ROCKLAND

Fluorescent TrueBlot® Anti-Mouse IgG Secondary Antibodies

Protocol

Fluorescent TrueBlot® monoclonal secondary antibodies combine the power and specificity of HRP-conjugated TrueBlot® with the versatility of fluorescent and near-infrared (NIR) dyes. TrueBlot® antibodies are designed for use in the Western Blot (WB) of Immunoprecipitated (IP) samples (IP-Western blot), in which the same antibody (or same species) is used for both applications. The unique detection properties of TrueBlot® antibodies recognize the native, non-reduced form of IgG over the reduced form of IgG to enable detection of immunoblotted target protein bands without hindrance by interfering immunoprecipitating immunoglobulin heavy and light chains. Fluorescent TrueBlot® easily accommodates the detection of proteins with overlapping molecular weights to the heavy and light chain to provide accurate, publication-quality, IP-Western blots.

This protocol is intended for use in IP-Western blot procedures with TrueBlot® Anti-Mouse Ig IP Agarose Beads (00-8811-25) and the compatible fluorescent TrueBlot® anti-mouse IgG secondary antibodies listed below.

I. Compatible Products

Product
Fluorescent TrueBlot® Anti-Mouse Fluorescein (18-0217-32)
Fluorescent TrueBlot® Anti-Mouse Dylight™ 680 (18-4417-32)
Fluorescent TrueBlot® Anti-Mouse DyLight™ 800 (18-4517-32)

Note: All fluorescent TrueBlot® anti-mouse IgG secondary antibodies are supplied as a lyophilized powder. Reconstitution with 100 µL of deionized water (or equivalent) will result in a concentration of 1 mg/mL. Be sure the powder is not at the top of the vial before opening. See data sheet for proper handling and storage conditions.

II. Reagents and Materials Required but Not Provided

Method	Product
Collection of Cell Lysate	UltraPure Sterile Water (MB-009-1000)
	1X PBS, pH 7.2: Dilute 10X PBS, pH 7.2 (MB-008) to 1X with UltraPure Sterile Water; keep on ice.
	Cell Lysis Buffer[1] **Note**: Add appropriate volume of protease/phosphatase inhibitors to recommended final concentration from manufacturer prior to use.
	Cell Scraper
	Protease and Phosphatase Inhibitors[2]
Immunoprecipitation	TrueBlot Anti-Mouse Ig IP Agarose Beads[3] (00-8811-25)
	Wide-bore Pipet Tips (or cut pipet tip for pipetting agarose bead suspension)
	IP Buffer[4]
	IP Antibody
SDS-PAGE/ Western Blot	2X SDS-PAGE Sample Buffer without DTT or b-ME (MB-018) or Laemmli equivalent
	1 M DTT or 14.3 M β-Mercapoethanol
	2X SDS-PAGE Sample Buffer with reducing agent[5]: Add DTT to a final concentration of 50 mM or add β-Mercaptoethanol to a final concentration of 2% (v/v)
	Opal Pre-Stained Standard 10–245 kDa (MB-210-0500) or 10–180 kDa (MB-209-0500)
	10X SDS-PAGE Running Buffer (MB-017) or running buffer equivalent
	Transfer Buffer[6]
	Ponceau S Solution (MB-072-0500)
	Blocking Buffer for Fluorescent Western Blotting (MB-070) or 5% BLOTTO (B501-0500) in 1X TBS[7]
	Primary Antibody (for WB)

Method	Product
SDS-PAGE/ Western Blot (Cont.)	1X TBS, pH 7.5: Dilute 10X TBS, pH 7.5 (MB-012) with UltraPure Sterile Water or equivalent to 1X
	1X TBS-T (0.1% Tween): Dilute 10X TBS, pH 7.5 (MB-012) with UltraPure Sterile Water or equivalent to 1X, add TWEEN® 20[8]
	0.20 µm or 0.5 µm PVDF or nitrocellulose membrane
	Forceps (blunt end)
	Membrane Incubation Trays (WIB-2875-010 or WIB-4625-005)

[1]RIPA (MB-030-0050) and NP-40 (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1% NP-40) are commonly used lysis buffers. For optimal results, choose a lysis buffer that is most compatible with your protein of interest and downstream IP application. If running protein assays prior to SDS-PAGE, be sure the components of your lysis buffer are compatible with your choice of assay.

