Instructions for Use

For the semi-quantitative measurement of GSK-3β (pS9) and Total GSK-3β in Human and mouse cell lysates.

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

1. BACKGROUND

Abcam’s GSK-3β (pS9) and GSK-3β (Total) in vitro SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit (ab205711) is designed for the semi-quantitative measurement of GSK-3β (pS9) and Total GSK-3β protein in Human and mouse cells.

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB substrate is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

Glycogen synthase kinase-3 (GSK3) was initially identified as an enzyme that regulated glycogen synthesis in response to insulin, through its ability to phosphorylate and inactivate glycogen synthase. GSK3, expressed as 2 closely related and similarly regulated isoforms, GSK3A and GSK3B. GSK3B is now known to be involved in a diverse array of signaling cellular processes, including glycogen synthesis, cellular adhesion, and it has been implicated in Alzheimer's disease. GSK3B is an important element of the PI3 kinase/Akt signaling pathway, and its kinase activity is down-regulated by Akt-mediated phosphorylation at Ser9.
2. **ASSAY SUMMARY**

Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all reagents, samples, and standards as instructed.

Add standard or sample to appropriate wells.

Add Antibody Cocktail to all wells. Incubate at room temperature.

Aspirate and wash each well. Add TMB Substrate to each well and incubate. Add Stop Solution at a defined endpoint. Alternatively, record color development kinetically after TMB substrate addition.
3. **PRECAUTIONS**

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. **STORAGE AND STABILITY**

Store kit at 2-8°C immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in sections 9 & 10.

5. **MATERIALS SUPPLIED**

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK-3β (pS9) Capture Antibody</td>
<td>1.5 mL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>GSK-3β (pS9) Detector Antibody</td>
<td>1.5 mL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>GSK-3β (Total) Capture Antibody</td>
<td>1.5 mL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>GSK-3β (Total) Detector Antibody</td>
<td>1.5 mL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Lyophilized GSK-3β Control Lysate</td>
<td>1 Vial</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>10X Wash Buffer PT</td>
<td>15 mL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>5X Cell Extraction Buffer PTR</td>
<td>10 mL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>50X Cell Extraction Enhancer Solution</td>
<td>1 mL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>TMB Substrate</td>
<td>12 mL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>12 mL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>SimpleStep Pre-Coated 96 Well Microplate (12 x 8 well strips)</td>
<td>96 Wells</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Plate Seal</td>
<td>1</td>
<td>+2-8°C</td>
</tr>
</tbody>
</table>
6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- An alternative fluorescent substrate, ADHP, can be used with this assay. Please see ab205810 ADHP HRP Substrate Kit for more information. A microplate reader capable of measuring fluorescence is required if using this product.
- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- PBS (1.4 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$, 140 mM NaCl, 2.7 mM KCl, pH 7.4).
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers (e.g. 1X Cell Extraction Buffer/1X Cell Extraction Enhancer Solution mix).
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical values for commonly used sample types are shown below in Figure 2 (section 16).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- The provided 5X Cell Extraction Buffer contains phosphatase inhibitors. Protease inhibitors can be added if required.
- The provided 50X Cell Extraction Enhancer Solution may precipitate when stored at + 4°C. To dissolve, warm briefly at + 37°C and mix gently. The 50X Cell Extraction Enhancer Solution can be stored at room temperature to avoid precipitation.
- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
9. REAGENT PREPARATION

- Equilibrate all reagents to room temperature (18 - 25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the formulations provided.

9.1 1X Cell Extraction Buffer PTR

Prepare 1X Cell Extraction Buffer PTR by diluting 5X Cell Extraction Buffer PTR and 50X Cell Extraction Enhancer Solution to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 7.8 mL deionized water, 2 mL 5X Cell Extraction Buffer PTR and 200 µL 50X Cell Extraction Enhancer Solution. Mix thoroughly and gently. If required protease inhibitors can be added.

Alternative – Enhancer may be added to Cell Extraction Buffer after extraction of cells or tissue. Refer to note in Section 19.

