IgG TrueBlot® Protocol for Mouse, Rabbit or Goat-derived Antibodies - *For Research Use Only*

Introduction

The IgG TrueBlot® for mouse, rabbit, or goat-derived antibodies represents unique series of respective anti-species IgG immunoblotting reagents conjugated to HRP (horse radish peroxidase) designed for use in IP western blot procedures in which the same species antibody is used for both the IP and immunblotting steps. Respective IgG TrueBlots® enable detection of immunoblotted target protein bands without hindrance by interfering immunoprecipitating immunoglobulin heavy and light chains.

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

Negative control: *Samples containing 0.5-2.0µg 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.*

Additional Controls:

1. Omit the cell extract during the IP
2. Omit the IP antibody during the IP
3. Omit the immunoblotting antibody.

Materials

- Immobilized species-specific Ig IP beads or immobilized Protein A* are included in respective kits and are available separately.

Species	Ig IP beads	Set	Kit
Mouse	(Cat. No. 00-8811-25)	(Cat. No. 88-7788-31)	(Cat. No. 88-8887-31)
Rabbit	(Cat. No. 00-8800-25)	(Cat. No. 88-1688-31)	(Cat. No. 88-8886-31)
Goat*	(Cat. No. 00-8844-25)	(Cat. No. 88-1488-31)	(Cat. No. 88-8884-31)

- Immunoprecipitation antibody
- PVDF or nitrocellulose membrane (0.2 of 0.45 µm)
- Immunoblotting species-specific monoclonal antibody
- Chemiluminscent Substrate
- Signal Detection Platform
- Secondary species-specific IgG TrueBlot® antibodies are also included in respective kits and are available separately in a variety of sizes.

Species	IgG HRP	Set	Kit
Mouse	(Cat. No. 18-8817-30, 18-8817-31, 18-8817-33)	(Cat. No. 88-7788-31)	(Cat. No. 88-8887-31)
Rabbit	(Cat. No. 18-8816-31, 18-8816-33)	(Cat. No. 88-1688-31)	(Cat. No. 88-8886-31)
Goat*	(Cat. No. 18-8814-31, 18-8814-33)	(Cat. No. 88-1488-31)	(Cat. No. 88-8884-31)

Buffers (see recipe below)

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

Instruments

- Centrifuge
- Rocking platform or orbital shaker
- SDS PAGE and Immunoblotting equipment and reagents

Step I: Preparation of Cell Lysate

1. Harvest approximately $1x10^7$ cells by using a 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 the Bradford/Lowry or other protein assay.

2. Wash cells with ~10 ml of cold PBS and centrifuge at 400xg 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 (see recipe below). Final concentration of cells should be about 1x107 cells/ml.

Note: If using adherent cells, the cold Lysis Buffer can be added directly to the plate and placed on a rocker at 4°C. Harvest by either scraping the cells or collecting the supernatant only 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 10,000xg 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 g/L.
10. The cell lysate can be frozen at this point for long-term storage at -80°C.

Step II: Cell Lysate Preclearing

1. Resuspend the immobilized Anti-species-specific 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 (~5x106 cells or ~500 g protein) to the pre-equilibrated bead slurry and incubate on a rocking platform or an orbital shaker for 30-60 minutes at 4°C.
3. Centrifuge at 2,500xg 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.

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 of antibody are sufficient to efficiently immunoprecipitate most antigens contained in a 1 ml extract derived from 1x10^7 cells. Using as little IP antibody as possible minimizes potential contamination of SDS reduced samples with non-reduced immunoprecipitating antibody light chain. It is not recommended to use more than 10 µg (per ml) or 5 µg per lane.

2. Incubate at 4°C for 1 hour on a rocking platform or orbital shaker.
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 orbital shaker.

Note: Step 1 and 3 can be combined into a single incubation step.

5. Centrifuge the tube at 2,500xg 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, centrifuge to pellet beads in between washes. In order to minimize background, care should be given to remove the supernatant completely following 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: It is critical to add reducing agent. Prior to use, prepare 2X SDS Reducing Sample Buffer by adding 1M DTT to 2X SDS Sample Buffer resulting in a final concentration of 50 mM DTT. NuPAGE or standard Laemmli buffer may also be used with the addition of reducing agent (50 mM DTT or 2% β-mercaptoethanol, final).

8. Vortex and heat to 90-100°C for 10 minutes.
9. Centrifuge at 10,000xg for 5 minutes, carefully collect supernatant and load onto the gel.
10. Alternatively, the supernatant samples can be collected, transferred to a clean tube and frozen at -80°C if the gel is to be run at a later stage.
11. Follow manufacturer's instructions for SDS-PAGE.

Step III: 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 the membrane, stain with a 0.1% Ponceau S solution. Protein bands can be visualized after staining for 5 minutes. Prior to blocking the membrane, remove the Ponceau S stain by rinsing the membrane with distilled water or TBS-T until the dye has been removed. Residual dye will not affect subsequent steps
3. Place the membrane into the blocking buffer (enough to cover the membrane) and incubate for 2 hours at room temperature or overnight at 4°C on a rocking platform. See recipe below.

Note: it is recommended to use BLOTTO (non-fat milk) as the blocking reagent as BSA does not effectively block the reduced Ig chain recognition.

4. Remove the blocking buffer and rinse the blot with TBS-T.
5. Prepare the primary mouse 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 hybridoma tissue culture supernatant or serum for immunoblotting; preliminary experiments should be performed to evaluate whether dilution of the supernatant or serum is needed to obtain the optimal results.
6. Incubate the blot in primary antibody for at least 2 hours at room temperature of overnight at 4°C on rocking platform.

Note: For optimal results, shorter incubation times should be determined empirically.

7. Following an overnight incubation of the membrane in the primary antibody, wash the blot at least 3-5 times in TBS-T for a minimum of 5-10 minutes each. Total should be more than 1 hour.
8. Prepare the secondary species-specific IgG TrueBlot® antibody at a 1:1,000 dilution in Blocking Buffer.

Note: Please avoid the presence of sodium azide in this step as it is deleterious to the HRP enzyme.

9. Incubate the blot in TrueBlot® secondary antibody for 1 hour at room temperature on a rocking platform.
10. Wash the blot at least 3-5 times in TBS-T for at least 5 minutes each. Total should be more than 1 hour.

11. Develop the blot using the Chemiluminescent HRP substrate following the manufacturer's instructions.
12. Electronically capture the signal for an appropriate time period. For optimal results, capture exposures after ten seconds, one minute, five minutes and 20 minutes or as determined otherwise.

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, pH 6.5
- 10% glycerol
- Bromophenol 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 thawing.

TBS-Tween (TBS-T)
- 25 mM Tris-HCl, pH 8.0
- 125 mM NaCl
- 0.1% Tween 20

Blocking Buffer:
- 5% non-fat dry milk in TBS-T, referred to as BLOTTO (Cat. No. B552-0500)

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 Na3VO4
- 1mM NaF

TrueBlot® Troubleshooting Guide

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 species-specific 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 species-specific IgG as primary antibody for species-specific 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 does not dry during immunoblotting procedure. Immobilon-P and other PVDF membranes must be saturated in methanol and equilibrated in buffer 4. 5% (w/v) non-fat 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