

#9306 Store at -20°C

PhosphoPlus® Rb (Ser780, Ser807/811) Antibody Kit

1 Kit
 (10 western blots)



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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-Rb (Ser780) Antibody	9307	100 µl	110 kDa	Rabbit IgG
Phospho-Rb (Ser807/811) Antibody	9308	100 µl	110 kDa	Rabbit IgG
Rb (4H1) Mouse mAb	9309	100 µl	110 kDa	Mouse IgG2a
Rb Control Proteins	9303	100 µl	76 kDa	
Anti-rabbit IgG, HRP-linked Antibody	7074	100 µl		Goat
Anti-biotin, HRP-linked Antibody	7075	100 µl		Goat
Anti-mouse IgG, HRP-linked Antibody	7076	100 µl		Goat
20X LumiGLO® Reagent and 20X Peroxide	7003	5 ml		
Biotinylated Protein Ladder Detection Pack	7727	100 µl		

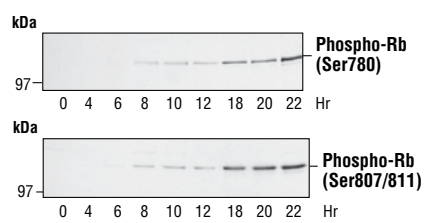
See www.cellsignal.com for individual component applications, species cross-reactivity, dilutions and additional application protocols.

Description: The PhosphoPlus® Rb (Ser780, Ser807/811) Antibody Kit provides reagents and protocols to investigate cell cycle progression within cells. The kit contains a total Rb antibody, two distinct phospho-Rb antibodies, and Rb control proteins along with secondary antibodies and reagents to perform up to 10 western blots.

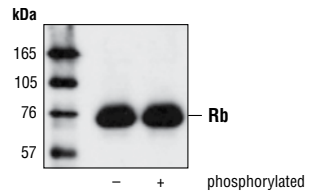
Background: The retinoblastoma tumor suppressor protein, Rb, regulates cell proliferation by controlling progression through the restriction point within the G1-phase of the cell cycle (1). Rb has three functionally distinct binding domains and interacts with critical regulatory proteins including the E2F family of transcription factors, c-Abl tyrosine kinase and proteins with a conserved LXCXE motif (2-4). Cell cycle-dependent phosphorylation by a CDK inhibits Rb target binding and allows cell cycle progression (5). Rb inactivation and subsequent cell cycle progression likely requires an initial phosphorylation by cyclin D-CDK4/6 followed by cyclin E-CDK2 phosphorylation (6). Specificity of different CDK/cyclin complexes has been observed *in vitro* (6-8) and cyclin D1 is required for Ser780 phosphorylation *in vivo* (9).

Specificity/Sensitivity: Phospho-Rb (Ser780) and Phospho-Rb (Ser807/811) antibodies detect endogenous levels of Rb only when phosphorylated at Ser780 and Ser807/811, respectively. These antibodies do not cross-react with Rb phosphorylated at other sites. Rb (4H1) Mouse mAb detects endogenous levels of total Rb protein. The antibody does not cross-react with the Rb homologues p107 or p130, or with other proteins.

Source/Purification: Polyclonal antibodies are produced by immunizing animals with a synthetic phosphopeptide corresponding to residues around Ser780 and Ser807/811 of human Rb. Antibodies are purified by protein A and



Western blot analysis of Rb phosphorylation in human fibroblasts synchronized by serum deprivation using **Phospho-Rb (Ser780) Antibody #9307** (upper) and **Phospho-Rb (Ser807/811) Antibody #9308** (lower). Cells were synchronized for 24 hours then released by addition of serum and harvested at the times indicated. Cell cycle progression was verified by cyclin analysis and FACS. (Provided by John Boylan, Dupont/Merck, Delaware.)



Western blot analysis of **Rb Control Protein #9303** using **Rb (4H1) mAb #9309**.

peptide affinity chromatography. Rb (4H1) monoclonal antibody is produced by immunizing animals with Rb-C Fusion Protein #6022, which contains residues 701-928 of human Rb.

Antibody 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.

20X LumiGLO® Reagent and 20X Peroxide Storage: Store at 4°C.

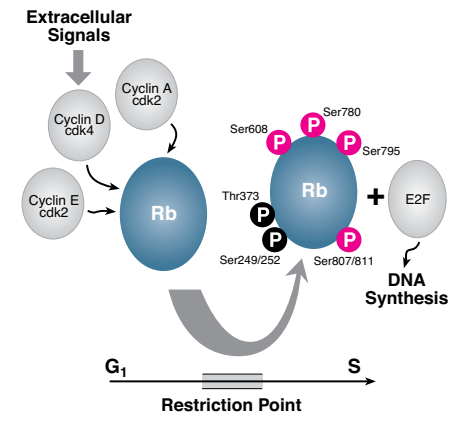
Biotinylated Protein Ladder Detection Pack Storage: Supplied in 65 mM Tris-HCl (pH 7.0 at 25°C), 35 mM NaCl, 1 mM Na₂EDTA, 2% SDS (w/v), 1 mM NaN₃, 40 mM dithiothreitol (DTT), 0.01% (w/v) phenol red and 10% glycerol. Store at -20°C.

Recommended Antibody Dilutions:
 Western blotting 1:1000

Please visit www.cellsignal.com for a complete listing of recommended companion products.

Background References:

- (1) Sherr, C.J. (1996) *Science* 274, 1672-1677.
- (2) Nevins, J. R. et al. (1992) *Science* 258, 424-429.
- (3) Welch, P.J. and Wang, J.Y. (1993) *Cell* 75, 779-790.
- (4) Hu, Q.J. et al. (1990) *EMBO J.* 9, 1147-1155.
- (5) Knudsen, E.S. and Wang, J.Y. (1997) *Mol. Cell. Biol.* 17, 5771-5783.
- (6) Lundberg, A.S. and Weinberg, R.A. (1998) *Mol. Cell. Biol.* 18, 753-761.
- (7) Connell-Crowley, L. et al. (1997) *Mol. Cell. Biol.* 8, 287-301.
- (8) Kitagawa, M. et al. (1996) *EMBO J.* 15, 7060-7069.
- (9) Geng, Y. et al. (2001) *Proc. Natl. Acad. Sci. USA* 98, 194-199.



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Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine
 Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—horse All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

page 1 of 3

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[®]-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.
- 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 cm²) 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[®] (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.

- 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.