

#2330 Store at -20°C

# Raf Family Antibody Sampler Kit

✓ 1 Kit  
(8 x 40 µl)



**Orders** ■ 877-616-CELL (2355)  
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**Support** ■ 877-678-TECH (8324)  
info@cellsignal.com  
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This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

| Products Included                        | Product # | Quantity | Mol. Wt.     | Isotype    |
|--|-----------|----------|--------------|------------|
| Phospho-A-Raf (Ser299) Antibody          | 4431      | 40 µl    | 68 kDa       | Rabbit IgG |
| A-Raf Antibody                           | 4432      | 40 µl    | 68 kDa       | Rabbit IgG |
| Phospho-c-Raf (Ser338) (56A6) Rabbit mAb | 9427      | 40 µl    | 74 kDa       | Rabbit IgG |
| Phospho-c-Raf (Ser289/296/301) Antibody  | 9431      | 40 µl    | 74 kDa       | Rabbit IgG |
| Phospho-c-Raf (Ser259) Antibody          | 9421      | 40 µl    | 74 kDa       | Rabbit IgG |
| c-Raf Antibody                           | 9422      | 40 µl    | 65 to 75 kDa | Rabbit IgG |
| Phospho-B-Raf (Ser445) Antibody          | 2696      | 40 µl    | 86 kDa       | Rabbit IgG |
| B-Raf (L12G7) Mouse mAb                  | 9434      | 40 µl    | 86 kDa       | Mouse IgG1 |
| Anti-rabbit IgG, HRP-linked Antibody     | 7074      | 100 µl   |              | Goat       |
| Anti-mouse IgG, HRP-linked Antibody      | 7076      | 100 µl   |              | Goat       |

**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 antibodies.*

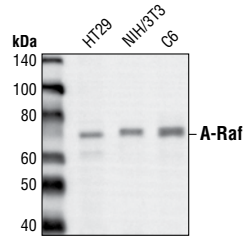
**Recommended Antibody Dilutions:**  
Western blotting 1:1000

**Please visit [www.cellsignal.com](http://www.cellsignal.com) for a complete listing of recommended companion products.**

See [www.cellsignal.com](http://www.cellsignal.com) for individual component applications, species cross-reactivity, dilutions, and additional application protocols.

**Background:** A-Raf, B-Raf and c-Raf (Raf-1) are the main effectors recruited by GTP-bound Ras to activate the MEK-MAP kinase pathway (1). Activation of c-Raf is the best understood and involves phosphorylation at multiple activating sites including Ser338, Tyr341, Thr491, Ser494, Ser497 and Ser499 (2). p21-activated protein kinase (PAK) has been shown to phosphorylate c-Raf at Ser338 and the Src family phosphorylates Tyr341 to induce c-Raf activity (3,4). Ser338 of c-Raf corresponds to similar sites in A-Raf (Ser299) and B-Raf (Ser445), although this site is constitutively phosphorylated in B-Raf (5). Inhibitory 14-3-3 binding sites on c-Raf (Ser259 and Ser621) can be phosphorylated by Akt and AMPK, respectively (6,7). While A-Raf, B-Raf and c-Raf are similar in sequence and function, differential regulation has been observed (8). Of particular interest, B-Raf contains three consensus Akt phosphorylation sites (Ser364, Ser428 and Thr439) and lacks a site equivalent to Tyr341 of c-Raf (8,9). The B-Raf mutation V600E results in elevated kinase activity and is commonly found in malignant melanoma (10). Six residues of c-Raf (Ser29, Ser43, Ser289, Ser296, Ser301 and Ser642) become hyperphosphorylated in a manner consistent with c-Raf inactivation. The hyperphosphorylation of these six sites is dependent on downstream MEK signaling and renders c-Raf unresponsive to subsequent activation events (11).

