein assay to confirm the purity and quantity of the starting material.
	Presence of protein carriers such as BSA or gelatin may be contaminating antibody sample	Remove and purify the antibody sample of all protein carriers such as BSA or gelatin or glycerol using affinity chromatography or another method before proceeding.
	Concentration of S- HyNic modification reagent	Make sure to thoroughly dissolve the S-HyNic reagent before adding it to the antibody.
		Use a calibrated pipette to ensure accuracy with small- volume additions.
	Presence of non-protein amine contaminants	Remove all non-protein amine contaminants such as glycine or Tris before modifying the antibody with S-HyNic reagent.
	Improper storage of S-HyNic reagent can lead to hydrolysis of the NHS ester	Keep and store the S-HyNic reagent sealed in the pouch provided below 4°C.
	Initial antibody concentration is low	Confirm initial antibody concentration prior to S-HyNic modification on the spectrophotometer. If in doubt, perform a Bradford assay.
High background	Overexposed film	Decrease exposure and develop time
	Insufficient washing	Add an additional wash cycle
	Incubation tray is contaminated with HRP	Use a clean incubated tray after every step of the procedure
	Insufficient blocking	Increase nonfat dry milk or BSA in blocking agent.

Weak signal	Used insufficient quantities of antigen or primary antibody	Load higher concentration of sample onto the gel or strip and re-probe blot using higher concentration of antibody
	Inefficient protein transfer	Optimize transfer procedure
Spots within the protein bands	Inefficient protein transfer	Optimize transfer procedure
	Unevenly hydrated membrane	Hydrate membrane according to manufacturer's instructions
	Bubble between ECL film and membrane	Remove all bubbles before exposing blots to film
Speckling	Over-heating during electrophoresis or transfer	Control temperature during electrophoresis and transfer
Too much nonspecific bands	Poor primary antibody: low signal/high noise	Use optimized primary antibodies (high signal)
	Heated sample stored at 4°C too long	Use freshly heated sample
White band with too many nonspecific bands	Primary antibody-HRP concentration too high	Decrease primary antibody-HRP concentration 2–5 times

V. Appendix A

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

Mouse LLC Lysate

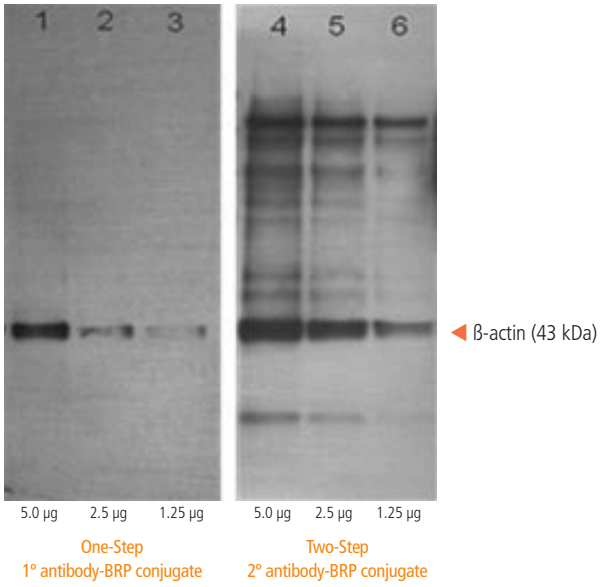


Mouse LLC Lysate

Lanes 1-5
Primary antibody-HRP Western Blot:
Mouse LLC lysate loading from 10 µg to 0.625 µg, nitrocellulose membrane blocked with 4% BSA, 1 hour at room temperature; mouse ß-actin antibody-HRP conjugate, 0.25 µg/mL, 1 hour at room temperature; ECL development.

Lanes 6-9
Secondary antibody-HRP Western Blot:
Mouse LLC lysate loading from 10 µg to 1.25 µg, nitrocellulose membrane blocked with 4% BSA, 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.

Mouse Spleen Whole Cell Lysate

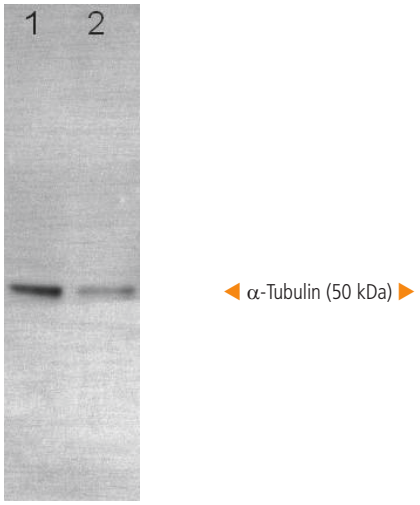


Mouse Spleen Whole Cell Lysate

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.

