solulink™

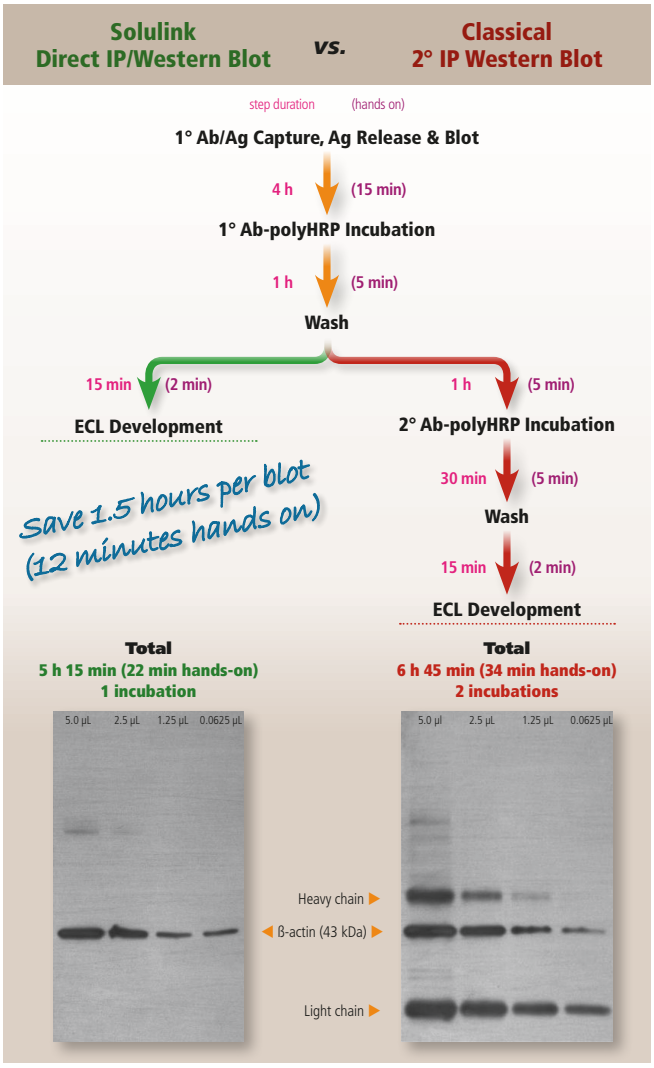
Rapid Direct IP/Western Blot Kit

Cat. Nos. A-9403-001A, A-9403-001B, and A-9403-001C

April 2011

I. Introduction

Solulink's two-component, high-performance Rapid Direct IP/Western Blot Kit includes α-species IgG immobilized on NanoLink™ Magnetic Beads for antigen/antibody pulldown and reagents to prepare a primary α-antigen antibody-HRP conjugate in near quantitative yield. The α-species IgG-immobilized beads efficiently capture the primary antibody/antigen complex and the primary α-antigen antibody-HRP conjugate yields a highly sensitive, one-step detection reagent. This novel method leads to increased sensitivity and eliminates bands resulting from the binding of detection reagents to heavy and light chains of antibodies and other background proteins on the blot membrane.



Advantages gained from this kit include:

- **Ease-of-Use:** The primary capture antibody/antigen complexes are captured using anti-species secondary antibodies immobilized on magnetic beads, avoiding the use of protein A or protein G beads.
- **Significant Time Savings:** The western blot is developed with a primary α-antigen antibody-HRP conjugate, eliminating the secondary antibody incubation and wash steps, saving 1.5 hours per blot.
- **Higher Sensitivity:** Only the antigen band is developed as the primary α-antigen antibody-HRP conjugate delivers more conjugate to the antigen band, not to the interfering heavy and light chain IgG bands.

This kit converts 75–100 µg of primary antibody to primary antibody-HRP conjugate in near-quantitative yield on one's benchtop, requiring only pipettes and a microcentrifuge, requiring less than 30 minutes hands-on time. The final conjugate allows, at minimum, development of 10–20 western blots, depending on the inherent affinity of the primary antibody. Examples of results using the Rapid Direct IP/Western Blot Kit from both splenocyte and mouse LLC cell lysates are presented in Appendix A.

