



Rabbit (polyclonal) Anti-Retinoblastoma Protein (Rb) [pSpT^{249/252}] Phosphospecific Antibody, Unconjugated

PRODUCT ANALYSIS SHEET

Catalog Number/Size:	44-584G (10 mini-blot size)
Lot Number:	See product label
Volume/Concentration:	See product label
Form of Antibody:	Rabbit polyclonal immunoglobulin in Dulbecco's phosphate buffered saline (without Mg ²⁺ and Ca ²⁺), pH 7.3 (+/- 0.1), 50% glycerol with 1.0 mg/mL BSA (IgG, protease free) as a carrier.
Preservative:	0.05% sodium azide (Caution: sodium azide is a poisonous and hazardous substance. Handle with care and dispose of properly.)
Purification:	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 serine 249 and threonine 252.
Immunogen:	The antiserum was produced against a chemically synthesized phosphopeptide derived from a region of human Rb that contains serine 249 and threonine 252 (based on Swiss Protein database, accession number P06400). The sequence is conserved in human, mouse and rat.
Specificity:	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). Phosphorylation of serine 249 and threonine 252 is catalyzed by Cyclin D-cdk4. Rb contains at least 16 consensus sequences for cdk phosphorylation, but the significance of many of these sites is unclear. Cdk4 becomes active prior to cdk2 during G1, and recent evidence suggests that phosphorylation of Rb by cdk4 may be required for its subsequent phosphorylation by cdk2. It has been shown that cdk4 phosphorylation inhibits HDAC binding which is involved in DNA acetylation and active repression by Rb.
Species Reactivity:	Human Rb. Mouse and rat (100% homologous), and chicken (92% homologous) Rb were not tested but are expected to react.
Applications:	The antibody has been used in Western blotting.
Suggested Working Dilutions:	For Western blotting applications, we recommended using the antibody at a 1:1000 starting dilution. The optimal antibody concentration should be determined for each specific application.
Storage:	Store at -20°C. We recommend a brief centrifugation before opening to settle vial contents. Then apportion into working aliquots and store at -20°C. For short term storage (up to one week), 2-8 °C is sufficient.
Expiration Date:	Expires one year from date of receipt when stored as instructed.
Positive Control Used:	Jurkat cells in high growth phase.

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Related Products:**Antibodies:**Rb [pT³⁵⁶], Cat. # 44-578Rb [pS⁶¹²], Cat. # 44-572**Antibodies (continued):**Rb [pS⁷⁸⁰], Cat. # 44-574**ELISAs:**

Rb (total), Cat. # KHO0011

Rb [pS⁸⁰⁷], Cat. # 44-579Rb [pS⁸¹¹], Cat. # 44-581GRb [pSpS^{807/811}], Cat. # 44-580Rb [pT⁸²¹], Cat. # 44-582GRb [pT⁸²⁶], Cat. # 44-576Rb [pSpT^{249/252}], Cat. # KHO0031Rb [pT⁸²¹], Cat. # KHO0021**References:**

Lechpammer, M., et al. (2005) Lavopiridol reduces malignant transformation of the esophageal mucosa in p27 knockout mice. *Oncogene* 24(10):1683-1688 (cites the use of cat. # 44-584G).

Brown, K.A., et al. (2004) Transforming growth factor-beta induces Cdk2 relocalization to the cytoplasm coincident with dephosphorylation of retinoblastoma tumor suppressor protein. *Breast Cancer Res.* 6(2):R130-R139 (cites the use of cat. # 44-582G and 44-584G).

Whittaker, S.R., et al. (2004) The cyclin-dependent kinase inhibitor CYC202 (R-Roscovitin) inhibits retinoblastoma protein phosphorylation, causes loss of cyclin D1, and activates the mitogen-activated protein kinase pathway. *Cancer Res.* 64:262-272 (cites the use of cat. # 44-582G).

Gutzkow, K.B., et al. (2002) Forskolin-mediated G1 arrest in acute lymphoblastic leukaemia cells: phosphorylated pRB sequesters E2Fs. *J. Cell Sci.* 115(Pt 5):1073-1082 (cites the use of cat. # 44-582G).

