Version 02-05/25

User's Manual

GSK3beta (Phospho-Ser9) and total GSK3beta ELISA Kit

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This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

GSK3beta (Phospho-Ser9) and total GSK3beta ELISA Kit is a very rapid, convenient and sensitive assay kit that can monitor the activation or function of important biological pathways in Human, Mouse and Rat cell lysates. By determining phosphorylated GSK-3 beta protein in your experimental model system, you can verify pathway activation in your cell lysates.

General Description

Glycogen synthase kinase 3β (GSK-3β) is a unique serine/threonine kinase that is inactivated by phosphorylation. In response to insulin binding, PKB/Akt phosphorylates GSK-3β on serine, which prevents GSK-3β from phosphorylating glycogen synthase. Unphosphorylated glycogen synthase is active and able to synthesize glycogen. GSK-3β is also unique in that it requires a substrate that has been phosphorylated by a distinct kinase before it can phosphorylate the substrate. This phosphate priming mechanism explains why phosphorylation of serine 9 inactivates GSK-3β. The phosphorylated serine binds to the GSK-3β priming phosphate position and prevents binding of alternative substrates 3. In addition to insulin signaling, GSK-3β participates in the Wnt signaling pathway, where it forms a complex with axin, β-catenin and adenomatous polyposis coli (APC) protein. In the presence of Wnts, GSK-3β is unable to phosphorylate β-catenin, which leads to stabilization of β-catenin. The Wnt pathway inactivates GSK-3β via the proteins, Dishevelled and FRAT, which disrupt the interaction of GSK-3β with axin, β-catenin, and APC. Clinically, there is considerable interest in GSK-3β inhibitors because they may mimic the effect of insulin or reduce the hyperphosphorylation of Tau that is observed in Alzheimer's Disease.

Principles of Testing

This Sandwich ELISA kit is an in vitro enzyme-linked immunosorbent assay for the measurement of Human, Mouse and Rat phospho-GSK-3 beta and total GSK-3 beta. An anti-pan GSK-3 beta antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and GSK-3 beta present in a sample is bound to the wells by the immobilized antibody and the wells are washed. In select wells, rabbit anti-phospho-GSK-3 beta (Ser9) antibody is added to detect phosphorylated GSK-3 beta. In the remaining wells, rabbit anti-pan-GSK-3 beta antibody is used to detect pan GSK-3 beta. After washing away unbound antibody, HRP-conjugated anti-rabbit IgG is pipetted into the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of GSK-3 beta (Ser9) or pan GSK-3 beta bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Reagents And Materials Provided

1. Pan GSK-3 beta Microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-pan-GSK-3 beta.
2. Wash Buffer Concentrate (20X) (Item B): 25 mL of 20X concentrated solution.
3. Positive Control-3T3S001-1 (Item K): 1 vial of lyophilized powder from NIH 3T3 cell lysate.
4. Phospho Detection Antibody GSK-3 beta (Ser9) (Item C-1): 1 vial of rabbit anti-phospho-GSK-3 beta (Ser9)

(1 vial is enough to assay half of the microplate).

5. Pan Detection Antibody GSK-3 beta (Item C-2): 1 vial of rabbit anti-pan-GSK-3 beta (1 vial is enough to assay half microplate).
6. HRP-conjugated anti-rabbit IgG concentrate (Item D-1): 1 vial (25 µL) of 1000X concentrated HRP conjugated anti-rabbit IgG.
7. TMB One-Step Substrate Reagent (Item H): 12 mL of 3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution.
8. Stop Solution (Item I): 8 mL of 0.2 M sulfuric acid.
9. Assay Diluent (Item E2): 15 mL of 5X concentrated buffer. For diluting cell lysate samples, detection antibodies (Items C-1 and C-2), and HRP-conjugated anti-rabbit IgG concentrate.
10. Cell Lysate Buffer (Item J): 10 mL 2X cell lysis buffer (does not include protease and phosphatase inhibitors).

Materials Required But Not Supplied

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Protease and Phosphatase inhibitors.
3. Shaker.
4. Precision pipettes to deliver 2 µL to 1 mL volumes.
5. Adjustable 1-25 mL pipettes for reagent preparation.
6. 100 mL and 1 liter graduated cylinders.
7. Absorbent paper.
8. Distilled or deionized water.
9. Log-log graph paper or computer and software for ELISA data analysis.
10. Tubes to prepare the positive control or sample dilutions.