II. Kit Components and Storage

Components	Quantity	Storage conditions
Secondary Antibody Beads	100 µL	2–8°C
S-HyNic	1 x 100 µg	2–8°C or RT
4FB-modified HRP	1 x 50 µL	2–8°C
Modification Buffer	10 mL	2–8°C
Spin Columns	3	2–8°C
Collection Tubes	6	2–8°C or RT
DMF	200 µL	2–8°C or RT
2-HP Quenching Reagent	100 µL	2–8°C
Antibody Working Buffer	120 mL	2–8°C
20X Wash Buffer	80 mL	2–8°C

III. Experimental Protocols

Materials required, but not included

- Primary antibody
- 1 M DTT and SDS-PAGE sample loading buffer
- UV spectrophotometer
- PVDF or nitrocellulose membrane
- Microcentrifuge
- SDS-PAGE gel and immunoblotting equipment and 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 concentrations of glycerol before proceeding.*

Note: *This protocol is specifically designed to conjugate 75–100 µg of antibody to HRP.*

Step 1: Antibody Preparation

1.1 **Lyophilized antibody:** Reconstitute the lyophilized antibody (75–100 µg) in 50–130 µL of 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 50–150 µL to a labeled microcentrifuge tube for use.
- If the antibody concentration is greater than 2 mg/mL, transfer a volume equivalent to 75–100 µg of antibody to a labeled tube.

Note: *Use 75 µg primary antibody for HRP conjugate preparation.*

Note: *Retain 25 µg to use for immunoprecipitation.*

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 provided collection tube. 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 back into a new, empty collection tube (provided).

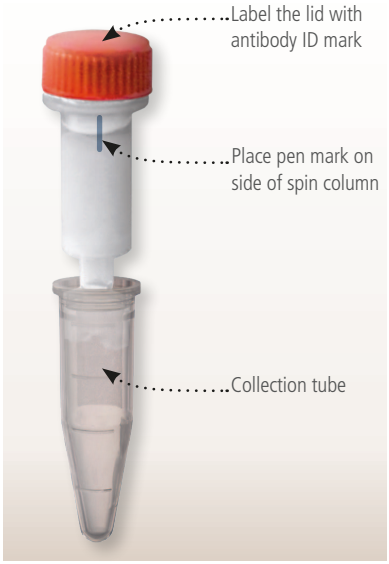
1.4 Load the antibody sample from step 1.2 to the top of the dry resin bed.

Note: *Briefly spin down sample before loading.*

1.5 Orient the spin column mark outward and centrifuge at 1,500X g for 2 minutes. Transfer the buffer-exchanged antibody solution (50–150 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 the A_{280} using an appropriate spectrophotometer. Record the 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).

Spin Column Setup



Step 2: Antibody Modification

2.1 Add 20 µL of 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 of dissolved S-HyNic reagent to the buffer exchanged antibody solution from step 1.5. 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 aiming outward and away from the center of the rotor and centrifuge at 1,500X g for 2 minutes.

2.5 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 4FB-modified HRP to collect the contents at the bottom of the tube. Transfer ~50 µL of the 4FB-modified HRP to the HyNic-modified antibody and mix well. Incubate at room temperature for 3 hours or overnight at 4°C by covering the tube with aluminum foil to avoid light.

3.2 Add $^{1}/_{10}$ volume of 2-HP quench reagent to antibody/HRP solution (*e.g.*, add 10 µL of 2-HP Quencher Solution to 100 µL of antibody/HRP solution). Pipette up and down to mix well. Incubate at room temperature for 2 hours and cover the tube with aluminum foil to avoid light.

3.3 Prepare another **red 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).

3.4 Load the completed antibody/HRP conjugate reaction from step 3.2 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. 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.

3.5 The final antibody concentration is based on the total starting amount of antibody from step 1.6 and the final conjugated volume from step 3.4. (*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).

3.6 For long term storage, add the same volume of glycerol to the antibody/HRP conjugate solution and mix well from step 3.5 and mix well. 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: Immunoprecipitation

4.1 Take 50–300 µg of cell lysate, dilute to 1 mg/mL by addition of 1X Modification Buffer.

4.2 Add 2–10 µg (normally 2 µg) of primary antibody to diluted cell lysate, mix well, and incubate at room temperature for 1 hour.