[2]The type of protease and phosphatase inhibitors used, as well as the final working concentration, varies and should be optimized by the end user. However, a suggestion of final concentrations for a general protease inhibitor cocktail is 1.0 mM PMSF, 10 µM leupeptin, 0.1 µM aprotinin, and 1.0 µM pepstatin. The recommended final concentration for phosphatase inhibitors is 1.0 mM Na_3VO_4 and 1 mM NaF. A variety of cocktails are also available commercially.

[3]Do not vortex the agarose IP beads prior to the elution step as this may cause damage. Agarose IP beads are in suspension and settle upon storage. Prior to use, mix the vial well by gentle inversion to ensure delivery of the proper bead volume. Ig IP beads give the best results; however, if using Protein A or G beads, follow manufacturer's recommended IP protocol. It is not recommended to use more than 10 µg (per mL) or a final of 5 µg of IP antibody per lane.

[4]The use of standard IP buffers for binding and wash steps, such as 1X PBS or 1X TBS with or without low concentrations of gentle detergents, as well as other IP buffers should be optimized by the end user for their specific immune complex. If using cell lysis buffer in these steps, take into consideration the detergent type and concentration, as these factors may disrupt native structure or protein-protein interactions.

[5]Prepare reducing 2X SDS sample buffer fresh and use within the hour. Discard remainder. Concentration of reducing agents can be increased, if needed.

[6]Choose transfer buffer based on protein of interest, gel type, membrane, and transfer type.

[7]Choice of buffer must be determined and optimized by end user. TWEEN 20 should not be included in the blocking step due to its autofluorescence properties.

[8]The final TWEEN 20 concentration used in the 1X TBS-T buffer may be adjusted if necessary (0.01–0.1%) when optimized by the end user.

III. Procedure for Cell Lysate Preparation

1. Aspirate or pipet off cell culture media. Wash with ice-cold 1X PBS, then remove 1X PBS.
2. Harvest approximately 1 x 10^7 cells by using a cell scraper and transfer to a conical tube. If working with adherent cells, you can skip this step and lyse directly on the plate (see step 5).
   Note: The total number of cells per mL and the cell equivalent loaded per lane of gel should be optimized specifically for each protein and antibody. Alternatively, protein concentration can be determined using a protein assay.
3. Wash cells with ~10 mL of cold 1X PBS and centrifuge at 400 x g for 10 minutes at 4°C.
   Note: If using adherent cells, wash cells on the plate while keeping the plate on ice.
4. Discard the supernatant and repeat step 3.
5. After the second wash, remove the supernatant and resuspend the cell pellet in 1 mL of cold Lysis buffer containing protease inhibitors. The final concentration of cells should be about 1 x 10^7 cells/mL.
   Note: If using adherent cells, the cold lysis buffer can be added directly to the plate and put on a rocker at 4°C. Harvest by either scraping or collecting just the supernatant. If collecting just the supernatant, proceed to step 8.
6. Gently vortex and transfer to a 1.5 mL microcentrifuge tube.
7. Place on ice for 30 minutes with occasional mixing.
8. Centrifuge at 10,000 x g for 15 minutes at 4°C.
9. Carefully collect the supernatant without disturbing the pellet and transfer to a new, clean 1.5 mL microcentrifuge tube and discard the pellet.
10. The protein concentration can be determined by a protein assay. Samples can be diluted to 1 mg/mL if desired.
11. The cell lysate can be frozen at this point for long-term storage at -80°C.