Storage

The entire kit may be stored at -20°C for up to 6 months from the date of shipment. Avoid repeated freeze-thaw cycles. For extended storage, it is recommended to store at -80°C.

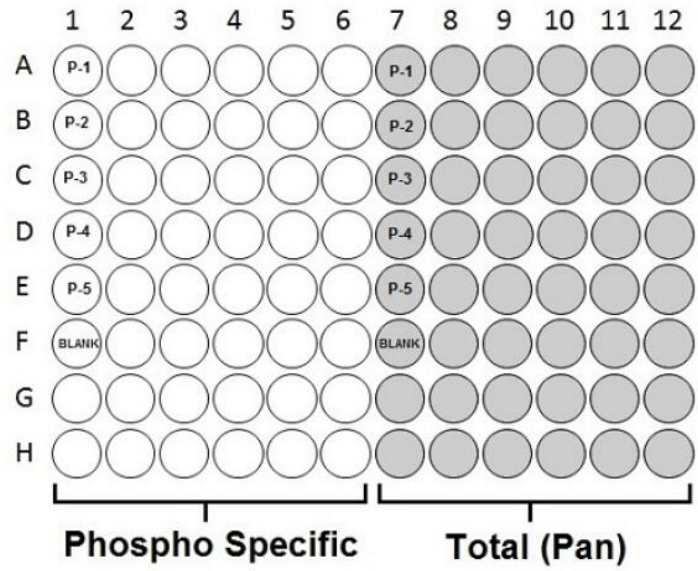
Specimen Collection And Preparation

Cell Lysate Preparation: Rinse the cells with PBS, making sure to remove any remaining PBS before adding the lysis buffer. Solubilize cells at 4 x 10^7 cells/mL in prepared Cell Lysate Buffer (Item J) (see Reagent Preparation step 3). Pipette up and down to resuspend the pellet. Incubate the lysates with shaking at 2-8°C for 30 minutes. Microcentrifuge at 13,000 rpm for 10 minutes at 2-8°C and transfer the supernatantes into a clean test tube. Lysates should be used immediately or aliquoted and stored at -70°C. Avoid repeated freeze-thaw cycles. Thawed lysates should be kept on ice prior to use.

For the initial experiment, we recommend a serial dilution, such as a 5-fold to 50-fold dilution, for your cell lysates with prepared Assay Diluent (Item E2) (see Reagent Preparation step 2) before use.

Note: The fold dilution of sample used depends on the abundance of phosphorylated proteins and should be

determined empirically. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.



Reagent Preparation

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. 5X Assay Diluent (Item E2) should be diluted 5-fold with deionized or distilled water before use.
3. Cell lysate buffer (Item J) should be diluted 2-fold with deionized or distilled water (for cell lysate and tissue lysate). We also recommend the addition of protease and phosphatase inhibitors (not included) to the lysis buffer prior to use.
4. Preparation of Positive Control: Briefly spin the Positive Control Vial (Item K). Add 450 µL of prepared 1X Assay Diluent (Item E2) into Item K to prepare a Positive Control (P-1) solution. Gently mix the powder to allow it to dissolve thoroughly. If a precipitate is seen in the solution after mixing, this can be removed by a quick centrifuge of the positive control vial, and then pipetting the supernate only for the assay. Pipette 300 µL 1X Assay Diluent into each tube. Use the Positive Control (P-1) solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. 1X Assay Diluent serves as the blank (P-0).
5. If the Wash Concentrate (20X) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to yield 400 mL of 1X Wash Buffer.
6. Preparation of Detection Antibodies:

a. Preparation of rabbit anti-phospho-GSK-3 beta (Ser9) antibody: Briefly spin the vial of rabbit anti-phospho-GSK-3 beta (Ser9) (Item C-1). Add 100 µL of 1X Assay Diluent into the vial to prepare a phospho detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days or at -80°C for one month). The concentrate should then be diluted 55-fold with 1X Assay Diluent and used in step 4 of the Assay Procedure.

b. Preparation of rabbit anti-pan-GSK-3 beta antibody: Briefly spin the vial of rabbit anti-pan-GSK-3 beta (Item

C-2). Add 100 µL of 1X Assay Diluent into the vial to prepare a pan detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days or at -80°C for one month). The concentrate should then be diluted 55-fold with 1X Assay Diluent and used in step 4 of the Assay Procedure.

7. Preparation of HRP-conjugated anti-rabbit IgG: Briefly spin the vial of HRP conjugated anti-rabbit IgG concentrate (Item D-1) before use. HRP-conjugated anti-rabbit IgG should be diluted 1000-fold with 1X Assay Diluent and used in step 7 of the Assay Procedure.

