Rabbit (polyclonal) Anti-Insulin/Insulin-Like Growth Factor-1 Receptor (IR/IGF1R) [pYpYpY$^{1158/1162/1163}$] Phosphospecific Antibody, Unconjugated

PRODUCT ANALYSIS SHEET

Catalog Number: 44806G (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 that is 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 1158, 1162 and 1163 (1131, 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 1158, 1162 and 1163 of the human insulin receptor (IR) as numbered according to Ebina, *et al.* (1146, 1150 and 1151 according to Ullrich, *et al.*). The corresponding residues in the IGF1R are 1131, 1135 and 1136. The sequence is conserved in mouse and rat for both the IR and IGF1R.

Target Summary: Biological actions of insulin and IGF1 are mediated by their respective cell surface receptors, both of which are receptor tyrosine kinases that regulate multiple signaling pathways through activation of a series of phosphorylation cascades. The IR and IGF1R (~95 kDa) are heterotetrameric proteins consisting of two ligand-binding α subunits and two β subunits that each contain a tyrosine kinase domain. Insulin/IGF1 binding to the extracellular domain leads to autophosphorylation of the receptor and activation of the intrinsic tyrosine kinase activity, which allows appropriate substrates to be phosphorylated. These two receptors differ in sequence in regions that confer specificity for the designated ligand as well as in certain intracellular signaling domains. These differences allow insulin and IGF-1 to regulate different physiological functions through receptors that share a very similar structure. Phosphorylation sites that are unique to each receptor presumably play a key role in these signaling differences.

The catalytic loops within the tyrosine kinase domains of the IR/IGF1R contain a three tyrosine motif. It is generally believed that autophosphorylation within the activation loop proceeds in a processive manner initiating at the second tyrosine (1162 for the IR or 1135 for the IGF1R), 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 and mouse IR/IGF1R. Rat (100% homologous) IR/IGF1R has 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 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.

Storage: Store at –20°C. Briefly centrifuge before opening to settle vial contents. Then, apportion into working aliquots and store at –20°C. For shipment or short-term storage (up to one week), 2-8°C is sufficient.

This product is for research use only. Not for use in diagnostic procedures.

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

Positive Controls Used: Chinese Hamster Ovary cells transfected with a vector encoding the human insulin receptor (CHO-T) and stimulated with insulin, or 3T3-L1 adipocytes +/- insulin.

Related Products:

Antibodies:

IR/IGF1R [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

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

References:

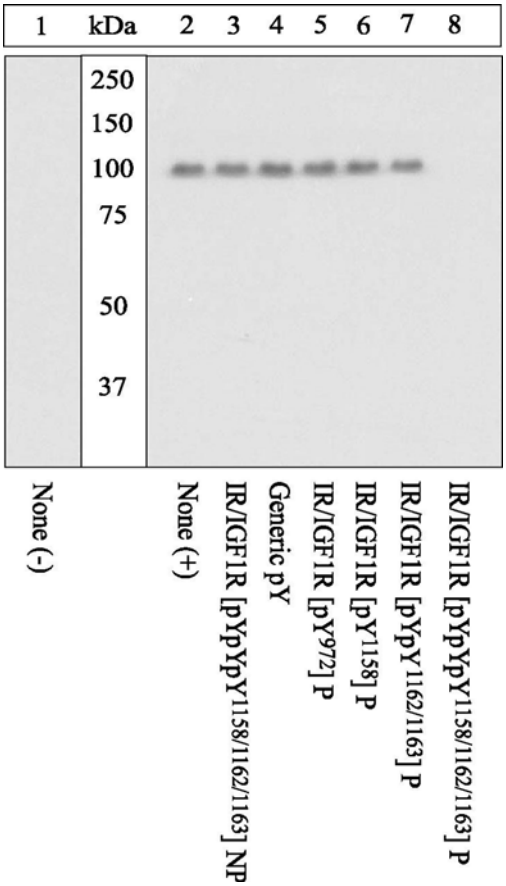
Wick, K.R., et al. (2003) Grb10 inhibits insulin-stimulated insulin receptor substrate (IRS)-phosphatidylinositol 3-kinase/Akt signaling pathway by disrupting the association of IRS-1/IRS-2 with the insulin receptor. J. Biol. Chem. 278(10):8460-8467 (cites the use of cat. # 44800G, 44802G and 44804G).

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).

Bevan, P. (2001) Insulin signalling. J. Cell. Sci. 114(Pt 8):1429-1430.

Skorey, K.I., et al. (2001) Development of a robust scintillation proximity assay for protein tyrosine phosphatase 1b using the catalytically inactive (c215s) mutant. Anal. Biochem. 291(2):269-278.

Playford, M.P., et al. (2000) Insulin-like growth factor 1 regulates the location, stability, and transcriptional activity of beta-catenin. Proc. Nat'l. Acad. Sci. USA 97(22):12103-12108.



Upregulation and Antibody-Peptide Competition

Extracts of CHO-T cells unstimulated (1) or stimulated with 100 nM insulin for 10 minutes (2-8) 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 [pYpYpY$^{1158/1162/1163}$] antibody for two hours at room temperature in a 3% BSA-TBST buffer, following prior incubation with: no peptide (1, 2), the non-phosphopeptide corresponding to the phosphopeptide immunogen (3), a generic phosphotyrosine-containing peptide (4), phosphopeptides corresponding to other IR/IGF1R sites (5-7) or the phosphopeptide immunogen (8). 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 [pYpYpY$^{1158/1162/1163}$] blocks the antibody signal, demonstrating the specificity of the antibody. The data also show the upregulation of IR/IGF1R [pYpYpY$^{1158/1162/1163}$] phosphorylation by 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 probes 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 at a dilution of 1:1000 and the peptides are used at a concentration of 167 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 667 nM 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. 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 of peptide solutions listed in Step 8.

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