IV. Procedure for Cell Lysate Preclearing

1. Resuspend the immobilized Ig IP bead slurry by repeated gentle inversion of the tube. Remove 50 µL of the bead slurry with a wide-bore pipet tip and wash in cell lysis buffer or IP buffer, if different. Resuspend in 50 µL IP buffer or cell lysis buffer.
2. Add 500 µL of cell lysate (~5 x 106 cells or ~500 µg of total protein) to the pre-equilibrated bead slurry and incubate on a rocking platform or rotator for 30–60 minutes at 4°C.
3. Centrifuge at 2,500 x g for 2–3 minutes at 4°C and transfer the supernatant to a new 1.5 mL microcentrifuge tube. If any of the bead slurry has been transferred, centrifuge again and carefully transfer the supernatant to another fresh 1.5 mL microcentrifuge tube.

V. Procedure for Immunoprecipitation

1. Add 1–10 µg of immunoprecipitation antibody to the tube containing the cold, precleared cell lysate.
   Note: This concentration of antibody is suggested as a starting point. Each investigator may desire to titrate the concentration of antibody and volume of cell lysate in preliminary experiments to determine the optimal conditions, e.g., 1–10 µg/1 x 10^7 cells/1 mL lysate. Typically, 2 µg is a sufficient amount of antibody to maximally immunoprecipitate most antigens in 1 mL of extract from 1 x 10^7 cells. Using as little IP antibody as possible minimizes potential contamination of SDS-reduced sample with non-reduced immunoprecipitating antibody light chain. It is not recommended to use more than 10 µg (per mL) or a final of 5 µg per lane.
2. Incubate at 4°C for 1 hour on a rocking platform or a rotator.
3. Add at least 50 µL of pre-equilibrated bead slurry to capture the immune complexes.
4. Incubate for 1 hour or overnight at 4°C on a rocking platform or a rotator.
   Note: Steps 1 and 3 can be combined for a single incubation.
5. Centrifuge the tube at 2,500 x g for 30 seconds at 4°C.
6. Carefully remove the entire supernatant and wash the beads 3–5 times with 500 µL of cold lysis buffer or IP buffer, centrifuging to pellet beads between each wash. To minimize background, be sure to completely remove the supernatant after each wash.
7. After the last wash, carefully aspirate the supernatant and add 50 µL of SDS reducing sample loading buffer or equivalent to the bead pellet.
   Note: 1X or 2X SDS reducing sample buffer can be added to the beads. In-house experiments use 2X. Please take into consideration the composition of the loading buffer. It is important that the sample is completely reduced.
8. Gently vortex and heat samples at 90–100°C for 10 minutes.
9. Centrifuge at 10,000 x g for 5 minutes, carefully collect the supernatant, and load onto the gel.
10. Alternatively, the supernatant samples can be collected, transferred to a clean 1.5 mL microcentrifuge tube and frozen at -80°C, if the gel is to be run at a later time.
11. Follow manufacturer's instructions for SDS-PAGE.