Law, B.K., et al. (2002) Rapamycin potentiates transforming growth factor beta-induced growth arrest in nontransformed, oncogene-transformed, and human cancer cells. *Mol. Cell. Biology* 22(23):8184-8198 (cites the use of cat. # 44-584G).

Glassford, J., et al. (2001) Vav is required for cyclin D2 induction and proliferation of mouse B lymphocytes activated via the antigen receptor. *J. Biol. Chem.* 276(44):41040-41048 (cites the use of cat. # 44-582G).

Parada, Y., et al. (2001) BCR-ABL and interleukin 3 promote haematopoietic cell proliferation and survival through modulation of cyclin D2 and p27Kip1 expression. *J. Biol. Chem.* 276(26):23572-23580 (cites the use of cat. # 44-582G).

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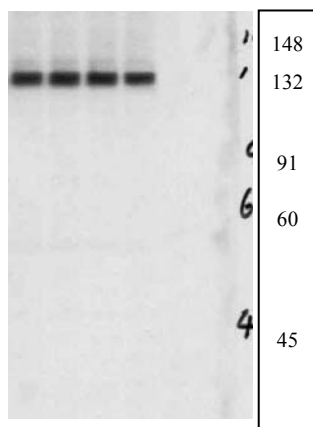
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Rb [pSpT^{249/252}] P
 Rb [pSpT^{249/252}] NP
 Generic pT
 Generic pS
 None

Peptide Competition

Extracts of Jurkat cells in high growth phase were resolved by SDS-PAGE on a 10% Tris-glycine gel and transferred to PVDF. The membrane was blocked with a 5% milk-TBST buffer for one hour at room temperature, then incubated with the Rb [pSpT^{249/252}] antibody for two hours at room temperature in a 1% milk-TBST buffer following its prior incubation with: no peptide (1), a generic phosphoserine-containing peptide (2), a generic phosphothreonine-containing peptide (3), the non-phosphopeptide corresponding to the phosphopeptide immunogen (4), or the phosphopeptide immunogen (5). After washing, the membrane was incubated with goat F(ab')₂ anti-rabbit IgG alkaline phosphatase (cat. # ALI4405) and signals were detected using the Tropix WesternStar™ detection method.

The data show that only the phosphopeptide corresponding to Rb [pSpT^{249/252}] blocks the antibody signal, 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 International 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 ddH₂O 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) overnight at 4°C or for one hour at room temperature.
12. Incubate the blocked blot with primary antibody at a 1:1000 starting dilution in Tris buffered saline supplemented with 1% non-fat dried milk and 0.1% Tween 20 overnight at 4°C or 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')₂ anti-rabbit IgG alkaline phosphatase conjugate (catalog number ALI4405) or goat F(ab')₂ 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 Na₄P₂O₇
2 mM Na₃VO₄
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 $\mu\text{g}/\text{mL}$ aprotinin
10 $\mu\text{g}/\text{mL}$ leupeptin
1 $\mu\text{g}/\text{mL}$ 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

Invitrogen's Phosphorylation Site Specific Antibodies (PSSAs) have been developed to enable the specific and sensitive detection of phosphorylation of particular amino acid residues in target proteins, while circumventing the need for protein purification, phosphopeptide mapping or handling radioactivity. The specificity of a PSSA in each experimental system can be confirmed through peptide competition. In this technique, aliquots of antibody are pre-incubated with peptide containing the sequence of the phosphopeptide immunogen used to raise the PSSA and the corresponding non-phosphopeptide. Following preincubation with the peptide, each antibody preparation is then used as a probe in antibody-based detection methods, such as Western blotting, immunocytochemistry, flow cytometry, or ELISA. With a PSSA specific for the phosphorylated target protein, pre-incubation with an excess of peptide containing the sequence of the phosphopeptide immunogen will block all antigen binding sites, while pre-incubation with the corresponding non-phosphopeptide will not affect the antibody.

Invitrogen has developed a line of control peptides specifically for use in peptide competition experiments with our PSSAs. These peptides, available as separate Invitrogen catalog items, are provided in pairs which contain the sequences of the phosphopeptide immunogen and the corresponding non-phosphopeptide.

