

# RIP Antibody

100 µl  
(10 Western mini-blots)

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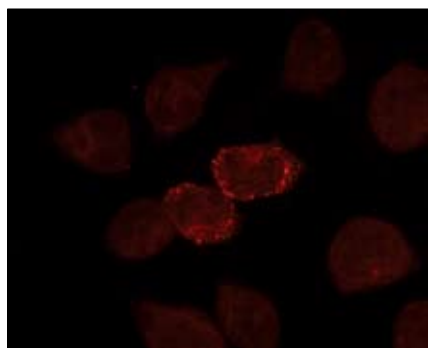
This product is for *in vitro* research use only and is not intended for use in humans or animals.

Applications	Species Cross-Reactivity	Molecular Wt.	Source
W, IP, IF-IC, F	H, M, R	78 kDa	Rabbit

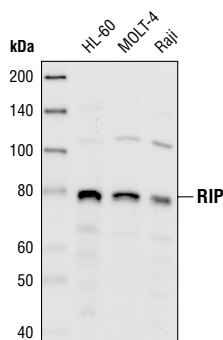
**Background:** The RIP (receptor-interacting protein) family of serine-threonine kinases (RIP, RIP2, RIP3 and RIP4) are important regulators of cellular stress that can trigger pro-survival and inflammatory responses through the activation of NF-κB as well as pro-apoptotic pathways (1). In addition to the kinase domain, RIP contains a death domain responsible for interaction with the death domain receptor Fas and for the recruitment to TNFR1 through interaction with TRADD (2,3). RIP also interacts with TNF-receptor-associated factors (TRAFs) and can recruit IKKs to the TNFR1 signaling complex via interaction with NEMO leading to IκB phosphorylation and degradation (6,7). Overexpression of RIP induces both NF-κB activation and apoptosis (2,3). Caspase-8 dependent cleavage of the death domain on RIP can trigger the apoptotic activity of RIP (8). RIP-deficient cells show a failure in TNF-mediated NF-κB activation making the cells more sensitive to apoptosis (4,5).

**Specificity/Sensitivity:** RIP Antibody detects endogenous levels of RIP protein. No cross reactivity was detected with other family members.

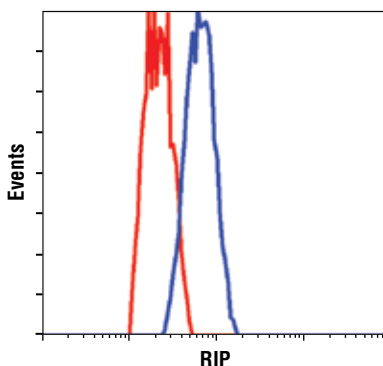
**Source/Purification:** Polyclonal antibodies were prepared by immunizing rabbits with a synthetic peptide (KLH-coupled) corresponding to residues surrounding arginine 413 of human RIP. Antibodies were purified by protein A and peptide affinity chromatography.



Immunofluorescent staining of HeLa cells transfected with RIP showing punctate cytoplasmic staining, using RIP Antibody.



Western blot analysis of extracts from HL-60, MOLT-4 and Raji cell lines, using RIP Antibody.



Flow cytometric analysis of Jurkat cells, using RIP antibody (blue) compared to a nonspecific negative control antibody (red).

**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
Immunoprecipitation	1:100
Immunofluorescence (IF-IC)	1:100
Flow Cytometry	1:100

**Companion Products:**

- RIP2 Antibody #4982
- NF-κB p65 Antibody #3034
- Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb #3033
- IKKα Antibody #2682
- IKKβ Antibody #2684
- IKKε Kinase #7553
- Phospho-IKKα/β (Ser176/180) (16A6) Rabbit mAb #2697
- Phospho-IκB-α (Ser32/36) (5A5) Mouse mAb #9246
- TRAF2 Antibody (Human Specific) #4724
- Phototope®-HRP Western Blot Detection System, Anti-rabbit IgG, HRP-linked Antibody #7071
- 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

**Background References:**

- (1) Meylan, E. and Tschopp, J. (2005) *Trends Biochem. Sci.* 30, 151-9.
- (2) Hsu, H. et al. (1996) *Immunity* 4, 387-96.
- (3) Stanger, B.Z. et al. (1995) *Cell* 81, 513-23.
- (4) Ting, A.T. et al. (1996) *EMBO J.* 15, 6189-96.
- (5) Kelliher, M.A. et al. (1998) *Immunity* 8, 297-303.
- (6) Devin, A. et al. (2000) *Immunity* 12, 419-29.
- (7) Zhang, S.Q. et al. (2000) *Immunity* 12, 301-11.
- (8) Lin, Y. et al. (1999) *Genes Dev.* 13, 2514-26.