VI. Procedure for Western Blot

1. Following SDS-PAGE, transfer proteins from the gel onto either a PVDF or nitrocellulose membrane.
   Note: For best protein transfer results, follow the instructions provided by the transfer system manufacturer.
   Optional: To determine whether the proteins have been transferred to the membrane, stain with a 0.1% Ponceau S solution. Protein bands can be visualized after staining for 5 minutes. To remove the Ponceau S stain, rinse with distilled water or 1X TBS-T until most of the dye is removed before moving on to blocking step. Residual dye will not affect subsequent steps.
2. Place the membrane into blocking buffer (enough to cover the membrane) and incubate for 2 hours at room temperature or overnight at 4°C on a rocking platform.
   Note: Use Rockland's blocking buffer for fluorescent Western blotting or 5% BLOTTO in 1X TBS. BSA may not effectively block the reduced chains.
3. Prepare the primary immunoblotting antibody in blocking buffer as recommended by the supplier.
   Note: If the recommended concentration is not known, use a standard concentration of 1–2 µg/mL. If using hybridoma tissue culture supernatant or serum for immunoblotting, preliminary experiments should be performed to evaluate whether dilution of the supernatant or serum is needed for best results.
4. Incubate the blot with primary antibody for at least 2 hours at room temperature or overnight at 4°C on a rocking platform.
   Note: Specific times should be determined empirically for optimal results.
5. After the incubation of the membrane with the primary antibody, wash the blot at least 3–5 times in 1X TBS-T, each wash for a minimum of 5–10 minutes each. Total should be more than 1 hour.
6. Prepare the fluorescent TrueBlot® secondary antibody at a 1:1000 dilution in the blocking buffer.
   Note: This dilution is intended as an initial recommendation. Specific conditions for each protein and antibody combination should be specifically optimized by the end user. To optimize the detection of mouse IgG1, we recommend performing a dot blot or titration to determine the ideal dilution factor (starting at 1:1000) for your desired application.
7. Incubate the blot with fluorescent TrueBlot® secondary antibody for 1 hour at room temperature on a rocking platform.
   Note: Specific times should be determined empirically for optimal results.
8. Wash the blot at least 3–5 times in 1X TBS-T, each wash for at least 5 minutes each. Total should be more than 1 hour.
9. Perform a final wash with 1X TBS to remove any residual Tween®.
10. Image the blot with an imaging system that is compatible with the fluorescent dye conjugated to the secondary antibody.

Notes:

Recommended Positive control: Species-dependent IgG TrueBlot® will detect SDS-denatured, non-reduced species-specific IgG. A 20–30 ng sample of non-reduced, immunoprecipitating antibody can be included in the immunoblot as a positive control to ensure positive performance of TrueBlot®.

Recommended Negative control: Samples containing 0.5–2.0 ug of reduced species-specific IgG (prepared and run immediately as described in Sample Preparation) can be included as a negative control to ensure that individual TrueBlot® assays do not detect heavy and light chains of the immunoprecipitating antibodies.

Recommended Additional Controls:

1. Omit the cell extract during the IP
2. Omit the IP antibody during the IP
3. For secondary antibody only, omit the primary immunoblotting antibody

TrueBlot® Troubleshooting Guide

Table 1. Common Troubleshooting Techniques for TrueBlot® Products

Issue	Resolution	Solution
No signal	1. Weak primary antibody	1. Use only primary antibodies optimized for immunoblotting
	3. Primary antibody is not a species-specific IgG	3. Use only species-specific IgG as primary antibody
	4. Target is not expressed in the sample or present at low levels	4. Use a positive control sample known to contain the target protein and optimize the amount of protein loaded
	5. Antigen is present in blocking solution	5. Change blocking reagents
High background	1. Non-optimized primary antibody	1. Use only primary antibodies optimized for immunoblotting
	2. Insufficient washing	2. Increase volume, number, and duration of washes or increase salt content of the wash buffer.
	3. Membrane was allowed to dry out and was not re-wet	3. Ensure membrane does not dry during immunoblotting procedure. Immobilon-P and other PVDF membranes must be saturated in methanol and equilibrated in buffer.
	4. Insufficient blocking	4. 5% (w/v) non-fat dry milk is the best blocking agent. BSA is specifically not recommended.
Ig and the specific band of interest are both seen	1. Improper sample preparation	1. Follow sample preparation procedure
	2. Sample not completely denatured/reduced	2. Increase amount of reducing/denaturing agent
Other bands besides the specific band of interest are seen	1. Poor primary antibody (low signal/high noise)	1. Use primary antibodies optimized for immunoblotting (high signal/low noise)
		2. Possible different isoforms/modification of the protein of interest

References

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227 (5259), 680.