9.2 5X Cell Extraction/Enhancer Buffer PTR

Prepare 5X Cell Extraction/Enhancer Buffer PTR by adding 1/10th volume of 50X Cell Extraction Enhancer Solution. To prepare 1 mL, add 100 µL of 50X Cell Extraction Enhancer Solution to 900 µL of 5X Cell Extraction Buffer PTR. This concentrated mix is used for lysing cells directly in cell culture medium.

9.3 1X Wash Buffer PT

Prepare 1X Wash Buffer PT by diluting 10X Wash Buffer PT with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL 10X Wash Buffer PT with 45 mL deionized water. Mix thoroughly and gently.
9.4 **Antibody Cocktail**

Prepare Antibody Cocktail by combining an appropriate volume of the capture and detector antibodies immediately prior to assay. To make 3 mL of the Antibody Cocktail combine 1.5 mL Capture Antibody with 1.5 mL Detector Antibody. Mix thoroughly and gently.
10. CONTROL LYSATE PREPARATION

Prepare serially diluted control lysates immediately prior to use. Always prepare a fresh set of positive controls for every use.

The following table describes the preparation of a lysate dilution series for duplicate measurements (recommended).

10.1 Reconstitute the Lyophilized GSK-3β Control Lysate* by adding 250 µL water by pipette. Mix thoroughly and gently. Hold at room temperature for 1 minute. This is the 100% **Stock Lysate** Solution. Remaining stock material should be aliquoted and stored at -80°C.

10.2 Label eight tubes with #1 - 8.

10.3 Add 125 µL 1X Cell Extraction Buffer PTR into tube #2 - 8.

10.4 Prepare **50% Control #1** by adding 125 µL of the 100% Stock Lysate Solution to 125 µL of 1X Cell Extraction Buffer PTR to tube #1. Mix thoroughly and gently.

10.5 Prepare **Control #2** by transferring 125 µL from Control #1 to tube #2. Mix thoroughly and gently.

10.6 Repeat for Tubes #3 through #7.

10.7 **Control #8** contains no protein and is the Blank control.

*Control lysates are supplied as a control reagent - not an absolute quantitation measure. A 3 - 4 point lysate dilution series is sufficient for this purpose.
## ASSAY PREPARATION

<table>
<thead>
<tr>
<th>Control #</th>
<th>Sample to Dilute</th>
<th>Volume to Dilute (µL)</th>
<th>Volume of Diluent (µL)</th>
<th>Starting Conc. (%)</th>
<th>Final Conc. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stock</td>
<td>125</td>
<td>125</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>Control #1</td>
<td>125</td>
<td>125</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>Control #2</td>
<td>125</td>
<td>125</td>
<td>25</td>
<td>12.5</td>
</tr>
<tr>
<td>4</td>
<td>Control #3</td>
<td>125</td>
<td>125</td>
<td>12.5</td>
<td>6.25</td>
</tr>
<tr>
<td>5</td>
<td>Control #4</td>
<td>125</td>
<td>125</td>
<td>6.25</td>
<td>3.13</td>
</tr>
<tr>
<td>6</td>
<td>Control #5</td>
<td>125</td>
<td>125</td>
<td>3.13</td>
<td>1.56</td>
</tr>
<tr>
<td>7</td>
<td>Control #6</td>
<td>125</td>
<td>125</td>
<td>1.56</td>
<td>0.78</td>
</tr>
<tr>
<td>8 (Blank)</td>
<td>none</td>
<td>-</td>
<td>125</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

![Diagram of assay preparation process](image-url)
11. SAMPLE PREPARATION

A cell density that yields 10,000 – 40,000 cells/well is suitable for the analysis of many cell lines. The lysis buffer volume should be adjusted so that lysates are in the range of 100-500 µg/mL of protein.

11.1 Preparation of extracts from adherent cells

11.1.1 Remove growth media and rinse adherent cells 2 times in PBS.

11.1.2 Solubilize the cells by addition of chilled 1X Cell Extraction Buffer PTR directly to the plate (for cells cultured in 96-well microplates, lyse the cells with 100 µL* of 1X Cell Extraction Buffer PTR).