For example: Briefly spin the vial. Add 5 µL of HRP-conjugated anti-rabbit IgG concentrate into a tube with 5.0 mL 1x Assay Diluent, pipette up and down to mix gently to prepare a 1000-fold diluted HRP-conjugated anti-rabbit IgG solution. Mix well.

Assay Procedure

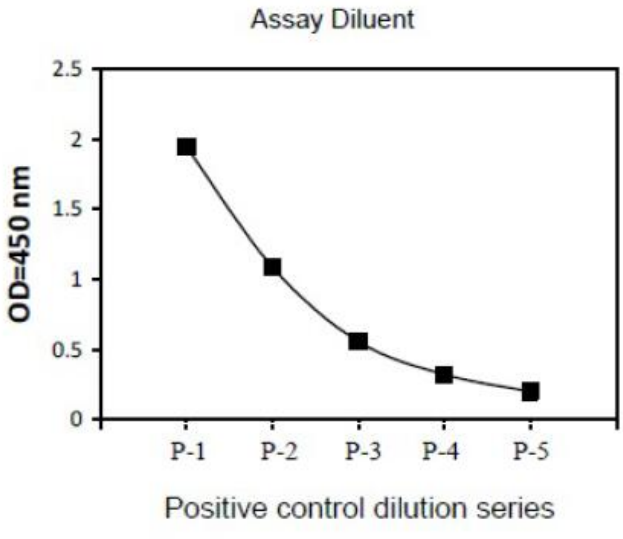
1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is strongly recommended to run all positive controls and samples in at least duplicate. It is also recommended to run the positive controls in singlet for each of the pan and phospho-specific antibodies.
2. See plate layout and label removable 8-well strips as appropriate for your experiment.
3. Add 100 µL of positive control or sample into appropriate wells. Cover the wells and incubate for 2.5 hours at room temperature or overnight at 4°C with gentle shaking.
4. Discard the solution and wash 4 times with 1X Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel Pipette or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 100 µL of prepared 1X rabbit anti-phospho-GSK-3 beta (Ser9) antibody (see Reagent Preparation step 6a) into the wells designated to detect phosphorylated protein. Add 100 µL of prepared 1X rabbit anti-pan-GSK-3 beta antibody (see Reagent Preparation step 6b) to the remaining wells to detect pan protein. Incubate for 1 hour at room temperature with gentle shaking.
6. Discard the solution. Repeat the wash as in step 4.
7. Add 100 µL of prepared HRP-conjugated anti-rabbit IgG solution (see Reagent Preparation step 7) to each well. Incubate for 1 hour at room temperature with gentle shaking.
8. Discard the solution. Repeat the wash as in step 4.
9. Add 100 µL of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
10. Add 50 µL of Stop Solution (Item I) to each well. Read at 450 nm immediately.

Typical Standard Curve

Calculate the mean absorbance for each sample. Then, subtract the average zero (blank) optical density from each sample mean and set of singlet positive controls.

A. Positive Control

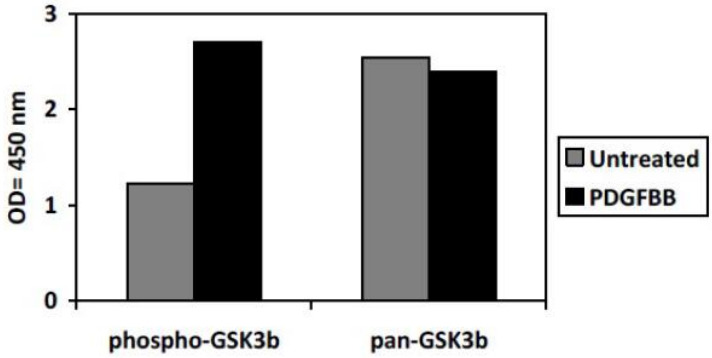
NIH 3T3 cells were treated with recombinant human PDGFBB at 37°C for 10 min. Cells were solubilzed at 4 x 10^7 cells/mL in Cell Lysate Buffer. Serial dilutions of lysates were analyzed in this ELISA (see Reagent Preparation step 4).



B. Recombinant Human PDGFBB Stimulation of NIH 3T3 Cell Lines

NIH 3T3 cells were untreated or treated with 50 ng/mL recombinant human PDGF BB for 10 min. Cell lysates were analyzed using this phosphoELISA and Western Blot.

(1) ELISA



(2) Western-Blot Analysis