

Protocol

TrueBlot® IP & Western Blot Kit for FLAG® Epitope Tag

Rockland's TrueBlot® Immunoprecipitation and Western Blot Kit for FLAG® Epitope Tag (88-8887-383) contains the critical supporting reagents, buffers, and substrates needed for the Western blot detection of immunoprecipitated proteins tagged with FLAG®. The HRP-conjugated TrueBlot® anti-mouse IgG secondary antibody supplied in the kit recognizes the native, non-reduced form of mouse IgG over the reduced form of mouse IgG. This unique detection property eliminates interference from the denatured/reduced heavy and light chain bands of the IP antibody allowing the same antibody to be used in both IP and Western Blot. Use of this kit easily allows researchers to generate accurate, publication-quality IP–Western Blots.

Components

Product	Size	Item No.
1000X Mouse TrueBlot® Anti-Mouse IgG Secondary Antibody (HRP)	50 µL	18-8817-31
TrueBlot® Blocker	10 g	88-888C-BC
20X TrueBlot® Assay Buffer	30 mL	88-888C-AB
TrueBlot® Enhancer Solution	25 mL	88-888C-ES
TrueBlot® Substrate A	12.5 mL	88-888C-SA
TrueBlot® Substrate B	12.5 mL	88-888C-SB
TrueBlot® Anti-Mouse Ig IP Beads ¹	25 µL	00-8811-25
Antibody for the detection of FLAG® ²	100 µg	200-301-383

¹Do 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. It is not recommended to use more than 10 µg (per mL) or a final of 5 µg of IP antibody per lane.

²A primary antibody has been provided to be used as a control at the user's discretion. Dilutions should be optimized by the user

Reagents Required

Collection of Cell Lysate

Product	Preparation	Item No.
UltraPure Sterile Water		MB-009-1000
1X PBS, pH 7.2	Dilute 10X PBS, pH 7.2 to 1X with UltraPure Sterile Water; keep on ice.	MB-008

Cell Lysis Buffer ¹	Add appropriate volume of protease/phosphatase inhibitors to recommended final concentration from manufacturer prior to use.
Cell Scraper	
Protease and Phosphatase Inhibitors ²	

Immunoprecipitation

Product	Preparation	Item No.
Wide-bore Pipet Tips	Or cut pipet tip for pipetting agarose bead suspension	
IP Buffer ⁴	Add appropriate volume of protease/phosphatase inhibitors to recommended final concentration from manufacturer prior to use.	

SDS-PAGE/Western Blot

Product	Preparation	Item No.
2X SDS-PAGE Sample Buffer without DTT	Or β -ME or Laemmli equivalent	MB-018
1 M DTT or 14.3 M β -Mercaptoethanol		
2X SDS-PAGE Sample Buffer with reducing agent ⁴	Add DTT to a final concentration of 50 mM or add β -Mercaptoethanol to a final concentration of 2% (v/v)	
Opal Prestained Protein Standard	10–245 kDa or 10–180 kDa	MB-201-0500 / MB-209-0500
10X SDS-PAGE Running Buffer or running buffer equivalent		MB-017
Transfer Buffer ⁶		
Ponceau S Solution		MB-072-500
1X TBS-T (0.1% Tween)	Dilute 10X TBS, pH 7.5 with UltraPure Sterile Water or equivalent to 1X, TWEEN® 20 ⁶	MB-012 / MB-009-1000
0.20 μ m or 0.5 μ m PVDF or nitrocellulose membrane		
Forceps (blunt end)		

¹RIPA 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.

²The 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 μ M leupeptin, 0.1 μ M aprotinin, and 1.0 μ M pepstatin. The recommended final concentration for phosphatase inhibitors is 1.0 mM Na₃VO₄ and 1 mM NaF. A variety of cocktails are also available commercially.

³Use of standard IP buffers for binding and wash steps, such as 1X PBS or 1X TBS with or without low concentrations of gentle detergent, 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.

⁴Prepare reducing 2X SDS sample buffer fresh and use within the hour. Discard remainder. Concentration of reducing agents can be increased, if needed.

⁵Choose transfer buffer based on protein of interest, gel type, membrane, and transfer type.

⁶The 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.

Equipment Required

1. Pre-cooled Centrifuge (4°C)
2. Rocking Platform
3. Rotator
4. Heat Block
5. SDS-PAGE and Western Blot Equipment
6. Imaging System (compatible with chemiluminescence) or dark room and equipment for x-ray film

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 1×10^7 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 1×10^7 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.

Procedure for Cell Lysate Preclearing

1. Resuspend the immobilized anti-species-specific IgG bead slurry. Remove 50 μL and wash in lysis buffer or IP buffer, if different. Resuspend in 50 μL of cell lysis buffer or IP buffer. Bead slurry by repeatedly inverting the vial several times. Do not vortex.
2. Add 500 μL of cell lysate ($\sim 5 \times 10^6$ cells or $\sim 500 \mu\text{g}$ protein) to the pre-equilibrated bead slurry and incubate on a rocking platform or orbital shaker for 30–60 minutes at 4°C .
3. Centrifuge at $2,500 \times g$ for 2–3 minutes at 4°C and transfer the supernatant to a new 1.5 mL tube. If any of the bead slurry has been transferred, centrifuge again and carefully transfer the supernatant to another fresh 1.5 mL tube.

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 $\mu\text{g}/1 \times 10^7$ cells/1 mL lysate.
2. Typically, 2 μg is a sufficient amount of antibody to maximally immunoprecipitate most antigens in 1 mL of extract from 1×10^7 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.
3. Incubate at 4°C for 1 hour on a rocking platform or a rotator.
4. Add at least 50 μL of pre-equilibrated bead slurry to capture the immune complexes.
5. 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. Centrifuge the tube at $2,500 \times g$ 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 $90\text{--}100^\circ\text{C}$ for 10 minutes.
9. Centrifuge at $10,000 \times g$ 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.

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 membrane in a container with transfer buffer. Under a chemical hood, prepare a container with TrueBlot® Enhancer Solution. Use tweezers to remove the membrane from the container with transfer buffer and allow residual buffer to drain off the membrane. Place the membrane in enhancer solution for 2 minutes, then wash with 1X TBS-T.
3. Place the membrane with 5% blocker solution (w/v) and incubate for 2 hours at room temperature or overnight at 4°C on a rocking platform.
Note: To prepare the 5% blocker solution (w/v), dilute 20X assay buffer with UltraPure water to 1X and dissolve appropriate amount of blocker powder. It is recommended to use milk or BLOTTO as the blocking reagent for HRP-conjugated TrueBlot® secondary antibodies, as BSA may not block the reduced chain as effectively.
4. 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.
5. 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.
6. 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.
7. Prepare the 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.
8. Incubate the blot with TrueBlot® secondary antibody for 1 hour at room temperature on a rocking platform.
Note: Specific times should be determined empirically for optimal results.
9. 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.
10. Prepare the chemiluminescent-HRP substrate working solution by mixing equal volumes of substrate A and substrate B.
11. Incubate the blot in chemiluminescent-HRP substrate working solution for 1–5 minutes.
12. Image the blot with an imaging system compatible with chemiluminescent detection or by exposing the blot to x-ray film.

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 µg 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 TrueBlots® do not detect heavy and light chains of the immunoprecipitating antibodies.

Recommended additional controls:

- Omit the cell extract during the IP
- Omit the IP antibody during the IP
- Omit the immunoblotting antibody

Troubleshooting Guide

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

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