*Lysis volume should be adjusted depending on the desired lysate concentration. Lysates in the range of 100 - 500 µg/mL protein are usually sufficient.

11.1.3 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.1.4 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

11.2 Preparation of extracts from non-adherent cells

11.2.1 Collect non-adherent cells by centrifugation and resuspend at an appropriate density in RPMI containing 10% FBS. Typical centrifugation conditions for cells are 500 x g for 5 minutes at RT.

11.2.2 Return cells to a 37°C incubator for 1 - 2 hours. For certain pathways, this can allow handling-mediated pathway activation to subside. This step is optional, and depends on the activation status of your cells following re-suspension.

11.2.3 At the completion of the cell treatment, harvest cells by centrifugation and lyse with 1X Cell Extraction Buffer PTR.*
11.2.4 Alternatively, in the absence of centrifugation cells may be lysed directly with a 20% volume of 5X Cell Extraction/Enhancer Buffer PTR (e.g. for 80 µL of cells, use 20 µL of 5X Cell Extraction/Enhancer Buffer PTR).

11.2.5 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.2.6 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

*For best results, we recommend centrifugation and lysis of cells with 1X Cell Extraction Buffer PTR (11.2.3). Matrix effects may be observed in different cell media using the direct lysis approach (11.2.4).

11.3 Preparation of extracts from tissue homogenates

11.3.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).

11.3.2 Homogenize 100 to 200 mg of wet tissue in 500 µL - 1 mL of chilled 1X Cell Extraction Buffer PTR. For lower amounts of tissue adjust volumes accordingly.

11.3.3 Incubate on ice for 20 minutes.

11.3.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.3.5 Transfer the supernatants into clean tubes and discard the pellets.

11.3.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.3.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.
12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or “edge effects” have not been observed with this assay.
13. ASSAY PROCEDURE

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

13.1 Prepare all reagents, working standards, and samples as directed in the previous sections.
13.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.
13.3 Add 50 µL of all samples and standards to appropriate wells.
13.4 Add 50 µL of the Antibody Cocktail to each well.
13.5 Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.
13.6 Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.
13.7 Add 100 µL of TMB Substrate to each well and incubate for 15 minutes in the dark on a plate shaker set to 400 rpm.
13.8 Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:
**ASSAY PROCEDURE**

<table>
<thead>
<tr>
<th>Mode</th>
<th>Kinetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>600 nm</td>
</tr>
<tr>
<td>Time</td>
<td>up to 15 minutes</td>
</tr>
<tr>
<td>Interval</td>
<td>20 sec - 1 minute</td>
</tr>
<tr>
<td>Shaking</td>
<td>Shake between readings</td>
</tr>
</tbody>
</table>

*Note that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450nm.*

13.9 Analyze the data as described below.
14. CALCULATIONS

14.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.

14.2 Create a standard curve by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.

Note: Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.

14.3 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted absorbance values against the standard curve. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.

14.4 Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

*Control Lysates are supplied as a control reagent - not an absolute quantitation measure
15. **TYPICAL DATA**

**TYPICAL CELL LYSATE DILUTION SERIES** – Data provided for demonstration purposes only.

**Figure 1.** Example of a typical GSK-3β (pS9) and GSK-3β (Total) cell lysate dilution series. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

<table>
<thead>
<tr>
<th>Conc. (µg/mL)</th>
<th>GSK-3β (pS9)</th>
<th>GSK (Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O.D. 450 nm</td>
<td>Mean O.D.</td>
</tr>
<tr>
<td>0.000</td>
<td>0.055</td>
<td>0.058</td>
</tr>
<tr>
<td>7.813</td>
<td>0.150</td>
<td>0.152</td>
</tr>
<tr>
<td>15.63</td>
<td>0.204</td>
<td>0.216</td>
</tr>
<tr>
<td>31.25</td>
<td>0.342</td>
<td>0.362</td>
</tr>
<tr>
<td>62.50</td>
<td>0.594</td>
<td>0.620</td>
</tr>
<tr>
<td>125.0</td>
<td>1.143</td>
<td>1.176</td>
</tr>
<tr>
<td>250.0</td>
<td>2.104</td>
<td>2.160</td>
</tr>
<tr>
<td>500.0</td>
<td>2.886</td>
<td>2.922</td>
</tr>
</tbody>
</table>
### 16. TYPICAL SAMPLE VALUES

**SENSITIVITY** –

The calculated minimal detectable (MDD) dose of GSK-3β (pS9) and GSK-3β (Total) protein in activated MCF-7 cell extracts is 3 µg/mL and 5 µg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=16) and adding 2 standard deviations then extrapolating the corresponding concentrations. Data for GSK-3β (pS9) is indicative only – the proportion of total protein that is phosphorylated is unknown.

