Store at –20°C

#3141

Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Antibody

☐ Small 100 µl
(10 Western mini-blots)

☐ Large 300 µl
(30 Western mini-blots)

Cell Signaling TECHNOLOGY®

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Applications	Species Cross-Reactivity	Molecular Wt.	Source
W, IF-IC	H, M, R, Mk	75 kDa Moesin. 80 kDa Ezrin, Radixin.	Rabbit

Background: The ezrin, radixin and moesin (ERM) proteins function as linkers between the plasma membrane and the actin cytoskeleton and are involved in cell adhesion, membrane ruffling and microvilli formation (1). ERM proteins undergo intra or intermolecular interaction between their amino- and carboxy-terminal domains, existing as inactive cytosolic monomers or dimers (2). Phosphorylation at a carboxy-terminal threonine residue (Thr567 of ezrin, Thr564 of radixin, Thr558 of moesin), which disrupts their amino- and carboxy-terminal association, may play a key role in modulating the conformation and function of ERM proteins (3,4). Phosphorylation at Thr567 of ezrin is required for cytoskeletal rearrangements and oncogene-induced transformation (5). Ezrin is also phosphorylated at tyrosine residues upon growth factor stimulation. Phosphorylation of Tyr353 of ezrin transmits a survival signal during epithelial differentiation (6).

Specificity/Sensitivity: Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Antibody detects endogenous levels of ezrin, radixin and moesin only when phosphorylated at threonine 567, 564 or 558, respectively. This antibody does not cross-react with related phospho-proteins such as merlin or band 4.1.

Source/Purification: Polyclonal antibodies are produced by immunizing rabbits with a synthetic phospho-peptide (KLH-coupled) corresponding to residues surrounding Thr567 of human ezrin. Antibodies are purified by protein A and peptide affinity chromatography.

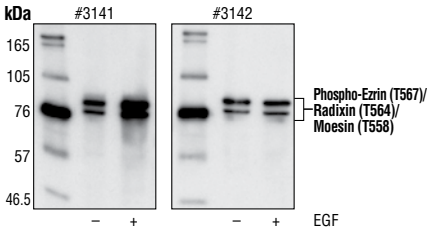
Selected Application References:

Wu, K.L. et al. (2004) The NHE1 Na/H Exchanger Recruits Ezrin/Radixin/Moesin Proteins to Regulate Akt-dependent Cell Survival. *J. Biol. Chem.* 279 (25), 26280–26286. Application: W.

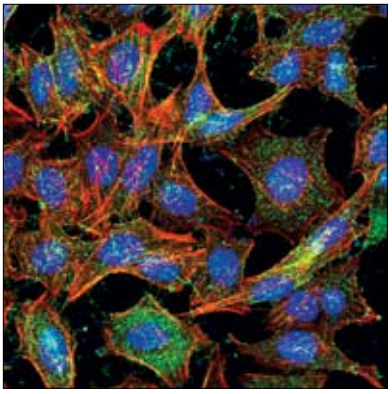
Gatto, C.L. et al. (2003) Local ERM activation and dynamic growth cones at Schwann cell tips implicated in efficient formation of nodes of Ranvier. *J. Cell Biol.* 162 (3), 489–498. Application: IC-IF.

John, G.R. et al. (2004) Interleukin-1β Induces a Reactive Astroglial Phenotype via Deactivation of the Rho GTPase-Rock Axis. *J. Neurosci.* 24 (11), 2837–2845. Applications: IC-IF, W.

Fievet, B.T. et al. (2004) Phosphoinositide binding and phosphorylation act sequentially in the activation mechanism of ezrin. *J. Cell Biol.* 164 (5), 653–659. Application: W.



Western blot analysis of extracts from A431 cells, untreated or EGF-treated, using Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Antibody (left) or Ezrin/Radixin/Moesin Antibody #3142 (right).



Confocal immunofluorescent analysis of HeLa cells labeled with Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Antibody (green). Actin filaments have been labeled with Alexa Fluor® 555 phalloidin (red). Blue pseudocolor = DRAQ5™ (fluorescent DNA dye).

Background References:

(1) Tsukita, S. and Yonemura, S. (1999) *J. Biol. Chem.* 274, 34507–34510.

(2) Mangeat, P. et al. (1999) *Trends Cell Biol.* 9, 187–192.

(3) Matsui, T. et al. (1998) *J. Cell Biol.* 140, 647–657.

(4) Gautreau, A. et al. (2000) *J. Cell Biol.* 150, 193–203.

(5) Tran Quang, C. et al. (2000) *EMBO J.* 19, 4565–4576.

