Anti-Phospho-Rb (Ser612) mAb

Cat# CY-M1013

100 µg (1 mg/mL x 100 µL)

Clone Name	Applications	Species Cross-Reactivity	Molecular Wt.	Source Isotype
3C11	WB, IP, E	H	115 kDa	Mouse IgG2a

Background

The retinoblastoma protein (Rb) is a nuclear phosphoprotein that regulates growth in the G1 phase of the cell cycle. Rb exerts its growth-inhibitory effects in part by binding to and inhibiting critical regulatory proteins, including members of the E2F family of transcription factors; E2F activation is necessary for the G1-S transition. E2F selectively associates with hypophosphorylated Rb, and phosphorylation of Rb appears to release E2F from an inhibitory complex, enabling it to promote the transcription necessary for progression into late G1 and S.

Rb is phosphorylated on a still imprecisely defined number of threonine and serine residues during G1 (1, 2). A temporal sequence of modifications has been defined through use of both Rb variants in which certain of these residues have been replaced and monoclonal antibodies (MAbs) specific for certain phosphorylated domains of Rb. Both serine 608 (S608) and S780 have been identified as among the sites that are initially phosphorylated. These phosphorylations have distinct effects on the ability of Rb to interact with its various partner proteins. Thus, Rb phosphorylated on S780 appears to lose its ability to bind to E2F. Phosphorylation of S807 and/or S811 is required to abolish Rb binding to c-Abl, while modification of threonine 821 (T821) and/or T826 is required to abolish Rb binding to LXCXE-containing proteins such as simian virus 40 large T antigen. However, these four sites do not appear to be involved in regulating Rb binding to the E2F transcription factors.

Phosphorylation of Rb also has effects on cell physiology, ostensibly by changing its association with these and other interacting partner proteins. For example, phosphorylation of S795 is required to inactivate Rb-imposed growth suppression in a microinjection assay. However, the relationship between growth inhibition and E2F binding is complex: phosphorylation of Rb in vitro by cyclin D-, cyclin E-, or cyclin A-associated kinase has been reported to release E2F, yet only action by cyclin D1–cyclin-dependent kinase 4 (cdk4) complexes, but not by cyclin E-cdk2 complexes, abrogates the growth-inhibitory property of Rb when microinjected into SaOS-2 cells.

Specificity/Sensitivity: Anti-Phospho-Rb (Ser612) mAb (3C11) detects endogenous Rb protein only when phosphorylated at serine612.

Source/Purification: Monoclonal antibody is produced by immunizing mice with a synthetic phosphopeptide corresponding to residues surrounding Ser612 of human Rb. IgG is purified by protein A Sepharose chromatography.

Recommended Antibody Dilutions: Western blotting: 1-2 µg/mL, Immunoprecipitation: 2-4 µg /sample, ELISA for detection of Cdk2 activity: 5-10 µg/mL

Storage: Supplied in 20 mM phosphate buffer (pH 7.5), 300 mM NaCl, 50 % glycerol. Store at –20°C.

Applications Key:
WB: Western blotting, **IP:** Immunoprecipitation, **IHC:** Immunohistochemistry, **IC:** Immunocytochemistry, **F:** Flow cytometry, **E:** ELISA, **FP:** Fluorescence polarization assay

Species Cross-Reactivity Key:
H: Human, **M:** Mouse, **R:** Rat, **Hm:** Hamster, **Mk:** Monkey, **Mi:** Mink, **C:** Chicken, **X:** Xenopus, **Z:** Zebra fish (Species enclosed in parentheses are predicted to react based on 100 % sequence homology.)

