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ProductInformation

GSK-3β Activity Assay Kit

Catalog Number **CS0990**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Synonym: Glycogen Synthase Kinase-3β Activity Assay Kit

Product Description

Glycogen synthase kinase-3 (GSK-3) is a multifunctional serine/threonine kinase found in all eukaryotes. This enzyme is a key regulator of numerous signaling pathways and is involved in a wide range of cellular processes, ranging from glycogen metabolism to cell cycle regulation and proliferation. GSK-3 is normally active in cells and is primarily regulated through inhibition of its activity.[1]

There are two mammalian GSK-3 isoforms, encoded by distinct genes, GSK-3α and GSK-3β with molecular masses of 51 kDa and 47 kDa, respectively. The difference in mass is due to a glycine-rich extension at the N-terminus of GSK-3α. Although highly homologous within their kinase domains (98% identity), the two isoforms share only 36% identity in the C-terminal residues.[1,2] Despite the similarity in their biochemical activity the two isoforms exhibit differences in relative activity toward substrates, other than glycogen synthase, and also in substrate specificity.[3,4] Moreover, the two enzymes may participate in different signaling pathways.[5]

In recent years GSK-3 is emerging as a prominent drug target.[6] In Alzheimer's disease, an abnormal increase in GSK-3 levels and activity has been associated with neuronal death, paired helical filament Tau formation, and neurite retraction, as well as a decline in cognitive performance. Abnormal activity of GSK-3 is also implicated in stroke patients.[2] Several GSK-3 inhibitors show effectiveness in normalizing blood glucose levels in animal models of type 2 diabetes. These findings have prompted efforts to develop GSK-3 inhibitors as drugs.

The GSK-3β Activity Assay Kit offers an easy, convenient, and sensitive assay of GSK-3β activity. It provides a means to examine the biological role of the GSK-3β signaling cascade and to explore new GSK-3β signaling stimuli, inhibitors, and activators. The assay is based on immunoprecipitation of GSK-3β using a specific anti-GSK-3β antibody. The immunoprecipitated kinase is incubated with γ–^{32}P-ATP and the incorporation of ^{32}P into the substrate is measured.

This kit was tested with cell extracts from different cell lines, HEK-293, MCF7, CHO, and CT26, as well as with the purified enzyme (Catalog Number G1663).

Components

The kit is sufficient for 50 assays.

Monoclonal Anti-Glycogen Synthase Kinase-3β (GSK-3β) Catalog Number G6414	100 µl
Assay Buffer Catalog Number A6230	1 ml
Wash Buffer, 8× Catalog Number W1142	16 ml
EZview™ Red Protein G Affinity Gel Catalog Number E3403	1 ml
GSK-3 Inhibitor Solution (SB 415286) Catalog Number G8670	100 µl
GSK-3β Peptide Substrate Catalog Number G8545	500 µg
P81 Cellulose Phosphate Squares Catalog Number P5497	10 each

Reagents and Equipment Required but Not Provided

- DMSO (Catalog Number D8418 or equivalent)
- CelLytic™ M Cell Lysis Reagent (for cell extraction, Catalog Number C2978)
- Microcentrifuge, e.g., Eppendorf® microcentrifuge 5415 Series (Catalog Numbers Z365998 and Z366005) or equivalent.
- Phosphoric acid 85% (Catalog Number 79617)
- Ethanol (Catalog Number 270741)
- Acetone (Catalog Number 179124)
- γ-^{32}P-ATP, ~3,000 Ci/mmole, 10 mCi/ml
- Microcentrifuge tubes (Catalog Numbers T6649 or T9661) or equivalent
- Scintillation vials (Catalog Number Z376825)
- Scintillation counter

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Use ultrapure water (17 MΩ·cm or equivalent) for preparation of reagents and throughout the procedure.

GSK-3β Substrate Solution - Reconstitute the GSK-3β Peptide Substrate (Catalog Number G8545) in 300 µl of ice-cold water, mix well, and keep on ice. For extended storage, freeze the GSK-3β Substrate Solution in working aliquots.

1× Wash Buffer – Before preparing the 1× Wash Buffer, make sure the concentrated solution (Wash Buffer, 8×) is completely dissolved. If required, vortex or warm the solution at 37 °C until homogenous. Dilute the Wash Buffer, 8× (Catalog Number W1142) 8-fold with water. Each reaction requires 2.2 ml of 1× Wash Buffer.