As a guide, GSK-3β (pS9) and GSK-3β (Total) is detectable in insulin treated MCF-7 cell extracts with a total cellular protein concentration < 5 µg/mL (see Figure 1).

**PRECISION** –

Mean coefficient of variations of interpolated values from 3 concentrations of MCF-7 extracts within the working range of the assay.

<table>
<thead>
<tr>
<th>Target</th>
<th>Intra-Assay</th>
<th>Inter-Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Target</td>
<td>n=</td>
</tr>
<tr>
<td>GSK-3β (pS9)</td>
<td>CV (%)</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>n=</td>
<td>6</td>
</tr>
<tr>
<td>GSK-3β (Total)</td>
<td>CV (%)</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>n=</td>
<td>6</td>
</tr>
</tbody>
</table>
**Figure 2.** Inhibition of GSK-3β (pS9) phosphorylation in MCF-7 cells in response to UCN-01 treatment. MCF-7 cells were cultured in 96-well tissue culture plates and treated (2 hours) with a dose-range of UCN-01. Cells were then stimulated (15 minutes) with 2.5 µg/mL insulin and lysed. Data from quadruplicate measurements of GSK-3β (pS9) are plotted and compared against total GSK-3β protein levels.
Figure 3. Cell line analysis for GSK-3β from 100 µg/mL preparations of cell extracts. Data from triplicate measurements (mean +/- SD) are plotted and compared to 1X Cell Extraction Buffer PTR (zero).

17. ASSAY SPECIFICITY

The GSK-3β (pS9) assay detects endogenous levels of GSK-3β (GenBank Accession NP_001139628) in cellular lysates, only when phosphorylated at Ser9.

The GSK-3β (Total) assay detects endogenous levels of GSK-3β (GenBank Accession NP_001139628) in cellular lysates, irrespective of phosphorylation status.

18. SPECIES REACTIVITY

This kit detects GSK-3β (pS9) in Human and mouse cell culture extracts. Detection in rat samples is also expected. Other species should be tested on a case-by-case basis.

Serum and plasma samples have not been tested with this kit. Please contact our Technical Support team for more information.
## 19. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difficulty pipetting lysate; viscous lysate.</td>
<td>Genomic DNA solubilized</td>
<td>Prepare 1X Cell Extraction Buffer PTR (without enhancer). Add enhancer to lysate after extraction.</td>
</tr>
<tr>
<td>Poor standard curve</td>
<td>Inaccurate pipetting</td>
<td>Check pipettes</td>
</tr>
<tr>
<td></td>
<td>Improper standard dilution</td>
<td>Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing</td>
</tr>
<tr>
<td>Low signal</td>
<td>Incubation times too brief</td>
<td>Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation</td>
</tr>
<tr>
<td></td>
<td>Inadequate reagent volumes or improper dilution</td>
<td>Check pipettes and ensure correct preparation</td>
</tr>
<tr>
<td></td>
<td>Incubation times with TMB too brief</td>
<td>Ensure sufficient incubation time until blue color develops prior addition of Stop solution</td>
</tr>
<tr>
<td>Large CV</td>
<td>Plate is insufficiently washed</td>
<td>Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.</td>
</tr>
<tr>
<td></td>
<td>Contaminated wash buffer</td>
<td>Prepare fresh wash buffer</td>
</tr>
<tr>
<td>Low sensitivity</td>
<td>Improper storage of the ELISA kit</td>
<td>Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB substrate solution protected from light.</td>
</tr>
</tbody>
</table>