



**Rabbit (polyclonal)  
Anti-Retinoblastoma Protein (Rb)  
[pT<sup>356</sup>] Phosphospecific Antibody,  
Unconjugated**

**PRODUCT ANALYSIS SHEET**

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<b>Catalog Number/Size:</b>	44-578 (10 blot size)
<b>Lot Number:</b>	See product label
<b>Volume/Concentration:</b>	See product label
<b>Form of Antibody:</b>	Rabbit polyclonal immunoglobulin in phosphate buffer, pH 7.4, with 1.0 mg/mL BSA (IgG, protease-free) as a carrier.
<b>Preservative:</b>	0.05% sodium azide (Caution: sodium azide is a poisonous and hazardous substance. Handle with care and dispose of properly.)
<b>Purification:</b>	Purified from rabbit serum by sequential epitope-specific chromatography. The antibody has been negatively preadsorbed using a non-phosphopeptide corresponding to the site of phosphorylation to remove antibody that is reactive with non-phosphorylated Rb protein. The final product is generated by affinity chromatography using a Rb-derived peptide that is phosphorylated at threonine 356.
<b>Immunogen:</b>	The antiserum was produced against a chemically synthesized phosphopeptide derived from a region of human Rb that contains threonine 356 (based on Swiss Protein database, accession number P06400).
<b>Target Group:</b>	Rb is a tumor suppressor gene which functions as a negative regulator of the cell cycle by interacting with transcription factors including E2F-1, PU.1, ATF-2, UBF, Elf-1 and c-abl. This ability to alter transcription is regulated by phosphorylation catalyzed by the cyclin-dependent protein kinases (cdks). Rb contains at least 16 consensus sequences for cdk phosphorylation, but the significance of all of these sites is unclear. Phosphorylation of threonine 356 is catalyzed by the cdk4 complex Cyclin D-cdk4, and plays a role in mediating growth suppression activity of Rb.
<b>Species Reactivity:</b>	Human Rb. Mouse (80% conserved) and rat (87%) Rb were not tested.
<b>Applications:</b>	The antibody has been used for Western blotting applications.
<b>Suggested Working Dilutions:</b>	For Western blotting, 0.25-0.75 µg/mL of buffer is recommended. At 0.50 µg/mL, the dilution provides 100 mL working solution, which at 10 mL/blot allows 10 blots to be performed. The optimal antibody concentration should be determined for each specific application.
<b>Positive Control Used:</b>	Jurkat cells in high growth phase.
<b>Storage:</b>	Store at -80°C. Upon initial thawing, apportion into working aliquots and store at -80°C. Avoid repeated freeze-thaw cycles to prevent denaturing the antibody.
<b>Expiration Date:</b>	Expires one year from date of receipt when stored as instructed.

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**Related Products:****Antibodies:**

Rb [pSpT<sup>249/252</sup>] antibody, Cat. # 44-584      Rb [pSpS<sup>807/811</sup>] antibody, Cat. # 44-580  
 Rb [pS<sup>612</sup>] antibody, Cat. # 44-572      Rb [pT<sup>821</sup>] antibody, Cat. # 44-582  
 Rb [pS<sup>807</sup>] antibody, Cat. # 44-579      Rb [pT<sup>826</sup>] antibody, Cat. # 44-576

**Peptides:**

Rb [pT<sup>356</sup>] control peptides, Cat. # 04-578Z

**References:**

Brantley, M.A., Jr. and J.W. Harbour (2001) The molecular biology of retinoblastoma. *Ocul. Immunol. Inflamm.* 9(1):1-8.

Tamrakar, S., et al. (2000) Role of pRB dephosphorylation in cell cycle regulation. *Front. Biosci.* 5:D121-D137.

Brown, V.D., et al. (1999) Cumulative effect of phosphorylation of pRB on regulation of E2F activity. *Mol. Cell. Biol.* 19:3246-3256.

Harbour, J.W., et al. (1999) Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell* 98:859-869.

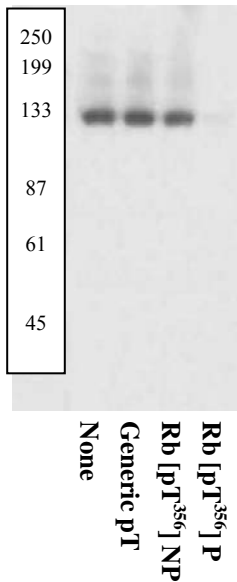
Driscoll, B., et al. (1999) Discovery of a regulatory motif that controls the exposure of specific upstream cyclin-dependent kinase sites that determine both conformation and growth suppressing activity of pRb. *J. Biol. Chem.* 274(14):9463-9471.

Zarkowska, T. and S. Mitnacht (1997) Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. *J. Biol. Chem.* 272:12738-12746.

Knudsen, E.S. and J.Y. Wang (1997) Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. *Mol. Cell. Biol.* 17:5771-5783.

