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Rabbit (polyclonal) Anti-Integrin β3 [pY785], Phosphospecific Antibody, Unconjugated

PRODUCT ANALYSIS SHEET

Catalog Number/Size: 44878 (10 mini-blot size)

Lot Number: See Product Label

Volume/Concentration: 100 µL

Form of Antibody: Rabbit polyclonal immunoglobulin in phosphate buffer, pH 7.4, 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 Integrin β3 receptor protein. The final product is generated by affinity chromatography using an Integrin β3 receptor-derived peptide that is phosphorylated at tyrosine 785 (tyrosine 759 in the chicken sequence for Integrin β3).

Immunogen: The antiserum was produced against a chemically synthesized phosphopeptide derived from the c-terminus of the Integrin β3 receptor (based on Swiss Protein database, accession number P05106). The sequence is conserved in human, mouse and rat.

Target Summary: Integrin β3, also known as CD61, is a 130 kDa transmembrane glycoprotein that binds non-covalently in complexes with integrin α subunits (α_{IIb}, α_v) to form the functional receptor that binds to specific extracellular matrix proteins (e.g., fibronectin, vitronectin, etc.). Integrin receptors are involved in the regulation of a variety of important biological functions, including embryonic development, wound repair, hemostasis, and prevention of programmed cell death. They are also implicated in abnormal pathological states such as tumor directed angiogenesis, tumor cell growth, and metastasis. These heterodimeric receptors bridge the cytoplasmic actin cytoskeleton with proteins present in the extracellular matrix and/or on adjacent cells. The clustering of integrin receptors on the cell surface and their binding to the extracellular matrix leads to the formation of focal contacts and the activation of various signal transduction pathways. Phosphorylation of tyrosine 785 on Integrin β3 is essential for Shc and Grb2 binding, and promotes cell migration. Tyr785 is commonly referred to as Tyr759, the corresponding site in the chicken Integrin β3 protein.

Reactivity: Human Integrin β3. Mouse and rat (100% homology), chicken (85% homology), and frog (77% homology) Integrin β3 have not been tested.

Applications: The antibody has been used in Western blotting. Other applications may work but have not been tested.

Suggested Working Dilutions: For Western blotting applications, we recommend using the antibody at 0.1-1.0 µg/mL. The optimal antibody concentration should be determined for each specific application.

Storage: Store at –80°C. We recommend a brief centrifugation before opening to settle vial contents. Then, apportion into working aliquots and store at –80°C. Avoid repeated freeze/thaw cycles.

Expiration Date: Expires one year from date of receipt when stored as instructed.

Positive Control Used: K562 cells transfected with human Integrin α_v and wild-type (WT) or mutant (Y785F) human Integrin β3, immunoprecipitated with 1A2 (human Integrin β3) mAb.

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Related Products:

Antibodies:

Integrin β1 [pS^{785}], Cat. # 44870G
Integrin β1 [$pTpT^{788/789}$], Cat. #44872G
Integrin β3 [pY^{773}], Cat. # 44876G
FAK [pS^{722}], Cat. # 44588G
FAK [pS^{732}], Cat. # 44590G
FAK [pS^{910}], Cat. # 44596G
Paxillin [pY^{31}], Cat. # 44720G
Cortactin [pY^{421}] (mouse), Cat. # 44854G
Cortactin [pY^{466}] (mouse), Cat. # 44856

References:

Boettiger, D. et al. (2001) Distinct ligand-binding modes for integrin alpha(v)beta(3)-mediated adhesion to fibronectin versus vitronectin. J. Biol. Chem. 276(34):31684-31690.

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Li, Z. et al. (2001) A Mitogen-activated Protein Kinase-dependent Signaling Pathway in the Activation of Platelet Integrin alpha IIbbeta 3. J. Biol. Chem. 276(45):42226-42232.

Maile, L.A. et al. (2001) Structural analysis of the role of the beta 3 subunit of the alpha V beta 3 integrin in IGF-I signaling. J. Cell Sci. 114(Pt 7):1417-1425.

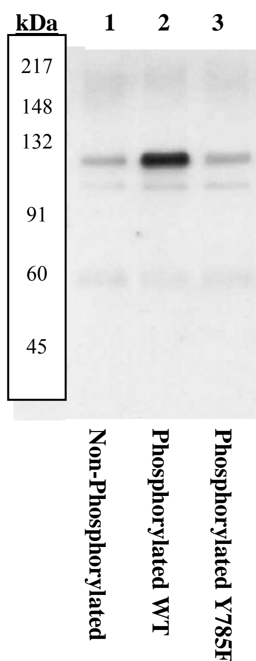
Sanjay, A. et al. (2001) Cbl associates with Pyk2 and Src to regulate Src kinase activity, alpha(v)beta(3) integrin-mediated signaling, cell adhesion, and osteoclast motility. J. Cell Biol. 152(1):181-195.

Patil, S. et al. (1999) Identification of a talin-binding site in the integrin beta(3) subunit distinct from the NPLY regulatory motif of post-ligand binding functions. The talin n-terminal head domain interacts with the membrane-proximal region of the beta(3) cytoplasmic tail. J. Biol. Chem. 274(40):28575-28583.

Schlaepfer, D.D. and Hunter, T. (1998) Integrin signalling and tyrosine phosphorylation: just the FAKs? Trends Cell Biol. 8(4):151-157.

Blystone, S.D. et al. (1997) Requirement of integrin beta3 tyrosine 747 for beta3 tyrosine phosphorylation and regulation of alphavbeta3 avidity. J. Biol. Chem. 272(45):28757-28761.

Richardson, A. and Parsons, J.T. (1995) Signal transduction through integrins: a central role for focal adhesion kinase? Bioessays 17(3):229-236.



Mutant Analysis by Immunoprecipitation-Western Blot

Extracts of K562 cells transfected with human Integrin α_v and wild-type (WT) or mutant (Y785F) human Integrin β3 immunoprecipitated with the 1A2 (human Integrin β3) mAb were resolved by SDS-PAGE on a 10% Tris-glycine gel and transferred to PVDF. The membrane was blocked with a 5% BSA-TBST buffer overnight at 4°C, then incubated with the Integrin β3 [pY^{785}] antibody for two hours at room temperature in a 3% BSA-TBST buffer with samples corresponding to: non-phosphorylated Integrin β3 (1), phosphorylated WT Integrin β3 (2), or phosphorylated Y785F mutant Integrin β3 (3). 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™ method.

The data show detection of Integrin β3 [pY^{785}] only with the phosphorylated WT human Integrin β3, not the nonphosphorylated WT nor the phosphorylated mutant Y785F human Integrin β3, demonstrating the specificity of the Integrin β3 [pY^{785}] 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 cat. # 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^oC.
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_2O 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^oC or for one hour at room temperature.
12. Incubate the blocked blot with primary antibody in Tris buffered saline supplemented with 3%Ig-free BSA and 0.1% Tween 20 overnight at 4^oC 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 $Na_4P_2O_7$
2 mM Na_3VO_4
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^oC 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 Ig-freeBSA
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 μg/mL)(1000 mL/L)/(150,000 μg/μmole) = 0.00333 μM.

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 μM) = 1.334 μM.

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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