Goat IgG TrueBlot® Western Blot Kit Protocol - *Research Use Only*

Catalog No. 88-8884-31

Materials Included

- Goat TrueBlot®: Anti-Goat IgG HRP - 50 µl. An HRP-conjugated second step reagent reacting with Goat IgGs for optimal signal detection in immunoprecipitation/immunoblotting experiments (1000X) - 18-8814-31
- TrueBlot® Enhancer Solution: 25 ml - 88-888C-ES
- TrueBlot® Blocker: 10 g - 88-888C-BC
- TrueBlot® Assay Buffer: 30 ml. 20X - 88-888C-AB
- TrueBlot® Substrate A: 12.5 ml - 88-888C-SA
- TrueBlot® Substrate B: 12.5 ml - 88-888C-SB

Materials Not Included

- Immobilized Protein A (Cat. No. PA50-00-0002) or Goat Ig IP beads (Cat. No. 00-8844-25) which is included in the kit (Cat. No. 88-1488-31)
- Immunoprecipitation antibody
- PVDF or nitrocellulose membrane (0.2 of 0.45 µm)
- Immunoblotting goat primary antibody
- Chemiluminscent Substrate
- X-ray film

Buffers (see recipe below)

- Lysis buffer (with protese inhibitors)
- Cold PBS
- SDS-PAGE sample buffer with reducing agent
- Protease inhibitors
- Blocking Buffer
- TBST-T

Instruments

- Centrifuge
- Rocking platform or rotator
- SDS PAGE and Immunoblotting equipment and reagents

Procedure Step I: Preparation of Cell Lysate

1. Harvest approximately $1x10^7$ cells by using cell scraper and transfer to conical tube. If working with adherent cells you can skip this step and lyse directly on the plate (see Step 6)
   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 Bradford/Lowry or other protein assay.
2. Wash cells with ~10 mL of cold PBS and centrifuge at 400 x*g* for 10 minutes at 4°C.
3. Discard the supernatant and repeat step 2.
4. After the second wash, remove the supernatant and resuspend the cell pellet in 1 mL of cold Lysis Buffer containing protease Inhibitors (such as a cocktail- see recipe below). Final concentration of cells should be about $1x10^7$ 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 and proceed to Step 8.

5. Gently vortex/mix and transfer to 1.5 mL tube.
6. Place on ice for 30 minutes, with occasional mixing.
7. Centrifuge at 10,000 x*g* for 15 minutes at 4°C.
8. Carefully collect the supernatant, without disturbing the pellet and transfer to a new clean tube and discard pellet.
9. The protein concentration can be determined by Bradford or other assay. Samples can be diluted to ~1 µg/ µL.
10. The cell lysate can be frozen at this point for long-term storage at -80°C.

Procedure Step II: Cell Lysate Preclearing

1. Resuspend the immobilized Protein A or Anti-Goat IgG bead slurry by gently vortexing. Remove 50 µL and wash in Lysis buffer or IP buffer, if different. Resupend in 50 µL IP buffer.
2. Add 500 µL of cell lysate (~5x10^6 cells or ~500µg protein) to the pre-equlibrated bead slurry to and incubate on a rocking platform or a 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 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 Step III: Immunoprecipitation

1. Add 1-10 µg of immunoprecipitation antibody to the tube containing the cold precleared cell lysate.
   Note: This concentration of monoclonal 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/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 1x10^7 cells. Using as little IP antibody as possible minimizes potential contamination of SDS reduced sample with nonreduced 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: Step 1 and 3 can combined for a single incubation.
5. Centrifuge the tube at 2,500 xg for 30 seconds at 4°C.
6. Carefully remove supernatant completely and wash the beads 3-5 times with 500 µL of cold Lysis Buffer, centrifuging to pellet beads in between each wash. In order to minimize background, care should be given to remove the supernatant completely after each wash.
7. After the last wash, carefully aspirate supernatant and add 50 µL of 1X Laemmli sample buffer (or any equivalent SDS-PAGE sample loading Buffer) to bead pellet.
   Note: Please take into consideration composition of the Loading buffer. Reducing agents can be added.
8. Vortex and heat to 90-100°C for 10 minutes.
9. Centrifuge at 10,000 xg for 5 minutes, collect supernatant carefully and load onto the gel.
10. Alternatively, the supernatant samples can be collected, transferred to clean tube and frozen at -80°C if the gel is to be run later.
11. Follow manufacturer's instructions for SDS-PAGE.

Procedure Step IV: Immunoblotting (Western Blotting, WB)

1. Transfer proteins from the gel onto PVDF or nitrocellulose membrane following instructions provided by the transfer system manufacturer for best protein transfer results.
2. Optional: To determine whether the proteins have been transferred to 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 TBS-T until most of the dye is removed before moving on to blocking step. Residual dye will not affect subsequent steps.
3. Remove membrane and soak in transfer buffer.
4. Under chemical hood, place the membrane in TrueBlot® Enhancer Solution and soak for 2 -Tm inutes, then w ash w