Knudsen, E.S. and J.Y. Wang (1996) Differential regulation of retinoblastoma protein function by specific cdk phosphorylation sites. *J. Biol. Chem.* 271:8313-8320.

**kDa**    1    2    3    4

**Peptide Competition**

Extracts prepared from Jurkat cells in high growth phase were resolved by SDS-PAGE on a 10% Tris-glycine gel and transferred to PVDF. Membranes were incubated with 0.50 µg/mL Rb [pT<sup>356</sup>] antibody, following prior incubation with: no peptide (1), a generic phosphothreonine containing peptide (2), the non-phosphopeptide corresponding to the phosphopeptide immunogen (3), or, the phosphopeptide immunogen (4). After washing, membranes were incubated with goat F(ab')<sub>2</sub> anti-rabbit IgG alkaline phosphatase (cat.# ALI4405) and bands were detected using the Tropic WesternStar™ detection method.

The data show that only the peptide corresponding to Rb [pT<sup>356</sup>] blocks the antibody signal, thereby demonstrating the specificity of the antibody.

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## Western Blotting Procedure

1. Lyse approximately  $10^7$  cells in 0.5 mL of ice cold Cell Lysis Buffer (formulation provided below). This buffer, a modified RIPA buffer, is suitable for recovery of most proteins, including membrane receptors, cytoskeletal-associated proteins, and soluble proteins. This cell lysis buffer formulation is available as a separate product which requires supplementation with protease inhibitors immediately prior to use (Invitrogen catalog number FNN0011). Other cell lysis buffer formulations, such as Laemmli sample buffer and Triton-X 100 buffer, are also compatible with this procedure. Additional optimization of the cell stimulation protocol and cell lysis procedure may be required for each specific application.
2. Remove the cellular debris by centrifuging the lysates at 14,000 x g for 10 minutes. Alternatively, lysates may be ultracentrifuged at 100,000 x g for 30 minutes for greater clarification.
3. Carefully decant the clarified cell lysates into clean tubes and determine the protein concentration using a suitable method, such as the Bradford assay. Polypropylene tubes are recommended for storing cell lysates.
4. React an aliquot of the lysate with an equal volume of 2x Laemmli Sample Buffer (125 mM Tris, pH 6.8, 10% glycerol, 10% SDS, 0.006% bromophenol blue, and 130 mM dithiothreitol [DTT]) and boil the mixture for 90 seconds at 100°C.
5. Load 10-30 µg of the cell lysate into the wells of an appropriate single percentage or gradient minigel and resolve the proteins by SDS-PAGE.
6. In preparation for the Western transfer, cut a piece of PVDF membrane slightly larger than the gel. Soak the membrane in methanol for 1 minute, then rinse with ddH<sub>2</sub>O for 5 minutes.
7. Soak the PVDF membrane, 2 pieces of Whatman paper, and Western apparatus sponges in transfer buffer (formulation provided below) for 2 minutes.
8. Assemble the gel and membrane into the sandwich apparatus.
9. Transfer the proteins at 140 mA for 60-90 minutes at room temperature.
10. Following the transfer, rinse the membrane with Tris buffered saline for 2 minutes.
11. Block the membrane with blocking buffer (formulation provided below) for 2 hours at room temperature.
12. Incubate the blocked blot with primary antibody at a concentration of 0.25-1.0 µg/mL in Tris buffered saline supplemented with 1% non-fat dried milk and 0.1% Tween 20 for two hours at room temperature.
13. Wash the blot with several changes of Tris buffered saline supplemented with 0.1% Tween 20.
14. Detect the antibody band using an appropriate secondary antibody, such as goat F(ab')<sub>2</sub> anti-rabbit IgG alkaline phosphatase conjugate (catalog number ALI4405) or goat F(ab')<sub>2</sub> anti-rabbit IgG horseradish peroxidase conjugate (catalog number ALI4404) in conjunction with your chemiluminescence reagents and instrumentation.

### Cell Lysis Buffer

#### Formulation:

10 mM Tris, pH 7.4  
100 mM NaCl  
1 mM EDTA  
1 mM EGTA  
1 mM NaF  
20 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>  
2 mM Na<sub>3</sub>VO<sub>4</sub>  
0.1% SDS  
0.5% sodium deoxycholate  
1% Triton-X 100  
10% glycerol  
1 mM PMSF (made from a  
0.3 M stock in DMSO)  
or 1 mM AEBSF (water soluble version of  
PMSF)  
60 µg/mL aprotinin  
10 µg/mL leupeptin  
1 µg/mL pepstatin  
(alternatively, protease inhibitor cocktail such  
as Sigma catalog number P2714 may be used)

### Transfer Buffer

#### Formulation:

2.4 gm Tris base  
14.2 gm glycine  
200 mL methanol  
Q.S. to 1 liter, then add  
1 mL 10% SDS.  
Cool to 4°C prior to use.

