Store at RT, 4°C, and –20°C

#12957

Western Blotting Application Solutions Kit

1 Kit (10 western blots)

Support: +1-978-867-2388 (U.S.)
www.cellsignal.com/support

Orders: 877-616-2355 (U.S.)
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For Research Use Only. Not For Use In Diagnostic Procedures.

Applications	Species Cross-Reactivity
W	All

Components Ship As: 13005S	Item #	Kit Quantity	Storage Temp
Cell Lysis Buffer (10X)	9803	15 ml	–20°C
3X Blue Loading Buffer	56036	8 ml	Room Temp.
30X Reducing Agent	14265	1000 µl	–20°C
Blue Prestained Protein Marker, Broad Range (11-250 kDa)	59329	100 µl	–20°C
Bovine Serum Albumin (BSA)	9998	12 g	4°C
Anti-rabbit IgG, HRP-linked Antibody	7074	100 µl	–20°C
Anti-mouse IgG, HRP-linked Antibody	7076	100 µl	–20°C
Components Ship As: 13131S	**Item #**	**Kit Quantity**	**Storage Temp**
PMSF	8553	34.84 mg	Room Temp.
Tris-Glycine SDS Running Buffer (10X)	4050	300 ml	Room Temp.
Nitrocellulose Sandwiches	12369	10 Pack	Room Temp.
Tris-Glycine Transfer Buffer (10X)	12539	300 ml	Room Temp.
Tris Buffered Saline with Tween® 20 (TBST-10X)	9997	300 ml	Room Temp.
Nonfat Dry Milk	9999	50 g	Room Temp.
SignalFire™ ECL Reagent A	46935	50 ml	Room Temp.
SignalFire™ ECL Reagent B	74709	50 ml	Room Temp.

Description: The Western Blotting Application Solutions Kit is designed to conveniently provide reagents needed for western blotting, from cell lysis to protein detection. The reagents in this kit are thoroughly validated with our primary antibodies and will work optimally with the CST western immunoblotting protocol, ensuring accurate and reproducible results. This kit includes sufficient reagents to run 10 mini-gels and complete western blot assays with either rabbit or mouse primary antibodies. All reagents in this kit are available individually.

Storage: All components are stable for 12 months when stored properly.

Upon receipt, BSA and 3X Blue Loading Buffer should be removed from #13005S and stored at 4°C and at room temperature, respectively. The 30X Reducing Agent and remaining #13005S components should be stored at -20°C.

All #13131S reagents should be stored at room temperature.

Reagents not supplied:

1. Primary Antibody
2. 1X PBS (#9808, 20X)
3. Reverse Osmosis Deionized (RODI) Water
4. Isopropyl alcohol
5. BCA Protein Assay (#7780) (optional)
6. Biotinylated Protein Ladder Detection Pack (#7727) (optional)
7. Methanol
8. 1X TBS (#12498, 10X) (optional)
9. Streptavidin-HRP (#3999) (if necessary)

Recommended Antibody Dilutions:

Anti-rabbit IgG, HRP-linked Antibody #7074 1:1000-1:3000
Anti-mouse IgG, HRP-linked Antibody #7076 1:1000-1:3000

Please visit www.cellsignal.com for a complete listing of recommended companion products.

IMPORTANT: Please refer to the primary antibody datasheet or product webpage for recommended primary antibody dilution buffer and recommended antibody dilution.

Thank you for your recent purchase. If you would like to provide a review visit www.cellsignal.com/comments.

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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.

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Western Blotting Application Solutions Kit Protocol

NOTE: Please refer to the primary antibody datasheet or product webpage for recommended primary antibody dilution buffer and recommended antibody dilution.

A Solutions and Reagents

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

SUPPLIED REAGENTS

1. **10X Cell Lysis Buffer:** (#9803) To prepare 10 ml of 1X Cell Lysis Buffer: add 1 ml 10X Cell Lysis Buffer to 9 ml dH_2O. Chill buffer on ice and add 50 µl 200X PMSF just prior to use.
2. **200X PMSF:** (#8553) Reconstitute 34.84 mg of lyophilized PMSF in 1 ml isopropyl alcohol, to make a 200 mM solution.
3. **1X SDS Sample Buffer:** Blue Loading Buffer Pack (#7722). Prepare fresh 3X reducing loading buffer by adding 1/10 volume 30X DTT (#14265) to 1 volume of 3X SDS loading buffer (#56036). Dilute to 1X with dH_2O.
4. **Blue Prestained Protein Marker, Broad Range (11-250 kDa):** (#59329)
5. **10X Tris-Glycine SDS Running Buffer:** (#4050) To prepare 1 L 1X running buffer: add 100 ml 10X Running Buffer to 900 ml dH_2O, mix.
6. **10X Tris-Glycine Transfer Buffer:** (#12539) To prepare 1 L 1X transfer buffer: add 100 ml 10X Transfer Buffer to 200 ml methanol + 700 ml dH_2O, mix.
7. **Nitrocellulose Sandwiches:** (#12369)
8. **10X Tris Buffered Saline with Tween® 20 (TBST):** (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH_2O, mix.
9. **Nonfat Dry Milk:** (#9999)
10. **Blocking Buffer:** 1X TBST with 5% w/v nonfat dry milk; for 150 ml, add 7.5 g nonfat dry milk to 150 ml 1X TBST and mix well.
11. **Wash Buffer:** 1X TBST
12. **Bovine Serum Albumin (BSA):** (#9998)
13. **Primary Antibody Dilution Buffer:** 1X TBST with 5% BSA or 5% nonfat dry milk as indicated on primary antibody datasheet; for 20 ml, add 1.0 g BSA or nonfat dry milk to 20 ml 1X TBST and mix well.
14. **Secondary Antibody Conjugated to HRP:** anti-rabbit (#7074); anti-mouse (#7076)
15. **Detection Reagent:** SignalFire™ ECL Reagent (#6883). Reagents A (#46935) and B (#74709) should be combined just prior to use.