4.3 Ten minutes prior to end of incubation time, prepare secondary antibody NanoLink Magnetic Beads by pelleting the beads by centrifugation (10 seconds at 1,000X g), then resuspend the bead slurry by pipetting the solution up and down so that **all beads** are in solution.

4.4 Transfer 10 µL of resuspended magnetic beads to a 1.5 mL microcentrifuge tube.

4.5 Add 200 µL of 1X Modification Buffer and mix by pipetting up and down. Place the tube on a magnetic rack for 1 minute to clarify the supernatant. Remove the supernatant using a pipette. Repeat this step two additional times to wash beads.

4.6 Discard the supernatant, then briefly spin down beads.

Note: *You may see some beads left on the microcentrifuge tube wall.*

4.7 Once the incubation of the antibody antigen complex (step 4.2) is completed, add the reaction solution to the washed secondary antibody beads. Be sure to pipette the reaction solution over any beads stuck on the wall of the tube to ensure that all beads are in solution and well mixed. Sonicate for 20 seconds.

4.8 Incubate for 1 hour at room temperature. Mix the bead slurry every 20 minutes during incubation.

Note: *Alternatively, if the beads are prepared first (steps 4.3 to 4.6), the solutions in steps 4.2 and 4.7 can be combined into a single incubation for 1 hour at room temperature to simultaneously bind and immobilize.*

4.9 Place the tube on a magnetic rack for 1 minute, then discard the supernatant.

4.10 Add 400 µL of 1X Wash Buffer (diluted from 20X Wash Buffer), mix thoroughly, and incubate for 2 minutes.

4.11 Place the tube on a magnetic rack for 1 minute and discard the supernatant.

4.12 Repeat steps 4.9 to 4.11 twice more.

Note: *There may be some beads left on the microcentrifuge tube wall.*

4.13 Discard supernatant, then briefly spin down beads.

4.14 Prepare reducing loading buffer: add $^{1}/_{10}$ volume of 1 M DTT (not provided) to 1X SDS-PAGE sample loading buffer (*e.g.*, NuPAGE® sample buffer or standard Læmmli buffer, not provided) to create 100 mM DTT in solution.

Note: *It is critical to add reducing agent DTT. Immediately add to 1X SDS-PAGE loading buffer before use.*

4.15 Add 40 µL of reducing sample loading buffer to the beads, mix well, and make sure all beads get into solution. Heat at 90–100°C for 10 minutes.

4.16 Briefly spin down at 1,000X g for 30 seconds and place the tube on a magnetic rack for 1 minute. Collect the supernatant carefully and load 5–20 µL onto a gel, depending on target antigen expression.

4.17 Alternatively, transfer the supernatant sample to a clean tube and freeze at -80°C if the gel is to be run later.

Step 5: Procedure for Western Blotting

5.1 Prepare Blocking Buffer by adding 3–5% dry milk (not provided) in 1X Wash Buffer (diluted from 20X Wash Buffer).

5.2 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.

5.3 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.

5.4 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).

5.5 Add the Antibody Working Buffer from step 5.4 to the membrane and incubate for 1 hour at room temperature or overnight at 4°C on shaker.

5.6 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.