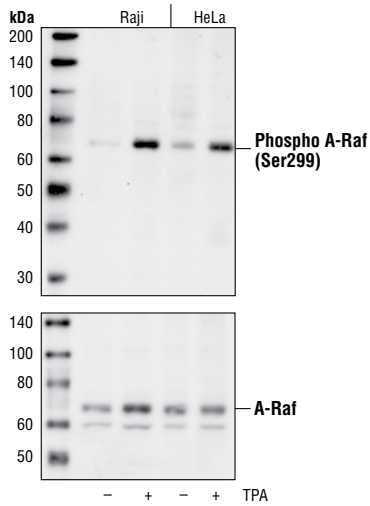
**Specificity/Sensitivity:** Each antibody in the Raf Family Antibody Sampler Kit recognizes only its specific target and does not cross react with other Raf family members.



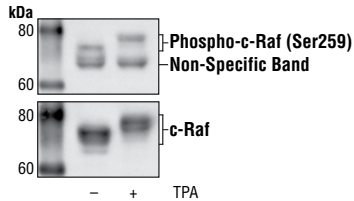
Western blot analysis of extracts from HT29, NIH/3T3 and C6 cell lysates using **A-Raf Antibody #4432**.

**Source/Purification:** The phospho-specific polyclonal antibodies are produced by immunizing rabbits with a synthetic phosphopeptide corresponding to residues surrounding Ser299 of human A-Raf, Ser445 of human B-Raf and Ser259, 289, 296 and 301 of c-Raf. The total polyclonal antibodies are produced by immunizing rabbits with a synthetic peptide corresponding to residues close to the linker domain of human A-Raf and a region surrounding Pro302 of human c-Raf. Polyclonal antibodies are purified by protein A and peptide affinity chromatography. The rabbit monoclonal antibody is produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding Ser338 of human c-Raf. The mouse monoclonal antibody is produced by immunizing animals with a recombinant fragment of B-Raf protein.

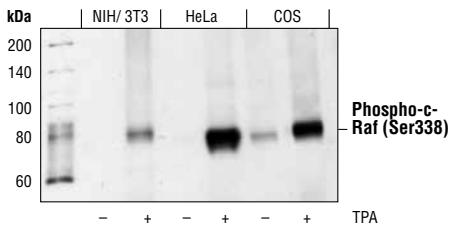
© 2011 Cell Signaling Technology, Inc. Rabbit monoclonal antibody is produced under license (granting certain rights including those under U. S. Patents No. 5,675,063 and 7,429,487) from Epitomics, Inc.



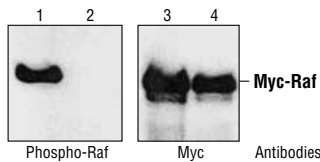
Western blot analysis of extracts from Raji and HeLa cells, untreated or TPA-treated (30 minutes), using **Phospho-A-Raf (Ser299) Antibody #4431** (upper) or **A-Raf Antibody #4432** (lower).



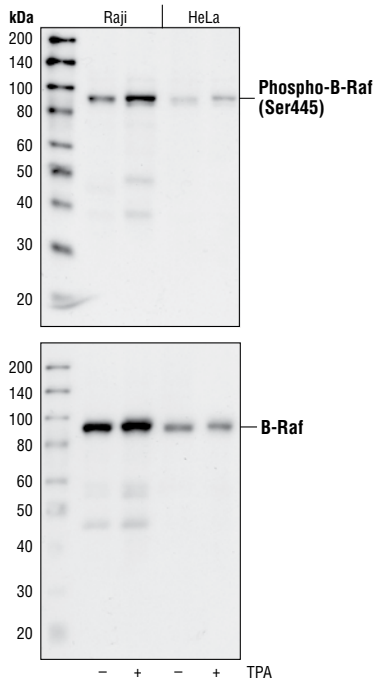
Western blot analysis of extracts from HeLa cells, untreated or TPA-treated, using **Phospho-c-Raf (Ser259) Antibody #9421** (upper), or a total c-Raf antibody (lower).



