Applications	Reactivity	Sensitivity	MW (kDa)	Isotype
W	All	Transfected Only	27	Rabbit IgG

Applications Key: W=Western Blotting

Reactivity Key: All=All species expected

Species cross-reactivity is determined by western blot.

Protocols

Western Immunoblotting Protocol

For western blots, incubate membrane with diluted primary antibody in either 5% w/v BSA or nonfat dry milk, 1X TBS, 0.1% Tween-20 at 4 ℃ with gentle shaking, overnight.

Reasons to use the Cell Signaling Technology western blotting protocol.

NOTE: Please refer to primary antibody datasheet for recommended primary antibody dilution buffer and recommended antibody dilution.

A. Solutions and Reagents

Learn about our Solutions and Reagents

NOTE: Prepare solutions with 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. **1X SDS Sample Buffer:** (#7722, #7723) 62.5 mM Tris-HCl (pH 6.8 at 25 ℃), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red.
3. **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5).
4. **10X Tris Buffered Saline:** (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH_2O, mix.
5. **Nonfat Dry Milk:** (#9999).
6. **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.
7. **Wash Buffer:** 1X TBST.
8. **Bovine Serum Albumin (BSA):** (#9998).
9. **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.
10. **Phototope®-HRP Western Blot Detection System:** (anti-rabbit #7071) (anti-mouse #7072) Includes biotinylated protein ladder (#7727), secondary antibody conjugated to horseradish peroxidase (HRP) (anti-rabbit #7074) (anti-mouse #7076), anti-biotin HRP-linked antibody (#7075), LumiGLO®chemiluminescent reagent and peroxide (#7003).
11. **Prestained Protein Marker, Broad Range (Premixed Format):** (#7720).
12. **Blotting Membrane:** This protocol has been optimized for Nitrocellulose Sandwiches (#12369). PVDF membranes may also be used. Pore size 0.2 µm is generally recommended.

B. Protein Blotting

A general protocol for sample preparation is described below.

Sample prep, SDS-PAGE and transfer

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with 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 ℃ 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 (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights are recommended.
8. Electrotransfer to nitrocellulose (#12369) membrane.

C. Membrane Blocking and Antibody Incubations

Block and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) of membrane; for different sized 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 one hr at room temperature.
3. Wash once for 5 min 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 buffer as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4 ℃.
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) (1:2000) and Anti-biotin, HRP-linked Antibody (#7075) (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for one 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 ℃.
2. Wash three times for 5 min each with 15 ml of TBST.

3. Incubate with Anti-biotin, HRP-linked Antibody (#7075) (1:1000) 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 ℃.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with Streptavidin-HRP (#3999) 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 secondary antibody will also visualize the biotinylated markers.

D. Detection of Proteins

Protein Detection

1. Incubate membrane with 10 ml LumiGLO® (0.5 ml 20X LumiGLO® #7003, 0.5 ml 20X Peroxide, and 9.0 ml purified water) or 10 ml SignalFire™ #6883 (5 ml Reagent A, 5 ml Reagent B) with gentle agitation for 1 min at room temperature. NOTE: LumiGLO® substrate can be further diluted if signal response is too fast.
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 following LumiGLO® incubation and declines over the following 2 hr.

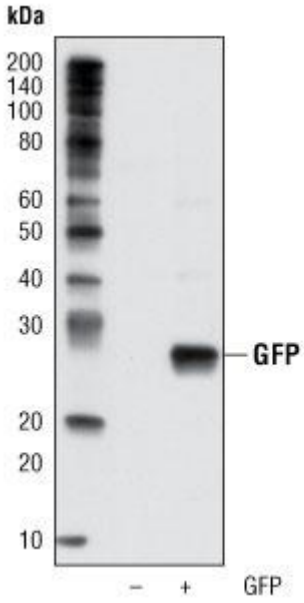
Specificity / Sensitivity

GFP (D5.1) XP® Rabbit mAb (HRP Conjugate) detects GFP-tagged proteins exogenously expressed in cells.

Source / Purification

Monoclonal antibody is produced by immunizing animals with a synthetic peptide corresponding to the amino terminus of GFP. This antibody was conjugated to HRP under optimal conditions.

Western Blotting



Western blot analysis of extracts from HCC827 cells, mock transfected or transfected with GFP, using GFP (D5.1) XP® Rabbit mAb (HRP Conjugate).