**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  
**Species Cross-Reactivity Key:** H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken X—Xenopus  
Species enclosed in parentheses are predicted to react based on 100% sequence homology.

F—Flow cytometry E—ELISA D—DELFIATM  
Z—zebra fish B—bovine All—all species expected

## 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<sup>®</sup>-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<sup>®</sup> 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<sup>2</sup>) 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<sup>®</sup> (0.5 ml 20X LumiGLO<sup>®</sup>, 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) with gentle agitation for 1 minute at room temperature.

**NOTE:** LumiGLO<sup>®</sup> 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<sup>®</sup> incubation and declines over the following 2 hours.

## Immunoprecipitation Protocol / (For Analysis By Western Immunoblotting)

### A Solutions and Reagents

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

- 1X Phosphate Buffered Saline (PBS)
- 1X Cell Lysis Buffer:** 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM  $\text{Na}_3\text{VO}_4$ , 1  $\mu\text{g/ml}$  Leupeptin

**NOTE:** CST recommends adding 1 mM PMSF before use\*.

- Transfer Buffer:** 25 mM Tris base, 0.2 mM glycine, 20% methanol (pH 8.5)
- Protein A or G Agarose Beads:** (Can be stored for 2 weeks at 4°C.) Please prepare according to manufacturer's instructions. Use Protein A for rabbit IgG pull down and Protein G for mouse IgG pull down.
- 3X SDS Sample Buffer:** 187.5 mM Tris-HCl (pH 6.8 at 25°C), 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue

### B Preparing Cell Lysates

- Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
- To harvest cells under non-denaturing conditions, remove media and rinse cells once with ice-cold PBS.

- Remove PBS and add 0.5 ml 1X ice-cold cell lysis buffer plus 1 mM PMSF\* to each plate (10 cm) and incubate the plates on ice for 5 minutes.
- Scrape cells off the plates and transfer to microcentrifuge tubes. Keep on ice.
- Sonicate samples on ice four times for 5 seconds each.
- Microcentrifuge for 10 minutes at 4°C, and transfer the supernatant to a new tube. If necessary, lysate can be stored at -80°C.

### C Immunoprecipitation

- Take 200  $\mu\text{l}$  cell lysate and add primary antibody. Incubate with gentle rocking overnight at 4°C.
- Add either protein A or G agarose beads (20  $\mu\text{l}$  of 50% bead slurry). Incubate with gentle rocking for 1–3 hours at 4°C.
- Microcentrifuge for 30 seconds at 4°C. Wash pellet five times with 500  $\mu\text{l}$  of 1X cell lysis buffer. Keep on ice during washes.
- Resuspend the pellet with 20  $\mu\text{l}$  3X SDS sample buffer. Vortex, then microcentrifuge for 30 seconds.
- Heat the sample to 95–100°C for 2–5 minutes.
- Load the sample (15–30  $\mu\text{l}$ ) on SDS-PAGE gel (12–15%).
- Analyze sample by Western blotting (see Western Immunoblotting Protocol).

## Flow Cytometry Protocol for Intracellular Staining Using Conjugated Secondary Antibodies

### A Solutions and Reagents

- 1X Phosphate Buffered Saline (PBS):** Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g  $\text{Na}_2\text{HPO}_4$  and 0.24 g  $\text{KH}_2\text{PO}_4$  in 800 mL distilled water ( $\text{dH}_2\text{O}$ ). Adjust the pH to 7.4 with HCl and the volume to 1 liter. Store at room temperature.
- Formaldehyde (methanol free)
- Incubation Buffer:** Dissolve 0.5 g bovine serum albumin (BSA) in 100 mL 1X PBS. Store at 4°C

### B Fixation

- Collect cells by centrifugation and aspirate supernatant.
- Resuspend cells briefly in 0.5–1 ml PBS. Add formaldehyde to a final concentration of 2–4% formaldehyde.
- Fix for 10 minutes at 37°C.
- Chill tubes on ice for 1 minute.