ADDITIONAL REAGENTS (NOT SUPPLIED)

1. **Primary Antibody**
2. **20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH_2O, mix.
3. **Reverse Osmosis Deionized (RODI) Water**
4. **Isopropyl Alcohol**
5. **BCA Protein Assay Kit:** (#7780) (OPTIONAL)
6. **Biotinylated Protein Ladder Detection Pack:** (#7727) (OPTIONAL)
7. **Methanol**
8. **10X Tris Buffered Saline (TBS):** (#12498) To prepare 1 L 1X TBS: add 100 ml 10X to 900 ml dH_2O, mix. (OPTIONAL)
9. **Streptavidin-HRP:** (#3999) (if necessary)

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 1X PBS; aspirate. If performing protein concentration quantification, proceed to step #3, if not, skip to step #8.
3. Lyse cells by adding 1X Cell Lysis Buffer (80 µl per well of 6-well plate or 400 µl for a 10 cm diameter plate). Incubate plate on ice for 5 min, then scrape cells off the plate and transfer lysate to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 sec to complete cell lysis and shear DNA (to reduce sample viscosity). Keep on ice.
5. Centrifuge extract for 10 min at 14,000 x g in a cold microcentrifuge. Transfer supernatant to a new tube and discard pellet.
6. Determine protein concentration using BCA Protein Assay Kit #7780.
7. Add 3X SDS Sample Buffer to a final concentration of 1X. Proceed to step #10.
8. Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl for a 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
9. Sonicate for 10–15 sec to complete cell lysis and shear DNA (to reduce sample viscosity). Keep on ice.
10. Heat a 20 µl sample to 95–100°C for 5 min; cool on ice.
11. Microcentrifuge for 5 min.
12. Prepare electrophoresis gel and apparatus using 1X running buffer.
13. Load 15 µl onto SDS-PAGE gel (10 cm x 10 cm). **NOTE:** Loading of prestained molecular weight markers (#59329, 5 µl/lane) to verify electrotransfer and biotinylated protein ladder (#81851, 10 µl/lane) to determine molecular weights are recommended.
14. Perform electrotransfer to nitrocellulose membrane (#12369) using 1X transfer buffer in a tank (wet) transfer system.

C Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) or smaller membrane; for larger membranes, adjust volumes accordingly.

I. Membrane Blocking

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 min at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hr at room temperature.
3. Wash three times for 5 min each with 15 ml of TBST.

II. Primary Antibody Incubation:

Proceed to one of the following specific set of steps depending on the primary antibody used.

For Unconjugated Primary Antibodies

1. Incubate membrane and primary antibody (at the appropriate dilution and diluent as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with the species appropriate HRP-conjugated secondary antibody (#7074 or #7076 at 1:2000) and, if necessary, anti-biotin, HRP-linked Antibody (#7075 at 1:1000–1:3000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.
5. Proceed with detection (Section D).

For HRP Conjugated Primary Antibodies

1. Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. If necessary, incubate with Anti-biotin, HRP-linked Antibody (#7075 at 1:1000–1:3000), to detect biotinylated protein markers, in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.
5. Proceed with detection (Section D).

For Biotinylated Primary Antibodies

1. Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with Streptavidin-HRP (#3999, not supplied) at 1:2000 in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.
5. Proceed with detection (Section D).
   Do not add Anti-biotin, HRP-linked Antibody for detection of biotinylated protein markers. There is no need. The Streptavidin-HRP will also visualize the biotinylated markers.

D Detection of Proteins

1. Incubate membrane in 10 ml SignalFire™ #6883 (5 ml Reagent A (#46935), 5 ml Reagent B (#74709) with gentle agitation for 1 min at room temperature.
2. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10 sec exposure should indicate the proper exposure time. **NOTE:** Due to the kinetics of the detection reaction, signal is most intense immediately after incubation.

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