Rabbit (polyclonal) Anti-Insulin/Insulin-Like Growth Factor-1 Receptor (IR/IGF1R) [pYpY$^{1162/1163}$] Phosphospecific Antibody, Unconjugated

PRODUCT ANALYSIS SHEET

Catalog Number: 44804G (10 mini-blot size)

Lot Number: See product label

Volume: 100 µL

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 reactive with non-phosphorylated insulin/insulin-like growth factor-1 receptor (IR/IGF1R). The final product is generated by affinity chromatography using an IR/IGF1R-derived peptide that is phosphorylated at tyrosines 1162 and 1163 (tyrosines 1135 and 1136 for IGF1R).

Immunogen: The antiserum was produced against a chemically synthesized phosphopeptide derived from the region of IR/IGF1R that contains tyrosines 1162 and 1163 of the human insulin receptor (IR) as numbered according to Ebina, *et al.* (1150 and 1151 according to Ullrich, *et al.*). The corresponding residues in the IGF1R are 1135 and 1136. The sequence is conserved in mouse and rat for both the IR and IGF1R.

Target Summary: Biological actions of insulin and IGF-1 are mediated by their respective cell surface receptor tyrosine kinases that regulate multiple signaling pathways through activation of a series of phosphorylation cascades. The IR and IGF-1R are heterotetrameric proteins consisting of two ligand-binding α subunits and two β subunits that each contain a tyrosine kinase domain. Insulin/IGF-1 binding to the extracellular domain leads to auto-phosphorylation of the receptor and activation of the intrinsic tyrosine kinase activity, leading to tyrosine phosphorylation of downstream target proteins. These two receptors differ in sequence in regions that confer specificity for the designated ligand as well as in certain intracellular signaling domains, resulting in significant differences in the functional consequences of activation of each receptor.

The catalytic loops within the tyrosine kinase domains of the IR/IGF1R contain a three tyrosine motif corresponding to tyrosines 1158, 1162 and 1163 (for the IR) and 1131, 1135 and 1136 (for the IGF1R). It is generally believed that autophosphorylation within the activation loop proceeds in a processive manner initiating at the second tyrosine (1162 or 1135), followed by phosphorylation at the first tyrosine (1158 or 1131), then the last (1163 or 1136), upon which the IR or IGF1R becomes fully active.

Reactivity: Human IR/IGF1R. Mouse and rat (100% homologous) IR/IGF1R have not been tested, but are expected to react. Although exhibiting a preference for IR/IGF-1R, this antibody has been shown by both peptide competition and protein blotting to react with other dual-phosphotyrosine motifs from proteins such as c-Met and Shc.

Applications: The antibody has been used in Western blotting. Previous lots of this antibody have been used in immunostaining. Other applications may work but have not been tested.

Suggested Working Dilutions: For Western blotting applications, we recommend using the antibody at a 1:1000 starting dilution. The exact concentration is not determined for each lot, however the typical range is 0.1-1.0 mg/mL. The optimal antibody concentration should be determined empirically for each specific application.

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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 shipment or short-term storage (up to two weeks), 2-8°C is sufficient.

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

Positive Control Used: Chinese hampster ovary cells transfected with a vector containing human insulin receptor (CHO-T) and stimulated with insulin, or 3T3-L1 cells stimulated with insulin.

Related Products:

Antibodies:

IR [pY^{972}], Cat. # 44800G
IR/IGF1R [pY^{1158}], Cat. # 44802G
IR/IGF1R [pYpYpY$^{1158/1162/1163}$], Cat. # 44806G
IRS-1 [pS^{312}], Cat. # 44814G
IRS-1 [pY^{612}], Cat. # 44816G
IRS-1 [pS^{616}], Cat. # 44550G
IRS-1 [pY^{896}], Cat. # 44818G
IRS-1 [pY^{941}], Cat. # 44820G
IRS-1 [pY^{1179}], Cat. # 44822G
IRS-1 [pY^{1229}], Cat. # 44824G
IRS-2 [pS^{731}], Cat. # 44828