GSK-3 Inhibitor Working Solution - Thaw the GSK-3 Inhibitor Solution (SB 415286, Catalog Number G8670) and dilute an aliquot 10-fold with 1× Wash Buffer. Each inhibition reaction requires 10 µl of GSK-3 Inhibitor Working Solution. Keep the prepared GSK-3 Inhibitor Working Solution on ice. The remaining concentrated GSK-3 Inhibitor Solution should be frozen in working aliquots. **Avoid repeated freeze-thaw cycles.**

10% DMSO Solution - Dilute an aliquot of DMSO (not provided) 10-fold with 1× Wash Buffer. Each reaction, except the inhibition reaction(s), requires 10 µl of 10% DMSO Solution. Keep the 10% DMSO Solution on ice.

0.5% Phosphoric Acid Solution - Add 11.8 ml of ~85% phosphoric acid to 2 liters of water and mix well.

Storage/Stability

The kit is shipped on dry ice and storage at –20 °C is recommended. After initial use store the GSK-3β antibody (Catalog Number G6414), the prepared GSK-3β Substrate Solution, and the GSK-3 Inhibitor Solution (Catalog Number G8670) at –20 °C in working aliquots.

Procedure

GSK-3β is immunoprecipitated from the sample with an anti-GSK-3β antibody and the EZview Red Protein G Affinity Gel. The immunoprecipitated kinase is incubated with the peptide substrate[7] in the presence of γ-^{32}P-ATP. Subsequently, the ^{32}P incorporated into the substrate is measured.

It is highly recommended to perform the assays in duplicate.

For each sample, it is suggested to perform the following negative control assays:

a) Negative Control 1 (no substrate) - Kinase activity control without substrate.

b) Negative Control 2 (no antibody) – Immunoprecipitation control without the immunoprecipitating antibody (or with an irrelevant antibody) in order to account for any activity arising from non-specific protein binding to the protein G affinity gel.

Table 1.
Reaction Scheme for Immunoprecipitation and Kinase Radioassay

	Immunoprecipitation			Kinase Activity			
	EZview Red Protein G Affinity Gel (E3403) 30 µl	**Cell Extract** 300–600 µl (>200 µg)	**GSK-3β antibody (G6414)** 2 µl	**Radioactive Reaction Mixture** 20 µl	**GSK-3 Inhibitor Working Solution** 10 µl	**10% DMSO Solution** 10 µl	**GSK-3β Substrate Solution** 5 µl
Sample	+	+	+	+	–	+	+
Inhibition Reaction	+	+	+	+	+	–	+
Negative Control 1 (no substrate)	+	+	+	+	–	+	–
Negative Control 2 (no antibody)	+	+	w/o antibody or with a different antibody	+	–	+	+

A. GSK-3β Immunoprecipitation

All steps of the Immunoprecipitation procedure should be performed on ice, unless otherwise stated.

1. Equilibration of the EZview Red Protein G Affinity Gel beads:
   a. Carefully mix the gel beads until uniformly suspended.
   b. Aliquot 30 µl of the 50% slurry into a clean 1.5 ml microcentrifuge tube. For dispensing the beads, use a wide orifice pipette tip or cut ~1 mm off a regular tip, to enlarge the opening and allow unrestricted flow of the bead suspension.
   c. Wash/equilibrate the beads with ice cold 1× Wash Buffer. Add 300 µl of 1× Wash Buffer to the tube, vortex, and centrifuge in a microcentrifuge for 30 seconds at ~8,000 x *g*. Carefully remove the supernatant with a micropipette or carefully aspirate the supernatant.
   d. Repeat step 1c. After removing the supernatant, set the washed bead pellet on ice.

   Note: For assaying multiple samples, it is possible to equilibrate the resin as a batch equal to the total volume required, according to the procedure described above. After step 1c add 1× Wash Buffer and dispense the required volume of resin per assay into clean 1.5 ml microcentrifuge tubes, centrifuge for 30 seconds at ~8,000 x *g*, and carefully remove the supernatant.
2. Add 2 µl of anti-GSK-3β antibody to each tube, except for the Negative Control 2 tube (no antibody).
3. Transfer >200 µg of cell lysate protein in a volume of 0.3–0.6 ml to each tube.
4. Incubate with thorough, gentle rocking at 2–8 °C for 3 hours to allow the antibody-antigen complexes to bind to EZview Red Protein G Affinity Gel beads.
5. Centrifuge the tubes in a microcentrifuge for 30 seconds at ~8,000 x *g* at 2–8 °C. Set the tubes on ice.
6. Aspirate the supernatant carefully or remove with a micropipette, and set the tubes containing the bead pellet on ice.
7. Wash the bead pellet by adding 500 µl of ice cold 1× Wash Buffer. Vortex briefly and incubate with thorough and gentle rocking at 2–8 °C for 1 minute.
8. Centrifuge the tubes in a microcentrifuge for 30 seconds at ~8,000 x *g* at 2–8 °C. Aspirate the supernatant carefully or remove with a micropipette, and set the tubes containing the bead pellet on ice.
9. Perform two additional washes by repeating steps 7–8.