References:

1. Kitagawa, M et al. The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2 EMBO J. 15: 7060-7069, 1996
2. Suzuki, S et al. Enzyme-Linked Immunosorbent Assay for Distinct Cyclin-Dependent Kinase Activities Using Phosphorylation-Site-Specific Anti-Rb Monoclonal Antibodies. Anal Biochem. 301(1):65-74, 2002
3. Taya, Y et al. Generation and Application of Phospho-specific Antibodies for p53 and Rb Mehtods in Molecular Biology Vol. 223, 17-26: Tumor Suppressor Genes: Regulation and Function, and Medical Applications

Fig.1 Western blot analysis of phosphorylated Rb in extracts from MOLT4 cell using Anti-Phospho-Rb (Ser612) mAb (3C11)

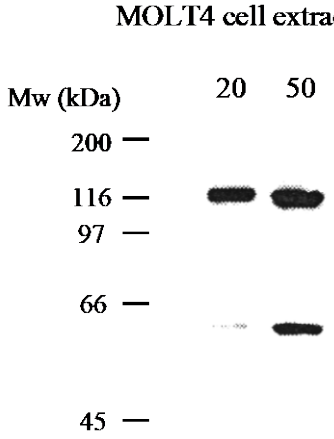
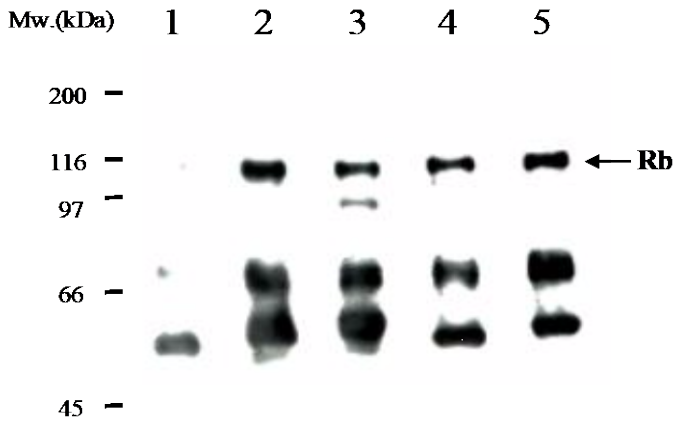


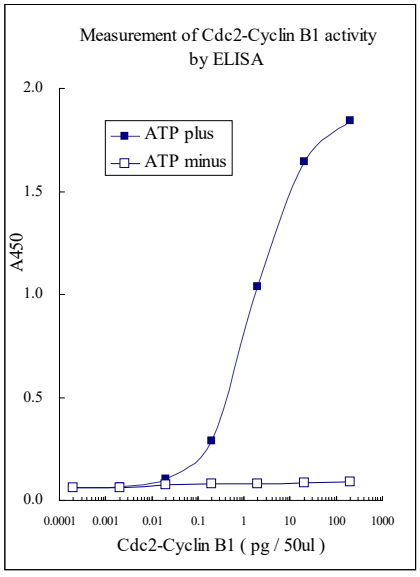
Fig.2 IP-Western blotting phosphorylated Rb in HL60 cell lysate using Anti-Phospho-Rb (Ser612) mAb (3C11: lane 2)



IP antibody:
1. Normal mouse IgG
2. Anti-phospho-pRb Ser612 clone 3C11
3. Anti-phospho-pRb Ser612 clone 4E4
4. Anti-phospho-pRb Ser807 clone 5H12
5. Anti-Rb mAb clone 3H9 (MBL, MK-15-3)

WB antibody:
Anti-Rb clone mAb 3H9 (MBL, MK-15-3)

Fig.3 ELISA for measurement of recombinant Cdc2-CyclinB1 kinase activity using Anti-Phospho-Rb (Ser612) mAb (3C11)



Western Immunoblotting Protocol

Solutions and Reagents

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

Transfer Buffer: 25 mM Tris base, 0.2 M glycine, 20 % methanol (pH 8.5)
SDS Sample Buffer (1X): 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
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 %).
10X TBS (Tris-buffered saline): 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).
Primary Antibody Dilution Buffer: 1X TBS, 0.1 % Tween-20 with 5 % blocking agent; 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 %).
Chemiluminescent HRP Detection: secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP), ECL™ chemiluminescent reagent (Amersham Pharmacia)
Wash Buffer TBS/T: 1X TBS, 0.1 % Tween-20
Blotting Membrane: This protocol has been optimized for nitrocellulose membranes, which we recommend. PVDF membranes may also be used.

