Store at 4°C

TrueBlack® Fluorescent Western Blot Blocking Buffer Kit

#40683

Cell Signaling TECHNOLOGY®

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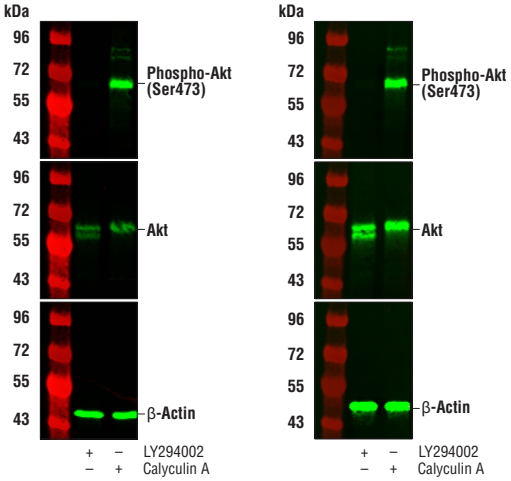
For Research Use Only. Not For Use In Diagnostic Procedures.

Product Includes	Product #	Kit Quantity	Storage Temp
TrueBlack® WB Blocking Buffer	57443	500 mL	4°C
TrueBlack® WB Antibody Diluent	78710	1 L	4°C

Description: The TrueBlack® Fluorescent Western Blot Blocking Buffer Kit is specifically formulated to increase the specificity and sensitivity of the fluorescent western blot assay by blocking non-specific interaction between fluorophore-labeled antibodies and the western blotting membrane. This kit provides the reagents needed to block non-specific protein binding and background caused by charged dyes. The reagents in the kit are compatible with both nitrocellulose and polyvinylidene difluoride (PVDF) membranes. This kit is not intended to be used for chemiluminescence detection.



Western blot analysis of extracts from Jurkat cells, untreated (-) or treated with LY294002 #9901 or Calyculin A #9902 (+), using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060 (upper), Akt (pan) (C67E7) Rabbit mAb #4691 (middle), or β-Actin (D6A8) Rabbit mAb #8457 (lower). The western blot membranes were treated with our standard antibody diluent and blocking buffers (left) or with the TrueBlack® Fluorescent Western Blot Blocking Buffer Kit (right). Increased antibody sensitivity and decreased background fluorescence can be seen when using the TrueBlack® Fluorescent Western Blot Blocking Buffer Kit.

Storage: Store at 4ºC. A slight precipitate may be present, but will not interfere with product performance. All components in this kit are stable for 24 months when stored at 4ºC. Recommend warming to room temperature and mixing before use.

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Applications: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **E-P**—ELISA-Peptide **Species Cross-Reactivity:** **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **Dm**—D. melanogaster **X**—Xenopus **Z**—zebrafish **B**—bovine **Dg**—dog **Pg**—pig **Sc**—S. cerevisiae **Ce**—C. elegans **Hr**—Horse **All**—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

Western Blotting Protocol (Fluorescent)

NOTE: The TrueBlack® Fluorescent Western Blot Blocking Buffer Kit (#40683) contains the necessary buffers to block the membrane and dilute the primary and secondary antibodies.

NOTE: Two-color western blots require primary antibodies from different species and appropriate secondary antibodies labeled with different dyes. Overlap of epitopes may cause interference and should be considered in two color western blots. If the primary antibodies require different primary antibody incubation buffers, test each primary individually in both buffers to determine the optimal one for the dual-labeling experiment.

A. Solutions and Reagents

NOTE: Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

1. **20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 mL 20X PBS to 950 mL dH_2O, mix.
2. **10X Tris Buffered Saline (TBS):** (#12498) To prepare 1 L 1X TBS: add 100 mL 10X TBS to 900 mL dH_2O, mix.
3. **1X SDS Sample Buffer:** Blue Loading Pack (#7722) or Red Loading Pack (#7723) Prepare fresh 3X reducing loading buffer by adding 1/10 volume 30X DTT to 1 volume of 3X SDS loading buffer. Dilute to 1X with dH_2O.
4. **10X Tris-Glycine SDS Running Buffer:** (#4050) To prepare 1 L 1X running buffer: add 100 mL 10X running buffer 900 mL dH_2O, mix.
5. **10X Tris-Glycine Transfer Buffer:** (#12539) To prepare 1 L 1X Transfer Buffer: add 100 mL 10X Transfer Buffer 200 mL methanol + 700 mL dH_2O, mix.
6. **10X Tris Buffered Saline with Tween® 20 (TBST-10X):** (#9997) To prepare 1 L 1X TBST: add 100 mL 10X TBST to 900 mL dH_2O, mix.
7. **Wash Buffer:** 1X TBST.
8. **TrueBlack® WB Blocking Buffer:** (#57443) Ready-to-use solution.
9. **TrueBlack® WB Antibody Diluent:** (#78710) Ready-to-use solution. (Secondary antibodies; anti-rabbit #5151 and #5366; anti-mouse #5257 and #5470).
10. **Blue Prestained Protein Marker, Broad Range (11-250 kDa):** (#59329).
11. **Blotting Membrane and Paper:** (#12369) This protocol has been optimized for nitrocellulose membranes (recommended). Pore size 0.2 µm is generally recommended.

B. Protein Blotting

A general protocol for sample preparation.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with cold 1X PBS; aspirate.
3. Lyse cells by adding 1X SDS sample buffer (100 µL per well of 6-well plate or 500 µL per plate of 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 sec to complete cell lysis and shear DNA (to reduce sample viscosity).
5. Heat a 20 µL sample to 95–100°C for 5 min; cool on ice.
6. Microcentrifuge for 5 min.
7. Load 20 µL onto SDS-PAGE gel (10 cm x 10 cm).

   NOTE: Loading of prestained molecular weight markers (#59329, 10 µl/lane) is recommended to verify electrotransfer and to determine molecular weights. Prestained markers are autofluorescent at near-infrared wavelengths.
8. Electrotransfer to nitrocellulose membrane (#12369).

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm2) of membrane; for different sized membranes, adjust volumes accordingly.

1. (Optional) After transfer, wash nitrocellulose membrane with 25 mL TBS for 5 min at room temperature.
2. Warm the TrueBlack® WB Blocking Buffer to room temperature and thoroughly mix prior to use.

   NOTE: The TrueBlack® WB Blocking Buffer may have a slight precipitate but this will not impact the performance.
3. Incubate membrane in 10 mL of TrueBlack® WB Blocking Buffer for 45 min at room temperature.
4. Remove the TrueBlack® WB Blocking Buffer.
5. Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 mL of TrueBlack® WB Antibody Diluent with gentle agitation overnight at 4°C.
6. Wash five times for 10 min each with 15 mL of TBST.
7. Incubate membrane with fluorophore-conjugated secondary antibody (#5470, #5257, #5366, #5151) (1:5000–1:25,000 dilution of 1 mg/mL stock) in 10 mL of TrueBlack® WB Antibody Diluent with gentle agitation for 2 hr at room temperature, protect from light.
8. Wash five times for 10 min each with 15 mL of TBST, protect from light.

D. Detection of Proteins

1. Drain membrane of excess TBST and allow to dry.

   CRITICAL STEP: Membrane must be dry for fluorescent staining.
2. Scan membrane using an appropriate fluorescent scanner following the manufacturer's recommendations.