Necroptosis Antibody Sampler Kit

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

Products Included

<table>
<thead>
<tr>
<th>Product</th>
<th>Quantity</th>
<th>Mol. Wt.</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIP (D94C12) XP® Rabbit mAb</td>
<td>20 µl</td>
<td>78 kDa</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>Phospho-RIP (Ser166) (D11L3S) Rabbit mAb</td>
<td>20 µl</td>
<td>78-82 kDa</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>MLKL (D216N) Rabbit mAb</td>
<td>20 µl</td>
<td>54 kDa</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>Phospho-MLKL (Ser358) (D6H3V) Rabbit mAb</td>
<td>20 µl</td>
<td>54 kDa</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>RIP3 (E1Z1D) Rabbit mAb</td>
<td>20 µl</td>
<td>46-62 kDa</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>P-RIP3 (S227) (D6W2T) Rabbit mAb</td>
<td>20 µl</td>
<td>46-62 kDa</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>Anti-Rabbit IgG, HRP-linked Antibody</td>
<td>100 µl</td>
<td></td>
<td>Goat</td>
</tr>
</tbody>
</table>

See www.cellsignal.com for individual component applications, species cross-reactivity, dilutions and additional application protocols.

Description: The Necroptosis Antibody Sampler Kit provides an economical means of detecting total and phosphorylated proteins associated with necrosis. The kit includes enough antibody to perform two western blots with each primary antibody.

Background: Necroptosis, a regulated pathway for necrotic cell death, is triggered by a number of inflammatory signals, including cytokines in the tumor necrosis factor (TNF) family, pathogen sensors such as toll-like receptors (TLRs), ischemic injury, and neurodegenerative diseases (1-3). The process is negatively regulated by caspases and is initiated through a complex containing the RIP and RIP3 kinases, typically referred to as the necrosome. Necroptosis is inhibited by a small molecule inhibitor of RIP, necrostatin-1 (Nec-1) (4). RIP is phosphorylated at several sites within the kinase domain that are sensitive to Nec-1, including Ser14, Ser15, Ser161, and Ser358 of human MLKL. Phospho-specific monoclonal antibodies are produced by immunizing rabbits with synthetic phosphopeptides corresponding to Ser166 of human RIP, Ser227 of human RIP3, and Ser358 of human MLKL.

Source/Purification: Monoclonal antibodies are produced by immunizing rabbits with synthetic peptides corresponding to Leu190 of human RIP, residues near the carboxyl terminus of human RIP3, and residues near the carboxyl terminus of human MLKL. Phospho-specific monoclonal antibodies are produced by immunizing rabbits with synthetic phosphopeptides corresponding to Ser166 of human RIP, Ser227 of human RIP3, and Ser358 of human MLKL.

Specificity/Sensitivity: Each antibody in the Necroptosis Antibody Sampler Kit detects endogenous levels of its target protein. MLKL (D216N) Rabbit mAb cross-reacts with an unidentified band at 130 kDa in some cell lines. Phospho-MLKL (Ser358) (D6H3V) Rabbit mAb may also bind to MLKL when dually phosphorylated at Thr357 and Ser358.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. Do not aliquot the antibody.

Background References:


U.S. Patent No. 5,675,063
Western Immunoblotting Protocol

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

NOTE: Please refer to 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.

1. 20X Phosphate Buffered Saline (PBS): (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH$_2$O, mix.
2. 10X Tris Buffered Saline (TBS): (#12498) To prepare 1 L 1X TBS: add 100 ml 10X to 900 ml dH$_2$O, 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$_2$O.
4. 10X Tris-Glycine SDS Running Buffer: (#4050) To prepare 1 L 1X running buffer: add 100 ml 10X running buffer to 900 ml dH$_2$O, mix.
5. 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$_2$O, mix.
6. 10X Tris Buffered Saline with Tween® 20 (TBST): (#9997) To prepare 1 L TBST: add 100 ml 10X TBST to 900 ml dH$_2$O, mix.
7. Nonfat Dry Milk: (#9999)
8. 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.
9. Wash Buffer: (#9997) 1X TBST
10. Bovine Serum Albumin (BSA): (#9998)
11. 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.
13. Prestained Protein Marker, Broad (Premixed Format): (#7720)
14. Blotting Membrane and Paper:
15. Secondary Antibody Conjugated to HRP:
   - anti-rabbit (#7074); anti-mouse (#7076)
16. Detection Reagents:
   - LumiGLO® chemiluminescent reagent and peroxide (#7003) or SignalFire™ ECL Reagent (#6883)

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.
3. 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.
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 (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights are recommended.
8. Electrophoresis 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.

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

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 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).

D. Detection of Proteins

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 at 4°C.
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 incubation and declines over the following 2 hr.