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^2 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).
8. Electrotransfer to nitrocellulose membrane.

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.

1. (Optional) After transfer, wash nitrocellulose membrane with 25 mL TBS for 5 minutes at room temperature.
2. Incubate membrane in 25 m of Blocking Buffer for 1 hour at room temperature.
3. Wash 3 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 3 times for 5 minutes each with 15 mL of TBS/T.
6. Incubate membrane with HRP-conjugated secondary antibody (1:3,000 in 10 mL of Blocking Buffer with gentle agitation for 1 hour at room temperature.
7. Wash 3 times for 5 minutes each with 15 mL of TBS/T.

Detection of Proteins

1. Incubate membrane with 4 mL ECL™ with gentle agitation for 1 minute at room temperature.
2. Drain membrane of excess developing solution, do not let dry, wrap in plastic wrap and expose to x-ray film. An initial tensecond exposure should indicate the proper exposure time.

Immunoprecipitation Followed by Western Immunoblotting Protocol

olutions and Reagents

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

Cell Lysis Buffer (1X): 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 Glycerolphosphate, 1 mM Na3VO4, 1 µg/mL Leupeptin
Note: We recommend adding 1 mM PMSF before use.
Protein A Agarose Beads: Add 5 mL of 1X PBS to 1.5 g of Protein A Agarose Beads. Shake 2 hours at 4°C; spin down. Wash pellet twice with PBS. Resuspend beads in 1 volume of PBS. (Can be stored for 2 weeks at 4°C)
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,
Transfer Buffer: 25 mM Tris base, 0.2 M glycine, 20 % methanol (pH 8.5)
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 %).
10X TBS (Tris-buffered saline): For 1 liter of 10X TBS, use 24.2 g Tris base and 80 g NaCl. Adjust pH to 7.6 with HCl (use at 1X).
Primary Antibody Dilution Buffer: 1X TBS, 0.05 % Tween-20 with 5 % nonfat dry milk. For 20 mL, add 2 mL 10X TBS to 18 mL water, mix. Add 1.0 g nonfat dry milk and mix well. While stirring, add 10 µL Tween-20 (100 %).
Wash Buffer TBS/T: 1X TBS, 0.1 % Tween-20
Chemiluminescent HRP Detection: secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP), ECL™ chemiluminescent reagent (Amersham Pharmacia)
Wash Buffer TBS/T: 1X TBS, 0.1 % Tween-20
Blotting Membrane: This protocol has been optimized for nitrocellulose membranes, which we recommend. PVDF membranes may also be used.

Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
2. To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 mL 1X ice-cold Cell Lysis Buffer plus 1 mM PMSF to each plate (10 cm^2) and incubate the plate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate 4 times for 5 seconds each on ice.
6. Microcentrifuge for 10 minutes at 4°C, and transfer the supernatant to a new tube. The supernatant is the cell lysate. If necessary, lysate can be stored at –80°C.

Immunoprecipitation

1. Take 200 µL cell lysate and add primary antibody; incubate with gentle rocking overnight at 4°C.
2. Add Protein A Agarose Beads (20 µL of 50 % bead slurry). Incubate with gentle rocking for 1–3 hours at 4°C.
3. Microcentrifuge for 30 seconds at 4°C. Wash pellet 2 times with 500 µL of 1X Cell Lysis Buffer. Keep on ice during washes.
4. Resuspend the pellet with 20 µL 3X SDS Sample Buffer. Votex, then microcentrifuge for 30 seconds.
5. Heat the sample to 95–100°C for 2–5 minutes.
6. Load the sample (15–30 µL) on SDS-PAGE gel (12–15 %).

7. Analyze sample by Western blotting (see Western Immunoblotting Protocol).

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