(6) Gautreau, A. et al. (1999) *Proc. Natl. Acad. Sci. USA* 96, 7300–7305.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at –20°C. *Do not aliquot the antibody.*

Recommended Antibody Dilutions:

Western blotting	1:1000
Immunofluorescence (IF-IC)	1:200

Companion Products:

Ezrin/Radixin/Moesin Antibody #3142

Ezrin Antibody #3145

Phospho-Ezrin (Tyr353) Antibody #3144

Anti-rabbit IgG, HRP-linked Antibody #7074

Prestained Protein Marker, Broad Range (Premixed Format) #7720

Biotinylated Protein Ladder Detection Pack #7727

20X LumiGLO® Reagent and 20X Peroxide #7003

IMPORTANT: For Western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

Applications Key: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **IC**—Immunocytochemistry **IF**—Immunofluorescence **F**—Flow cytometry **E**—ELISA **D**—DELFIA®

Species Cross-Reactivity Key: **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **X**—Xenopus **Z**—zebra fish **B**—bovine **All**—all species expected

Species enclosed in parentheses are predicted to react based on 100% sequence homology.

Western Immunoblotting Protocol (Primary Ab Incubation In BSA)

For Western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

A Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

1. 1X Phosphate Buffered Saline (PBS)
2. **1X SDS Sample Buffer:** 62.5 mM Tris-HCl (pH 6.8 at 25°C), 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 (TBS):** To prepare 1 liter of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X).
5. Nonfat Dry Milk (weight to volume [w/v])
6. **Blocking Buffer:** 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk; for 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g nonfat dry milk and mix well. While stirring, add 0.15 ml Tween-20 (100%).
7. **Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T)
8. Bovine Serum Albumin (BSA)
9. **Primary Antibody Dilution Buffer:** 1X TBS, 0.1% Tween-20 with 5% BSA; for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g BSA and mix well. While stirring, add 20 µl Tween-20 (100%).
10. **Phototope®-HRP Western Blot Detection System** #7071**:** Includes biotinylated protein ladder, secondary anti-rabbit (#7074) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO® chemiluminescent reagent and peroxide.
11. Prestained Protein Marker, Broad Range (Premixed Format) #7720
12. Biotinylated Protein Ladder Detection Pack #7727
13. **Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes, which CST recommends. PVDF membranes may also be used.

B Protein Blotting

A general protocol for sample preparation is described below.

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 seconds to shear DNA and reduce sample viscosity.
5. Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
6. Microcentrifuge for 5 minutes.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm).

NOTE: CST recommends loading prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights.

8. Electrotransfer to nitrocellulose or PVDF membrane.

C Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) of membrane; for different sized membranes, adjust volumes accordingly.

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
3. Wash three times for 5 minutes each with 15 ml of TBS/T.
4. Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
5. Wash three times for 5 minutes each with 15 ml of TBS/T.
6. Incubate membrane with HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature.
7. Wash three times for 5 minutes each with 15 ml of TBS/T.

D Detection of Proteins

1. Incubate membrane with 10 ml LumiGLO® (0.5 ml 20X LumiGLO®, 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) with gentle agitation for 1 minute 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-second 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 hours.

Immunofluorescence Protocol

***IMPORTANT:** Please refer to the **APPLICATIONS** section on the front page of the data sheet to determine **IF THIS PRODUCT** is validated and approved for the specific protocol you will be using.

A Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

1. **10X Phosphate Buffered Saline (PBS):** To prepare 1 L add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na_2HPO_4) and 2.4 g potassium phosphate, monobasic (KH_2PO_4) to 1 L dH_2O. Adjust pH to 7.4.
2. Formaldehyde, 16%, methanol free, Polysciences, Inc. (cat# 18814), use fresh, store opened vials at 4°C in dark, dilute in PBS for use.
3. Xylene
4. Ethanol, anhydrous denatured, histological grade, 100% and 95%
5. Distilled water (dH_2O)
6. **1X PBS/0.3% Triton X-100 (PBS/Triton):** To prepare 1 L, add 100 ml 10X PBS to 900 ml dH_2O. Add 3 ml Triton X-100 and mix.
7. **10 mM Sodium Citrate Buffer:** To prepare 1 L, add 2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O7 \bullet 2H_2O$) to 1 L dH_2O. Adjust pH to 6.0.
8. **1X PBS, high salt (0.4M) (high salt PBS):** To prepare 1L, add 100 ml 10X PBS to 900 ml dH20. Add 23.38 g NaCl and mix.
9. Fluorochrome-conjugated secondary antibody

NOTE: When using any primary or fluorochrome-conjugated secondary antibody for the first time, titrate the antibody to determine which dilution allows for the strongest specific signal with the least background for your sample.