5. Place the membrane into the 5% TrueBlot Blocker in TrueBlot® Assay Buffer (enough to cover the membrane) and incubate for 2 hours at room temperature or overnight at 4°C on a rocking platform. [Preparation of 5% TrueBlot Blocker in TrueBlot Assay Buffer: Dilute 20X TrueBlot Assay Buffer with $d_{H2}0$ to 1X. Using TrueBlot® Blocker Powder, make a 5% (w/v) solution.]
   Note: it is recommended to use Milk as the blocking reagent as BSA does not effectively block the reduced Ig chain recognition.
6. Remove the blocking buffer and rinse blot with TBS-T.
7. Prepare the primary goat immunoblotting antibody in Blocking Buffer as recommended by the supplier. If the recommended concentration is not known use a standard concentration of 1-2 µg/ml. If using hybrid- oma 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.
8. Incubate the blot with primary antibody for at least 2 hours at room temperature of overnight at 4°C on rocking platform.
   Note: Shorter times should be determined empirically for optimal results
9. After the overnight incubation of the membrane with the primary antibody, wash the blot at least 3-5 times in TBS-T, each wash for a minimum of 5-10 minutes each. Total should be more than 1 hour.
10. Prepare the secondary antibody Goat IgG TrueBlot® at a 1:1,000 dilution in the Blocking Buffer.
    Note: Please avoid the presence of sodium azide in this step as it is deleterious to the HRP enzyme.
11. Incubate the blot with the TrueBlot® secondary antibody for one hour at room temperature on a rocking platform.
12. Wash the blot at least 3-5 times in TBS-T, each wash for at least 5 minutes each. Total should be more than 1 hour.
13. Prepare Substrate: Mix equal volumes of Substrate A and B
14. Incubate the blot in chemiluminescent-HRP substrate working solution (combined A and B) for 1- 5 minutes.
15. Expose the blot to X-ray film for an appropriate time period. For best results, expose for ten seconds, one minute, five minutes and 20 minutes.

Related Products

Chemiluminescent FemtoMax™ Super Sensitive HRP Substrate for Microwell/Membrane (2 component sytem) - FEMTOMAX-020

BLOTTO Immunoanalytical Grade (Non-Fat Dry Milk) - B501-0500

10X TTBS pH 7.5 (1.0 M Tris HCl 1.5 M Sodium Chloride 0.1% (w/v) Tween-20) - MB-013

2X SDS-PAGE Sample Buffer without DTT or b-MeOH (125 mM Tris HCl, 20% Glycerol, 4% SDS, 0.1% bromphenol blue, pH 6.8) - MB-018

Related Phospho Specific Primary Antibodies

TrueBlot® is ideal for studying post-translational protein modifications, including phosphorylation, or protein-protein interactions. Our portfolio offers a large and diverse set of phospho-specific antibodies and reagents that detect phosphorylated targets with great precision. Additionally, Rockland phospho antibodies provide accuracy and dependability that clients within the life sciences have come to expect.

- Anti-AKT pS473 (Mouse) 200-301-268
- Anti-AKT pT308 (Mouse) 200-301-269
- Anti-ATM Protein Kinase pS1981 (Mouse) 200-301-400
- Anti-Histone H2AvD pS137 (Rabbit) 600-401-914
- Anti-Myosin pS19/pS20 (Rabbit) 600-401-416
- Anti-SMAD3 pS423 pS425 (Rabbit) 600-401-919

Solutions & Recipes

2X SDS Reducing Sample Loading Buffer (containing 50 mM DTT)

- 950 mL of 2X SDS sample buffer
- 50 mL of 1M DTT

Note: Use within 1 hour and discard remainder.

2X SDS Sample Buffer

- 6% SDS
- 25mM Tris base pHed to 6.5 with HCl
- 10% glycerol
- Bromphenol blue

Note: Can be stored long term at -20°C and for up to 1 month at room temperature.

1M DTT

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

TBS-Tween (TBS-T)

- 25 mM Tris-HCl, pH 8.0
- 125 mM NaCl
- 0.1% Tween 20

Blocking Buffer:

- 5% Carnation nonfat dry milk in TBS-T

Note: Milk solution should be stored at 4°C short term or -20°C for long term.

NP-40 Cell Lysis Buffer:

- 50mM Tris-HCl pH 8.0
- 150mM NaCl
- 1% NP-40

RIPA Buffer:

- 50mM Tris-HCl pH 7.4
- 1% NP-40
- 0.25% Na-deoxycholate
- 150mM NaCl
- 1mM EDTA

Protease Inhibitor Cocktail (100X):

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

Phosphatase Inhibitor (100X):

- 1mM Na_3VO_4
- 1mM NaF

Goat TrueBlot® Troubleshooting Chart

Problem	Possible Cause	Solution
A. No Signal	1. Weak primary antibody 2. NaN_3 is present during HRP-substrate incubation 3. Primary antibody is not a goat IgG 4. Target protein is not expressed in the sample or present at very low level 5. Antigen is present in blocking solution	1. Use only primary antibodies optimized for immunoblotting 2. Incubate HRP-substrate in NaN_3 free buffer 3. Use only goat IgG as primary antibody for Goat IgG TrueBlot® 4. Use as positive control, sample known to contain the target protein and optimize the amount of protein loaded 5. Change blocking reagents
B. High background	1. Non-optimized primary antibody 2. Insufficient washing 3. Membrane was allowed to dry and not re-wetted 4. Insufficient blocking	1. Use only primary antibodies optimized for immunoblotting 2. Increase volume, number and duration of washes; increase salt content of the wash buffer (see Appendix) 3. Ensure membrane is not dried during immunoblotting pro- cedures. Immobilon-P and other PVDF membranes must be wetted in methanol and equilibrated in buffer 4. 5% (w/v) nonfat dry milk is the best blocking agent. **BSA is specifically not recommended.**
C. I see Ig in addition to my specific band of interest	1. Improper sample preparation	1. Follow sample preparation procedure
D. I see other bands in addition to my specific band of interest	1. Poor primary antibody: low signal/high noise	1. Use primary antibodies optimized for immunoblotting (high signal/noise) 2. Possible different isoforms/modifications of the protein of interest