Rabbit (polyclonal) Anti-Glycogen Synthase (Muscle/Liver) [pSpS$^{641/645}$] Phosphospecific Antibody, Unconjugated

PRODUCT ANALYSIS SHEET

Catalog Number:	44-1092G (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 Glycogen Synthase. The final product is generated by affinity chromatography using a Glycogen Synthase-derived peptide that is phosphorylated at serine 641 and serine 645.
Immunogen:	The antiserum was produced against a chemically synthesized phosphopeptide derived from the region of human Glycogen Synthase that contains serine 641 and serine 645. The sequence is conserved in human, mouse and rat.
Target Summary:	Glycogen Synthase (GS), is an 85 kDa protein and a key enzyme in regulating glycogen synthesis by catalyzing the rate-limiting step of UDP-glucose incorporation into glycogen. The activity of glycogen synthase is regulated by hormonal stimuli (insulin, catecholamines and glucagons) and non-hormonal stimuli (blood glucose level and exercise). Muscle glycogen synthase is phosphorylated on at least nine sites by multiple kinases including glycogen synthase kinase-3 (GSK-3), mitogen-activated protein kinase-related protein kinase (DYRK), and SAPK2b/p38b which leads to its inactivation. GSK-3 phosphorylates glycogen synthase at four serine sites in the COOH terminus including serines 641 and 645. Serine 641 is also phosphorylated by DYRK, while serine 645 is phosphorylated efficiently by SAPK2b/p38b. SAPK2b/p38b is believed to represent a priming kinase that allows GSK-3 to phosphorylate glycogen synthase at serine 641 and therefore inhibits its activity.
Cross-Reactivity:	Human and mouse Glycogen Synthase. Rat (100% homologous) has not been tested, but is expected to react.
Applications:	The antibody has been used in Western blotting. Previous lots of this antibody have been used in ELISA. Other applications have not been tested at Invitrogen.
Suggested Working Dilutions:	For Western blotting applications, we recommend using the antibody at a 1:1,000 starting dilution. The optimal antibody concentration should be determined empirically 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 shipment or 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.

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

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Positive Control Used: Human HepG2 lysates; mouse 3T3-L1 adipocytes lysates

Related Products:

Antibodies:

GSK-3α/β, monoclonal, Cat. # 44-610
GSK-3β [pS^{9}], Cat. # 44-600G
GSK-3β [pY^{216}]/GSK-3α [pY^{279}], Cat. # 44-604G
IR/IGF-1R [pYpYpY$^{1158/1162/1163}$], Cat. # 44-806G
IRS-1 [pS^{616}], Cat. # 44-550G
IRS-1 [pS^{312}], Cat. # 44-814G
Akt/PKB, Cat. # 44-609G
Akt/PKB [pT^{308}], Cat. # 44-602G
Akt/PKB [pS^{473}] monoclonal, Cat. # 44-621G
PTEN [pSpTpS$^{380/382/385}$], Cat. # 44-1066G
ERK1&2 [pTpY$^{185/187}$], Cat. # 44-680G
JNK1&2 [pTpY$^{183/185}$], Cat. # 44-682G
p38 [pTpY$^{180/182}$], Cat. # 44-684G

References:

Takadera, T. and T. Ohyashiki (2004) Glycogen synthase kinase-3 inhibitors prevent caspase-dependent apoptosis induced by ethanol in cultured rat cortical neurons. Eur. J. Pharmacol. 499(3):239-245.

Nielson, J.N. and J.F. Wojtaszewski (2004) Regulation of glycogen synthase activity and phosphorylation by exercise. Proc. Nutr. Soc. 63(2):233-237.

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Grenn, E.R. et al. (2004) Protein-targeting-to-glycogen promotes glycogen storage in hepatocytes by inactivation of phosphorylase and enzyme translocation. J. Biol. Chem. [Epub ahead of print] .

Ugi, S. (2004) Protein Phosphatase 2A negatively regulates insulin's metabolic signaling pathway by inhibiting Akt (Protein Kinase B) activity in 3T3-L1 adipocytes. Mol. Cell. Biol. 24(19):8778-8789.

Green, A.R., et al. (2004) Protein-targeting-to-glycogen promotes glycogen storage in hepatocytes by inactivation of phosphorylase and enzyme translocation. J. Biol. Chem. [Epub ahead of print].

Kuma, Y., et al. (2004) Identification of glycogen synthase as a new substrate for stress-activated protein kinase 2b/p38beta. Biochem. J. 379(Pt 1):133-139.

Skurat, A.V. and A.D. Dietrich (2004) Phosphorylation of Ser640 in muscle glycogen synthase by DYRK family protein kinases. J. Biol. Chem. 279(4):2490-2498.

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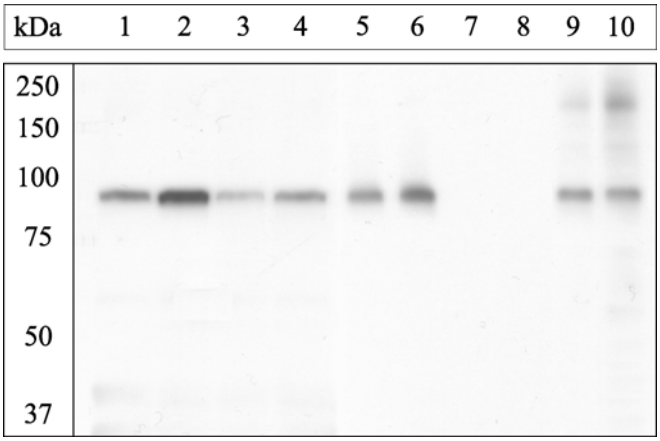

Peptide Competition and Phosphatase Treatment

Extracts of 3T3-L1 cells untreated (1), treated with 100 nM Wortmanin for 90 minutes (2), stimulated with 100 nM Wortmanin for 90 minutes plus 100 nM insulin for 60 minutes (3), stimulated with 100 nM insulin for 20 minutes (4), or stimulated with 100 nM insulin alone for 10 minutes (5-10), were resolved by SDS-PAGE on an 8% Tris-glycine gel and transferred to PVDF. The membrane was left untreated (1-7, 9, 10) or treated with lambda (λ) phosphatase (8), blocked with a 5% BSA-TBST buffer for one hour at room temperature, then incubated with the glycogen synthase [pSpS$^{641/645}$] antibody for two hours at room temperature in 3% BSA-TBST buffer, following prior incubation with: no peptide (1-4), the non-phosphopeptide corresponding to the phosphopeptide immunogen (5), a generic phosphoserine-containing peptide (6), the phosphopeptide immunogen (7) or the phosphopeptides corresponding to glycogen synthase sites [pSpS$^{8/11}$] (9) or [pSpS$^{649/653}$] (10). 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 Wortmanin activates the phosphorylated antibody signal (by inhibiting Akt and thus blocking inhibition of GSK3), while insulin inhibits the signal (by activating Akt and thus inhibiting GSK3). The data also show that only the phosphopeptide corresponding to glycogen synthase [pSpS$^{641/645}$] blocks the signal and that phosphatase stripping eliminates the signal, verifying that the antibody is indeed phosphorylation site-specific.

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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 (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 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 1:1,000 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.

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 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. 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 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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