### Tris Buffered Saline

#### Formulation:

20 mM Tris-HCl, pH 7.4  
0.9% NaCl

### Blocking Buffer

#### Formulation:

100 mL Tris buffered saline  
5 gm non-fat dried milk  
0.1 mL Tween 20

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### Peptide Competition Experiment

To demonstrate the specificity of a Phosphorylation Site Specific Antibody, we recommend the following peptide competition experiment which uses our control peptides. These control peptides have the sequences of the phosphopeptide immunogen used to raise the antibody and the corresponding non-phosphorylated peptide. In the competition experiment, 200-500 fold molar excess of the phosphorylated and non-phosphorylated peptides are pre-incubated with aliquots of the antibody prior to use in immunoassay procedures.

A sample calculation for the determination of the 200 fold molar excess of peptide to antibody is presented below. The following assumptions have been made:

- The molecular mass of an IgG molecule is 150,000 daltons.
- Each mole of antibody binds two moles of peptide.
- The Phosphorylation Site Specific Antibody is used at a concentration of 0.5 µg/mL.

The optimal antibody concentration for use in peptide competition experiments is below saturating as determined by previous experiments in your system. If an optimal concentration has not been determined, it is suggested that the concentration provided on the antibody Product Analysis Sheet be used. A final antibody concentration of 0.5 µg/mL is satisfactory for most applications.

The molarity of the 0.5 µg/mL antibody solution is:

$$(0.5 \mu\text{g/mL})(1000 \text{ mL/L}) / (150,000 \mu\text{g}/\mu\text{mole}) = 0.00333 \mu\text{M}.$$

Because each mole of antibody binds two moles of peptide, 0.5 µg/mL antibody can bind 0.00667 µM of peptide.

A 200 fold molar excess of peptide is  $(200)(0.00667 \mu\text{M}) = 1.334 \mu\text{M}$ .

The following procedure describes peptide competition experiments using antibody at a concentration of 0.5 µg/mL and a 200 fold molar excess of peptides based on the calculation above, in a total volume of 2 mL.

#### Procedure:

1. Prepare three identical test samples, such as identical nitrocellulose or PVDF strips with transferred protein. The test samples should be blocked with BSA or non-fat dried milk in a buffer compatible with an antibody based detection method, such as Tris buffered saline or phosphate buffered saline.
2. Slowly thaw the Phosphorylation Site Specific Antibody on ice.
3. Prepare 3 mL of a 2x (1 µg/mL) antibody stock solution in a buffer appropriate for the application. Suggested buffer formulations are TBS or PBS supplemented with blocking protein such as BSA or non-fat dried milk.
4. Apportion the unused Phosphorylation Site Specific Antibody into working aliquots and store at -80°C for future use.
5. The lyophilized control peptides should be warmed to room temperature, ideally under desiccation.
6. Reconstitute each of the control peptides to a concentration of 100 µM using nanopure water at room temperature. As indicated on the peptide labels, each vial contains 0.1 mg. For a peptide with a molecular mass of 1500, reconstitution with 0.67 mL water yields a solution with a concentration of 100 µM.
7. Allow the peptides to dissolve at room temperature, then gently triturate several times using a pipette. Avoid introducing air bubbles.
8. Label 3 test tubes as follows:
  - tube 1: water only no peptide control
  - tube 2: phosphopeptide
  - tube 3: non-phosphopeptide
9. Prepare 2x peptide stock solutions (2.66 µM) or water control by pipetting the following:
  - tube 1: water control stock: 0.973 mL buffer (TBS or PBS plus blocking protein) plus 27 µL water.
  - tube 2: phosphopeptide 2x stock: 0.973 mL buffer (TBS or PBS plus blocking protein) plus 27 µL reconstituted (100 µM) phosphopeptide.
  - tube 3: non-phosphopeptide 2x stock: 0.973 mL buffer (TBS or PBS plus blocking protein) plus 27 µL reconstituted (100 µM) non-phosphopeptide.
10. Apportion unused reconstituted peptide solutions into working aliquots and store at -20°C for future use.
11. Pipette 1 mL of the 2x antibody stock into each of the tubes marked 1, 2, and 3. The tubes should be incubated for 30 minutes at room temperature with gentle rocking.
12. The pre-incubated antibody in each of the three tubes is then ready for use. Pipette the contents of each tube onto the three identical test samples.

For Western blotting strips:

  - ◆ Incubate these strips for 2 hours at room temperature, followed by several washes to remove unbound antibody.
  - ◆ Transfer each strip to a new solution containing a labeled secondary antibody (example goat anti-rabbit IgG-alkaline phosphatase conjugate).
  - ◆ Remove unbound secondary antibody by thorough washing and develop bands.

The signals obtained with antibody incubated with “(1) water only no peptide control”, which represents the maximum signal, and the signals obtained with “(2) phosphopeptide” and “(3) non-phosphopeptide” are readily compared under these conditions.

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