### C Permeabilization

- Permeabilize cells by adding ice-cold 100% methanol slowly to pre-chilled cells, while gently vortexing, to a final concentration of 90% methanol. Alternatively, to remove fix prior to permeabilization, pellet cells by centrifugation and resuspend in 90% methanol.
- Incubate 30 minutes on ice.
- Proceed with staining or store cells at -20°C in 90% methanol.

### D Staining Using Unlabeled Primary and Conjugated Secondary Antibodies

**NOTE:** Allow for isotype matched controls for monoclonal antibodies or species matched IgG for polyclonal antibodies. Count cells using a hemacytometer or alternative method.

- Aliquot 0.5–1x10<sup>6</sup> cells into each assay tube (by volume).
- Add 2–3 ml Incubation Buffer to each tube and rinse by centrifugation. Repeat.
- Resuspend cells in 100  $\mu\text{l}$  Incubation Buffer per assay tube.
- Block in Incubation Buffer for 10 minutes at room temperature.
- Add the primary antibody at the appropriate dilution to the assay tubes (see individual antibody data sheet for the appropriate dilution).
- Incubate for 30–60 minutes at room temperature.
- Rinse as before in Incubation Buffer by centrifugation.
- Resuspend cells in fluorochrome-conjugated secondary antibody\*, diluted in Incubation Buffer according to the manufacturer's recommendations.
- Incubate for 30 minutes at room temperature.
- Rinse as before in Incubation Buffer by centrifugation.
- Resuspend cells in 0.5 ml PBS and analyze on flow cytometer.

\*Recommended Secondary Antibodies from Invitrogen.

A-11070 Alexa Fluor® 488 F(ab')<sub>2</sub> fragment of goat anti-rabbit IgG (H+L) (1:1000 dilution)  
A-11017 Alexa Fluor® 488 F(ab')<sub>2</sub> fragment of goat anti-mouse IgG (H+L) (1:1000 dilution)

# 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<sub>2</sub>HPO<sub>4</sub>) and 2.4 g potassium phosphate, monobasic (KH<sub>2</sub>PO<sub>4</sub>) to 1 L dH<sub>2</sub>O. Adjust pH to 7.4.
- Formaldehyde, 16%, methanol free, Polysciences, Inc. (cat# 18814), use fresh, store opened vials at 4°C in dark, dilute in PBS for use.
- Xylene
- Ethanol, anhydrous denatured, histological grade, 100% and 95%
- Distilled water (dH<sub>2</sub>O)
- 1X PBS/0.3% Triton X-100 (PBS/Triton):** To prepare 1 L, add 100 ml 10X PBS to 900 ml dH<sub>2</sub>O. Add 3 ml Triton X-100 and mix.
- 10 mM Sodium Citrate Buffer:** To prepare 1 L, add 2.94 g sodium citrate trisodium salt dihydrate (C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub>•2H<sub>2</sub>O) to 1 L dH<sub>2</sub>O. Adjust pH to 6.0.
- 1X PBS, high salt (0.4M) (high salt PBS):** To prepare 1L, add 100 ml 10X PBS to 900 ml dH<sub>2</sub>O. Add 23.38 g NaCl and mix.
- 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.

- Vectashield Mounting Medium (Vector Labs, Burlingame, CA, cat# H-1000) or Vectashield Mounting Medium with DAPI (cat# H-1200)

## 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.

- Rinse cells briefly in PBS.
- 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.

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

**OPTION:** 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.

- 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:

- Incubate sections in three washes of xylene for 5 minutes each.

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- Incubate sections in two washes of 100% ethanol for 10 minutes each.
- Incubate sections in two washes of 95% ethanol for 10 minutes each.
- Rinse sections twice in dH<sub>2</sub>O for 5 minutes each.

Antigen Unmasking:

- Place slides in room temperature 10 mM sodium citrate buffer pH 6.0.
- Bring slides to boiling in sodium citrate buffer using water bath or microwave, then maintain at 95-99°C for 10 minutes.
- Cool slides for 30 minutes on bench top.
- Rinse sections in dH<sub>2</sub>O three times for 5 minutes each.
- Rinse sections in PBS for 5 minutes.
- 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.

- Cover sections with 2-4% formaldehyde in PBS

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

- Allow cells to fix for 15 minutes at room temperature.
- 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.

- 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.
- 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).
- 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.

- Incubate overnight at 4°C.
- 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.

- 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.

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