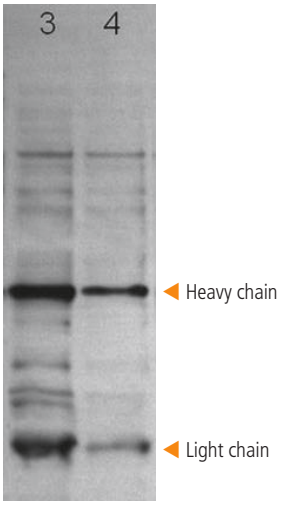
Lanes 4-6
Secondary antibody-HRP Two Step Western Blot: *Mouse spleen cell lysate loading from 5 µg to 1.25 µg, nitrocellulose membrane blocked with 3% milk, 1 hour at room temperature; mouse ß-actin, 0.25 µg/mL, 1 hour at room temperature; goat anti-mouse-HRP conjugate, 0.1 µg/mL, 1 hour at room temperature; ECL development.*

Mouse Splenocyte Lysate



Solulink Direct
IP/Western Blot

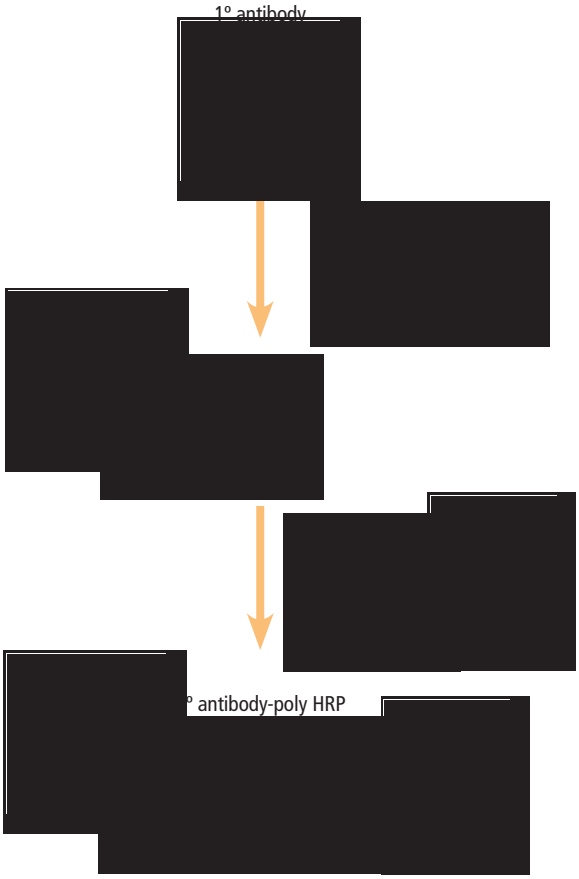
1. Load sample 5 µg (Lane 1) to 2.5 µg (Lane 2).
2. Transfer to nitrocellulose membrane, 3% milk block, 1 hour.
3. Incubate with 0.2 µg/mL α-Tubulin antibody-HRP conjugate, 1 hour.
4. ECL development.



2° Antibody
Western Blot Protocol

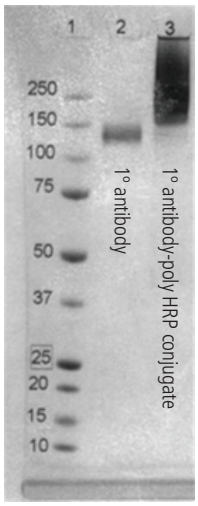
1. Load sample 5 µg (Lane 1) to 2.5 µg (Lane 2).
2. Transfer to nitrocellulose membrane, 3% milk block, 1 hour.
3. Incubate with 0.2 µg/mL α-Tubulin antibody-HRP conjugate, 1 hour.
4. Incubate with 0.15 µg/mL goat anti-mouse-HRP conjugate, 1 hour.
5. ECL development.

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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- 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.

RapidDirect™ Primary Antibody polyHRP Western Blot Labeling Kit

Cat. No. A-9401-001

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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.

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