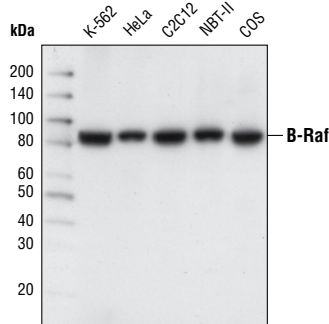
Western blot analysis of extracts from NIH3T3, HeLa and COS cells, untreated or treated with TPA using **Phospho-c-Raf (Ser338) (56A6) Rabbit mAb #9427**.



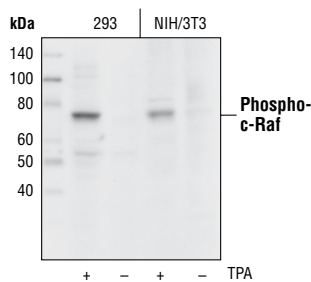
Western blot analysis of recombinant Myc-tagged c-Raf protein, wildtype (lanes 1 and 3) and S259A mutant (lanes 2 and 4), using **Phospho-Raf (Ser259) Antibody #9421** or a Myc antibody. (Provided by Dr. Guri Tzivion, Massachusetts General Hospital.)



Western blot analysis of extracts from Raji and HeLa cells treated with TPA (200 nM, 30 minutes) using **Phospho-B-Raf (Ser445) Antibody #2696** (top) and total B-Raf Antibody (bottom).



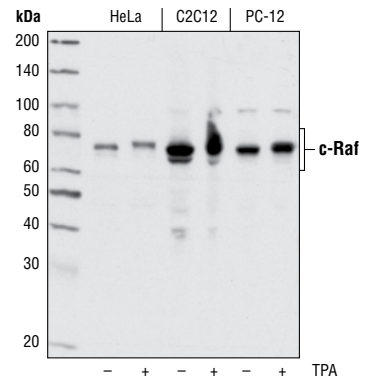
Western blot analysis of extracts from K-562, HeLa, C2C12, NBT-II and COS cells using **B-Raf (L12G7) Mouse mAb #9434**.



Western blot analysis of extracts from untreated or TPA-treated 293 and NIH/3T3 cells using **Phospho-c-Raf (Ser289/296/301) Antibody #9431**.

#### Background References:

- (1) Avruch, J. et al. (1994) *Trends Biochem. Sci.* 19, 279–283.
- (2) Chong, H. et al. (2001) *EMBO J.* 20, 3716–3727.
- (3) King, A.J. et al. (1998) *Nature* 396, 180–183.
- (4) Fabian, J.R. et al. (1993) *Mol. Cell Biol.* 13, 7170–7179.
- (5) Mason, C.S. et al. (1999) *EMBO J.* 18, 2137–2148.
- (6) Zimmermann, S. and Moelling, K. (1999) *Science* 286, 1741–1744.
- (7) Sprenkle, A.B. et al. (1997) *FEBS Lett.* 403, 254–258.
- (8) Marais, R. et al. (1997) *J. Biol. Chem.* 272, 4378–4383.
- (9) Guan, K.L. et al. (2000) *J. Biol. Chem.* 275, 27354–27359.
- (10) Davies, H. et al. (2002) *Nature* 417, 949–954.
- (11) Dougherty, M.K. et al. (2005) *Mol. Cell* 17, 215–224.



Western blot analysis of extracts from HeLa, C2C12 or PC12 cells, untreated or TPA-treated (200 nM for 30 minutes), using **c-Raf Antibody #9422**.

## 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.

- 1X Phosphate Buffered Saline (PBS)
- 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
- Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
- 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).
- Nonfat Dry Milk (weight to volume [w/v])
- 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%).
- Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T)
- Bovine Serum Albumin (BSA)
- 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%).
- 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.
- Prestained Protein Marker, Broad Range (Premixed Format) #7720
- Biotinylated Protein Ladder Detection Pack #7727
- 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.

- Treat cells by adding fresh media containing regulator for desired time.
- Aspirate media from cultures; wash cells with 1X PBS; aspirate.
- 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.
- Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
- Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
- Microcentrifuge for 5 minutes.
- 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.

- 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.

- (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
- Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- 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.
- Wash three times for 5 minutes each with 15 ml of TBS/T.

### D Detection of Proteins

- 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.

- 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.