B. Addition of Inhibitor

10. Add the GSK-3 Inhibitor Working Solution:
    a. Add 10 µl of the GSK-3 Inhibitor Working Solution to each inhibition reaction tube(s).
    b. As a control, add to the rest of the tubes 10 µl of the 10% DMSO Solution instead of the GSK-3 Inhibitor Working Solution.

11. Briefly centrifuge the tubes for several seconds at ~8,000 x *g* to collect all the liquid to the bottom of the microcentrifuge tube. Suspend the beads by gently tapping on the bottom of the tube. Incubate the samples at room temperature for ~5 minutes to allow proper inhibition. Add the peptide substrate as detailed in the next step.

C. Addition of the peptide substrate

12. Add 5 µl of the GSK-3β Substrate Solution to the relevant tubes [Reaction, Inhibition reaction, and Negative Control 2 (no antibody) tubes].

13. Add 5 µl of water to the Negative Control 1 tube (no substrate).

D. Substrate Phosphorylation and Detection

14. Radioactive Reaction Mixture – Combine the following:
    - 125 µl of Assay Buffer
    - 75 µl of 1× Wash Buffer
    - 2.5 µl of γ-^{32}P-ATP (specific activity of 10 mCi/ml).

    This volume of Radioactive Reaction Mixture is sufficient for 8-10 reactions.

15. Add 20 µl of the Radioactive Reaction Mixture to each tube, from steps 12–13, and mix the tube contents by gentle pipetting.

16. Incubate the tubes for 30 minutes at 37 °C. Mix the samples gently every 5 minutes (possibly by gentle tapping on the bottom of the tube). From this step on, work at room temperature.

17. Briefly centrifuge the tubes for several seconds at ~8,000 x *g*. Spot 25 µl of the upper liquid phase of the assay mixture on a P81 cellulose phosphate square. It is recommended to number the squares using a suitable pencil. Let the moist square air-dry (~1 minute).

18. Soak the cellulose phosphate squares in 0.5% Phosphoric Acid Solution. Wash the cellulose phosphate squares 4 times by soaking in 0.5% Phosphoric Acid Solution. For each wash, agitate gently for 5–6 minutes.

19. Wash once with ethanol for 1 minute.

20. Wash once with acetone for 1 minute.

21. Dry the cellulose phosphate squares at room temperature or under a heat lamp, and count the incorporated radioactivity using Cerenkov mode (i.e., count the emission without scintillation liquid, using tritium channel).

References

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Troubleshooting Guide

Problem	Possible cause	Solution
The signal is very poor or no signal is observed.	The amount of GSK-3β in the sample is very low.	• Increase the sample volume - increasing the sample volume up to 1 ml usually does not affect the interaction between the antibody and the GSK-3β kinase. • Increase the reaction incubation time from 30 minutes up to 90 minutes (Substrate Phosphorylation and Detection, step 16).
	There is no GSK-3β in the sample.	• Prepare a fresh lysate. • Add appropriate protease inhibitors to the sample (Catalog Number P8340) or increase their concentration to prevent degradation of GSK-3β. • Verify that the sample is appropriate. Determine the presence of GSK-3β in the sample by performing an immunoblot of the sample using anti-GSK-3β antibody prior to the performance of the immunoprecipitation.
	Incubation time is inadequate.	• 2 to 4 hour incubation is usually sufficient for the antigen-antibody complex to bind the protein G affinity resin. However, in some cases extended incubation may be needed to increase the signal. Therefore, prolong the incubation time of the anti-GSK-3β antibody with the EZview Red Protein G and cell lysate.
	Interfering substance is present in sample.	• Excessive detergent concentration may interfere with the interaction between the antibody and GSK-3β. • Make sure the extraction buffer is not interfering with the kinase activity. Some extraction buffers that can be used for immunoprecipitation are not suitable for kinase activity assays. **It is highly recommended to use the CelLytic M Cell Lysis Reagent (Catalog Number C2978) for cell extract preparation.**
Background is too high.	Proteins bind non-specifically to protein G, the resin beads, or the microcentrifuge tube.	• Pre-clear the sample once or several times by pre-incubation with EZview Red Protein G Affinity Gel (without the anti-GSK-3β antibody) to remove proteins that may bind non-specifically. • During the final wash (Immunoprecipitation Procedure, step 9), after suspending the resin, transfer the entire sample to a clean microcentrifuge tube before centrifuging the sample.
	Insufficient washes in the immunoprecipitation step	• Increase the number of washes • Prolong the duration of the washes to at least 15 minutes.