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

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

IV. Troubleshooting Guide

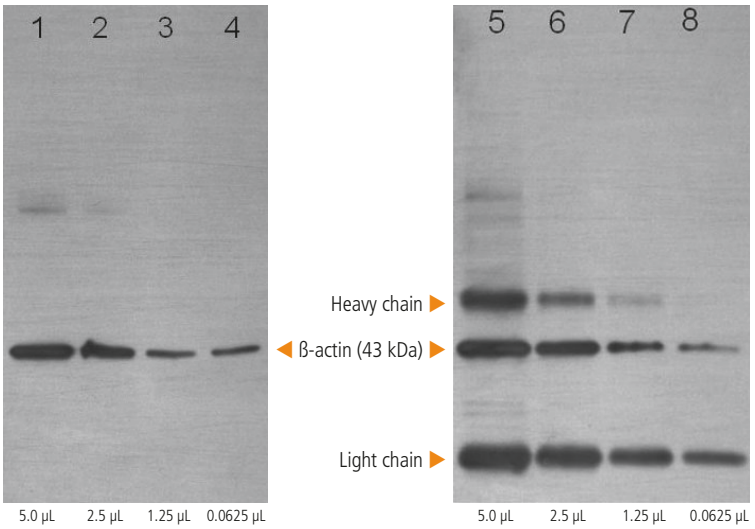
Problem	Possible Cause	Recommended Action
High background	Too much primary antibody-HRP was used	Reducing the amount of primary antibody-HRP in immunoblotting
	Non-optimized primary antibody	Use only primary antibodies optimized for immunoblotting
	Overexposed film	Decrease exposure and develop time
	Insufficient washing	Add an additional wash cycle or increase the salt concentration of the Wash Buffer
	Incubation tray is contaminated with HRP	Use a clean incubated tray after every step of the procedure
	Insufficient blocking	Increase nonfat dry milk in blocking agent.
Weak signal	Weak primary antibody	Use only primary antibodies optimized for immunoblotting
	Too little protein is loaded	Load more protein onto the gel
	The primary antibody has a low affinity for the antigen	Increase the antibody concentration or incubation time during immunoprecipitation
	Primary antibody is not a match IgG for secondary antibody beads	Use only match IgG as primary antibody for match secondary antibody beads
	Target protein is not expressed in the sample or present at very low level	Use positive control, and optimize the amount of protein loading
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 many 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 fold

V. Appendix A: Example Results/Comparisons

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.

Mouse LLC Lysate

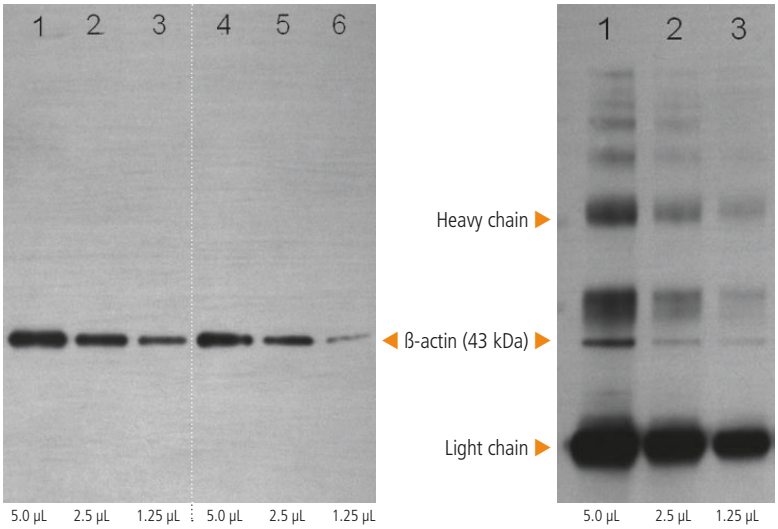
Capture Elution Protocols

One-Step Antigen (Lanes 1–3)

1. Incubate 25 µg mouse LLC lysate with 2 µg mouse ß-actin antibody and 10 µL goat α-mouse antibody immobilized NanoLink Magnetic Beads, 1 hour.
2. Elute actin from beads by incubation with 0.1 M DTT Loading Buffer, 90°C, 10 minutes.

Two-Step Antigen (Lanes 4–6)

3. Incubate 2 µg mouse ß-actin antibody with 25 µg mouse LLC lysate, 1 hour.
4. Add to 10 µL goat α-mouse antibody immobilized NanoLink Magnetic Beads, incubate, 1 hour.
5. 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 1.25 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.