In performing the Peptide Competition Experiment, it is important to note that the optimal dilutions of both antibody and peptide should be determined empirically for each specific application. The optimal dilution of antibody in these procedures is below saturating, as determined by previous experiments in your system. If an optimal antibody dilution has not been determined in your system, please refer to the Suggested Working Dilution on the antibody Product Analysis Sheet for guidance on an appropriate starting dilution. The optimal dilution of peptide used in these procedures will depend on the overall affinity or avidity of the antibody, as well as the quantity of the target antigen. A 50-150 fold molar excess of peptide to antibody is found to be effective for most peptide competition experiments.

In the example presented below, the PSSA is used as a dilution of 1:1000 and the peptides are used at a concentration of 333 nM. The total volume of the phosphopeptide and non-phosphopeptide-pre-incubated antibody preparations is 2 mL, sufficient for probing Western blot strips, as well as for use in other antibody-based detection methods. Under these conditions, the molar excess of peptide to antibody is ≥ 50 .

Procedure:

1. Prepare three *identical test samples*, such as identical PVDF or nitrocellulose strips to which the protein of interest has been transferred. The test samples should be blocked using a blocking buffer, such as Tris buffered saline supplemented with 0.1% Tween 20, and either 5% BSA or 5% non-fat dried milk.
2. Prepare 6.5 mL of *working antibody stock solution* (1:1000 in this example) by adding 6.5 μ L of antibody stock solution to 6.5 mL of buffer containing blocking protein, such as TBS supplemented with 0.1% Tween 20, and either 3% BSA or 3% non-fat dried milk.
3. Apportion the unused PSSA into working aliquots and store at -20°C for future use (the stock PSSA contains 50% glycerol and will not freeze at this temperature).
4. Allow the *lyophilized control peptides* to reach room temperature, ideally under desiccation.
5. Reconstitute each of the control peptides (supplied at 0.1 mg/vial) to a concentration of 66.7 μM with nanopure water. For a peptide with a molecular mass of 1500 (stated on the peptide Product Analysis Sheet), reconstitution with 1 mL water yields a solution with a concentration of 66.7 μM .
6. Apportion the unused reconstituted peptide solutions into working aliquots and store at -20°C for future use.
7. Label 3 test tubes as follows:
 - tube 1: water only no peptide control
 - tube 2: phosphopeptide
 - tube 3: non-phosphopeptide
8. Into each tube, pipette the following components
 - tube 1: 2 mL diluted PSSA solution plus 10 μ L nanopure water
 - tube 2: 2 mL diluted PSSA solution plus 10 μ L phosphopeptide
 - tube 3: 2 mL diluted PSSA solution plus 10 μ L non-phosphopeptide
9. Incubate the three tubes for 30 minutes at room temperature with gentle rocking. During this incubation, the peptides have the chance to bind to the combining site of the antibody.
10. At the end of the incubation step, transfer the contents of each of the three tubes to clean reaction vessels containing one of the three identical test samples.

For Western blotting strips:

- Incubate the strips with the pre-incubated antibody preparations for 1 hour at room temperature or overnight at 4°C .
- Wash each strip four times, five minutes each, to remove unbound antibody.
- Transfer each strip to a new solution containing a labeled secondary antibody [e.g., goat F(ab')₂ anti-rabbit IgG alkaline phosphatase conjugate (Cat. # ALI4405) or goat F(ab')₂ anti-rabbit IgG horseradish peroxidase conjugate (Cat. # ALI4404)].
- Remove unbound secondary antibody by thorough washing, and develop the signal using your chemiluminescent reagents and instrumentation.

The signals obtained with antibody incubated with the "Water Only, No Peptide Control" (Tube 1), represents the maximum signal in the assay. This signal should be eliminated by pre-incubation with the "Phosphopeptide" (Tube 2), while pre-incubation with the "Non-Phosphopeptide" (Tube 3) should not impact the signal. If the "Phosphopeptide" only partially eliminates the signal, repeat the procedure using twice the volume of water or peptide solutions listed in Step 8. If partial competition is seen following pre-incubation with the "Non-Phosphopeptide", repeat the procedure using half the volumes of water or peptide solutions listed in Step 8.

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