10. Prolong® Gold Antifade Reagent (Invitrogen, Eugene, OR, Cat# P36930)

B Specimen Preparation

I. Cultured Cell Lines (IF-IC)

IMPORTANT: Please check the **APPLICATIONS** section of the data sheet to verify that this product is validated and approved for **(IF-IC).**

NOTE: This general fixation protocol will work with most antibodies and cell lines. However, we recommend you try different IF/IC fixation methods (methanol or acetone alone, aldehyde alone, or combinations of these) to identify the optimal fixation protocol for each antibody and/or cell line.

NOTE: Cells should be grown, treated, fixed, and stained directly in multiwell plates, chamber slides, or on coverslips.

1. Rinse cells briefly in PBS.
2. Aspirate PBS, cover cells to a depth of 2-3 mm with 2-4% formaldehyde in PBS.

NOTE: Formaldehyde is toxic, use only in fume hood.

3. Allow cells to fix for 15 minutes at room temperature.
4. Aspirate fixative, rinse three times in PBS for 5 minutes each.

5.Methanol Permeabilization Step (if required, please refer to front page): After formaldehyde fixation, cover cells with ice-cold 100% methanol (use enough to cover cells completely to a depth of 3-5 mm, DO NOT LET CELLS DRY), incubate cells in methanol for 10 minutes in freezer, rinse in PBS for 5 minutes.

6. Proceed with Immunostaining section C.

II. Paraffin Sections (IF-P)

IMPORTANT: Please check the **APPLICATIONS** section of the data sheet to verify that this product is validated and approved for **(IF-P).**

Deparaffinization/Rehydration:

1. Incubate sections in three washes of xylene for 5 minutes each.
2. Incubate sections in two washes of 100% ethanol for 10 minutes each.
3. Incubate sections in two washes of 95% ethanol for 10 minutes each.
4. Rinse sections twice in dH_2O for 5 minutes each.

Antigen Unmasking:

1. Place slides in room temperature 10 mM sodium citrate buffer pH 6.0.
2. Bring slides to boiling in sodium citrate buffer using water bath or microwave, then maintain at 95-99°C for 10 minutes.
3. Cool slides for 30 minutes on bench top.
4. Rinse sections in dH_2O three times for 5 minutes each.
5. Rinse sections in PBS for 5 minutes.
6. Proceed with Immunostaining section C.

III. Frozen/Cryostat Sections (IF-F)

IMPORTANT: Please check the **APPLICATIONS** section of the data sheet to verify that this product is validated and approved for **(IF-F).**

NOTE: Fresh frozen/unfixed sections should be fixed immediately in 2-4% formaldehyde as follows to preserve signaling epitopes.

1. Cover sections with 2-4% formaldehyde in PBS

NOTE: Formaldehyde is toxic, use only in fume hood.

2. Allow cells to fix for 15 minutes at room temperature.
3. Rinse slides three times in PBS for 5 minutes each.

C Immunostaining

NOTE: All subsequent incubations should be carried out at room temperature unless otherwise noted in a humid light-tight box or covered dish/plate to prevent drying and fluorochrome fading.

1. Block specimen in 5% normal serum from same species as secondary antibody (eg. normal goat serum, normal donkey serum) in PBS/Triton for 60 minutes.
2. While blocking, prepare primary antibody by diluting as indicated on datasheet in PBS/Triton. You will need 50-100 µl per section, 25-50 µl per coverslip, chamber, or well (48 or 96 well plate).
3. Aspirate blocking solution, apply diluted primary antibody.

NOTE: For double-labeling, prepare a cocktail of mouse and rabbit primary antibodies at their appropriate dilutions in PBS/Triton.

4. Incubate overnight at 4°C.
5. Rinse three times in PBS for 5 minutes each.

OPTION: To decrease background stain, rinse in high salt PBS for two minutes between second and third PBS rinses. Be aware, this may reduce specific staining of some antibodies.

NOTE: If using primary antibodies directly conjugated with AlexaFluor® fluorochromes, then skip to step C8.

6. Incubate in fluorochrome-conjugated secondary antibody diluted in PBS/Triton for 1-2 hours at room temperature in dark.

NOTE: For double-labeling, prepare a cocktail of fluorochrome-conjugated anti-mouse and anti-rabbit primary antibodies at their appropriate dilutions in PBS/Triton.

7. Rinse in PBS/high salt PBS as in step 5.
8. Coverslip slides with Prolong® Gold Antifade Reagent or apply just enough to cover cells in multiwell plate.
9. Seal slides by painting around edges of coverslips with nail polish.
10. Examine specimens immediately using appropriate excitation wavelength, depending on fluorochrome for best results or store flat at 4°C in dark.