ELISAs: IR/IGF1R [pY^{1158}], KHR9121
IR/IGF1R [pYpY$^{1162/1163}$], Cat. # KHR9131

Luminex: IRS-1 [pS^{312}], Cat. # LHO0521
IR/IGF1R [pYpY$^{1162/1163}$], Cat. # LHR9131

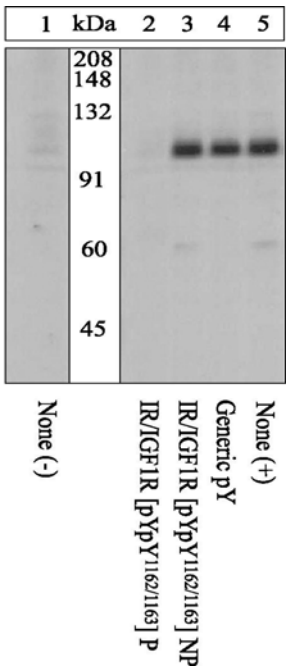
Extracts: CHO-T cell extracts +/- insulin, Cat. # 55150A

References:

Galic, S., et al. (2003) Regulation of insulin receptor signaling by the protein tyrosine phosphatase TCPTP. Mol. Cell. Biol. 23(6):2096-2108 (cites the use of cat. # 44804G).

Wick, K.R., et al. (2003) Grb10 inhibits insulin-stimulated insulin receptor substrate (IRS)-phosphatidylinositol 3-kinase/Akt signaling pathway by disrupting the association of IRS-1/IRS-2 with the insulin receptor. J. Biol. Chem. 278(10):8460-8467 (cites the use of cat. # 44800G, 44802G and 44804G).

Pender, C., et al. (2002) Regulation of insulin receptor function by a small molecule insulin receptor activator. J. Biol. Chem. 277(46):43565-43571 (cites the use of cat. # 44802G and 44804G).



Upregulation and Antibody-Peptide Competition

Extracts of CHO-T cells over-expressing the human insulin receptor unstimulated (1) or stimulated with 100 nM insulin for 10 min at 37°C (2-5) 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 and incubated with the IR/IGF1R [pYpY$^{1162/1163}$] antibody for two hours at room temperature in a 3% BSA-TBST buffer, following prior incubation with: no peptide (1, 5), the phosphopeptide immunogen (2), the non-phosphopeptide corresponding to the phosphopeptide immunogen (3), or a generic phosphotyrosine-containing peptide (4). After washing, the membrane was incubated with goat F(ab')$_2$ anti-rabbit IgG HRP-conjugate (Cat. # ALI4404) and signals were detected using the Pierce SuperSignal™ method.

The data show that only the phosphopeptide corresponding to IR/IGF1R [pYpY$^{1162/1163}$] completely blocks the antibody signal, demonstrating the specificity of the antibody. The data also show the up-regulation of this site upon stimulation with insulin in this cell system.

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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°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_2O for 5 minutes. Alternatively, nitrocellulose may be used.
7. Soak the 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 3% Ig-free BSA 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 (Cat. # ALI4405) or goat $F(ab')_2$ anti-rabbit IgG horseradish peroxidase conjugate (Cat. # 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 Cat. # 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 Ig-free BSA
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-antigen interaction.

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 at 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 µL 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 µL nanopure water
   - tube 2: 2 mL diluted PSSA solution plus 10 µL phosphopeptide
   - tube 3: 2 mL diluted PSSA solution plus 10 µL non-phosphopeptide
9. Incubate the three tubes for 30 minutes at room temperature with gentle rocking.
10. At the end of the incubation step, transfer the contents of each of the three tubes to three clean reaction vessels. Into each reaction vessel, transfer an identical test sample.

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 signal 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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