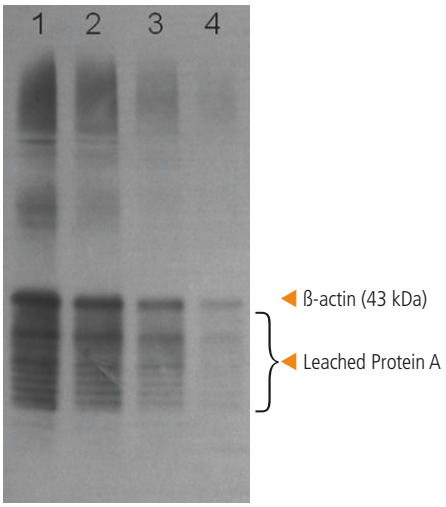
2° Antibody Western Blot Protocol

1. Load IP sample 5 µL to 1.25 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.

Mouse LLC Lysate

Protein A Beads Antigen Capture / Elution Protocol

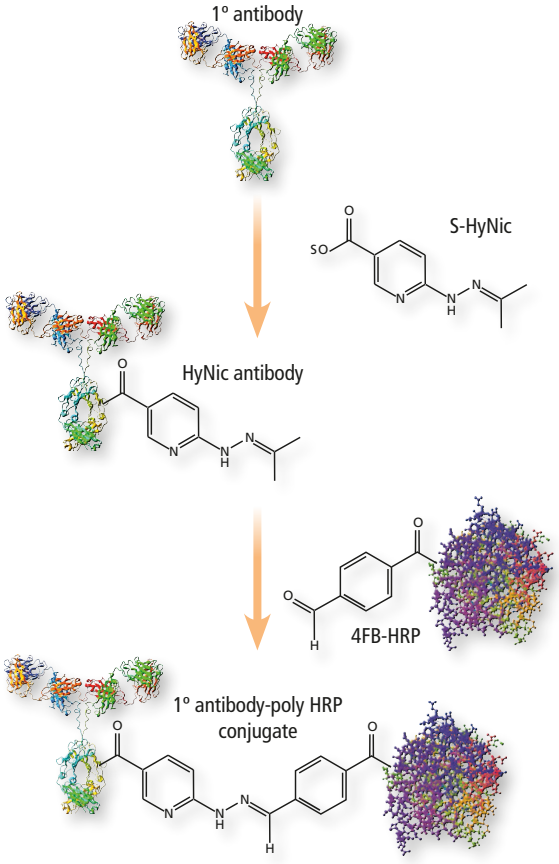
1. Mouse LLC lysate was incubated with 4 µg mouse ß-actin antibody 1 hour.
2. Added 50 µL protein A-agarose beads and incubated for 4 hours.
3. Elute ß-actin from beads by incubation with Loading Buffer, 90°C, 10 minutes.



Rapid Direct IP/Western Blot

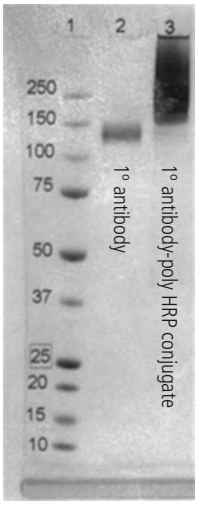
1. Load IP sample 5 µL to 0.625 µL.
2. Transfer to nitrocellulose membrane, 3% milk block, 1 hour.
3. Incubate with 0.2 µg/mL ß-actin antibody-HRP conjugate, 1 hour.
4. ECL development.

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 IP/Western Blot Kit yielding the primary antibody-poly-HRP conjugate.*



VI. Appendix B: Solutions & Recipes

Required, but not included:

1M DTT

- Can be made fresh or can be stored in aliquots at -20°C for 6 months or 4°C for 2 weeks. Avoid repeated freeze thaws.

1X Reducing Sample loading Buffer

(containing 0.1 M DTT)

- 3% SDS
- 25 mM Tris base, pH 6.5
- 10% glycerol
- 6.25% bromophenol blue
- 6.25% phenol red
- 0.1 M DTT
- 4X Sample Buffer without DTT is available from Life Technologies (Cat. No. NP0007)

IP Lysis Buffer

- 150 mM NaCl
- 50 mM Tris-HCl, pH 7.4
- 1–10% NP-40
- 0.25–0.5% Na-deoxycholate
- 1–5 mM EDTA

Protease Inhibitor Cocktail (100X)

- PMSF, 5 mg (50 µg/mL)
- Aprotinin, 100 µg (1 µg/mL)
- Leupeptin, 100 µg (1 µg/mL)
- Pepstatin, 100 µg (1 µg/mL)

Phosphatase Inhibitor (100X)

- 1 mM Na_3VO_4
- 1 mM NaF

Kit includes:

Modification Buffer, pH 7.4

- 100 mM Phosphate
- 150 mM NaCl

Antibody Working Buffer, pH 7.4

- 25 mM Tris-HCl
- 100 mM NaCl
- 0.05% Tween-20
- 1% BSA

20X Wash Buffer, pH 7.4

- 0.5 M Tris-HCl
- 2 M NaCl
- 1% Tween-20

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Rapid Direct IP/Western Blot Kit

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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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