



ab123454

**GSK3 beta (GSK3B) ELISA
Kit**

Instructions for Use

For the quantitative measurement of Human, Mouse, Rat GSK3 beta protein.

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

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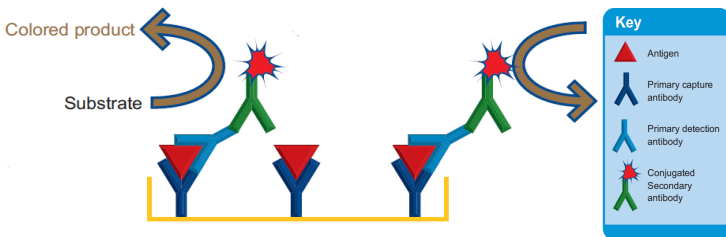
ab123454 GSK3 beta ELISA Kit

1. Table of Contents

1. Introduction	3
2. Assay Summary	6
3. Kit Contents	7
4. Storage and Handling	7
5. Additional Materials Required	8
6. Preparation of Reagents	9
7. Sample Preparation	10
8. Assay Procedure	13
9. Data Analysis	15
10. Specificity	20
11. Troubleshooting	21

2. Introduction

Principle: ab123454 GSK3 beta (GSK3B) Human ELISA (Enzyme-Linked Immunosorbent Assay) kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of GSK3 beta protein in cell lysates. The assay employs an antibody specific for the Human GSK3 beta coated onto well plate strips. Standards and samples are pipetted into the wells and analyte present in the sample is bound to the wells by the immobilized antibody. The wells are washed and an anti-GSK3 beta primary detector antibody is added. After washing away unbound primary detector antibody, HRP-conjugated secondary detector antibody specific for the primary detector antibody is pipetted to 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 analyte bound. The developing blue color is measured at 600 nm. Optionally the reaction can be stopped by adding hydrochloric acid which changes the color from blue to yellow and the intensity can be measured at 450 nm.



Background:

Glycogen synthase kinase 3 is a proline directed serine, threonine kinase originally identified due to its ability to phosphorylate and inactivate glycogen synthase. However, it has also been found of key importance in signaling pathways, cell fate determination, energy metabolism, transcription regulation, neuronal development and body pattern formation. There are two isoforms of GSK-3 (alpha and beta) which show a high degree of homology within their catalytic domains.

Activity of GSK3 beta is regulated by phosphorylation of serine 9 (inactivating), phosphorylation of tyrosine 216 (activating) and by protein complex formation which can activate (Axin, AAPC, B-catenin) or inactivate (Frat 1 and 2) the enzyme. GSK3 beta regulates by phosphorylation the activity of numerous metabolic and signaling proteins such as glycogen synthase, ATP citrate lyase, cyclic-AMP-dependent protein kinase, acetyl CoA carboxylase, cyclin D1, insulin receptor substrate-1, pyruvate dehydrogenase amongst others. Furthermore, it phosphorylates structural cytoskeletal proteins (MAPs and tau), making it a key component of neuronal structure and plasticity. GSK3 beta is also important for the regulation of transcription factors that modulate cell survival such as activator protein-1, cyclic AMP response element binding protein (CREB), Myc and NFkB.

ab123454 GSK3 beta ELISA Kit

Altered GSK3 beta function has been implicated in neuronal dysfunction. Polymorphisms in the GSK3B gene have been shown to be associated with an increased risk for Alzheimer disease and with modifications to the disease risk in Parkinson's disease due to its interaction with microtubule associated protein tau (MAPT) haplotypes. GSK3 beta has been found to be involved in the development of psychiatric disorders after the findings that lithium and valproate, both known to control bipolar disorder, are inhibitors of GSK3 beta. Furthermore the levels of GSK3 beta have been found significantly reduced in post-mortem frontal cortex samples from patients with schizophrenia. GSK3 beta has also been implicated in diabetes, cancer and cardiac hypertrophy. GSK3 beta has become a key target of pharmacological development in Alzheimer's, cancer diabetes, obesity, asthma, immune disorders, hypertension, AIDS and Glaucoma as indicated by filed patents in the last decade.

3. Assay Summary

Bring all reagents to room temperature.

Prepare all the reagents, samples and standards as instructed.



Add 50 μ L standard or sample to each well used.

Incubate 2 hours at room temperature.



Aspirate and wash each well three times.

Add 50 μ L prepared 1X Detector Antibody to each well.

Incubate 1 hour at room temperature.



Aspirate and wash each well three times.

Add 50 μ L prepared 1X HRP Label.

Incubate 1 hour at room temperature.



Aspirate and wash each well four times. Add 50 μ L TMB Development Solution to each well. Immediately begin recording the color development for 15 minutes at 600 nm.

Alternatively add a Stop solution at a user-defined time and read at 450 nm.

4. Kit Contents

Item	Quantity
2X Extraction Buffer	15 mL
20X Buffer	20 mL
10X Blocking Buffer	6 mL
GSK3 beta Microplate (12 x 8 antibody coated well strips)	96 Wells
10X GSK3 beta Detector Antibody	0.7 mL
10X HRP Label	1 mL
TMB Development Solution	6 mL

5. Storage and Handling

Store all components at 4°C. This kit is stable for at least 6 months from receipt. A GSK3 beta standard protein is not included with the kit, but it is available from the Abcam catalogue (ab63193). Unused microplate strips should be returned to the pouch containing the desiccant and resealed.

6. Additional Materials Required

- Microplate reader capable of measuring absorbance at 600 nm (or 450 nm after addition of Stop solution - not supplied).
- Method for determining protein concentration (BCA assay recommended).
- Deionized water
- Multi and single channel pipettes
- PBS (1.4 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 140 mM NaCl, 2.7 mM KCl, pH 7.3)
- Tubes for standard dilution
- Stop solution (optional) – 1N hydrochloric acid
- Optional plate shaker for all incubation steps
- Optional (highly recommended) GSK3 beta protein standard (ab63193) shipped and stored frozen
- Wellplate cover or seals

7. Preparation of Reagents

- 7.1 Equilibrate all reagents to room temperature (18-25°C) before use.
- 7.2 If preparing lysates from cell pellets prepare 1X Extraction Buffer by adding 15 mL 2X Extraction Buffer to 15 mL nanopure water.
- 7.3 Prepare 1X Wash Buffer by adding 20 mL 20X Buffer to 380 mL nanopure water. Mix gently and thoroughly.
- 7.4 Prepare 1X Incubation Buffer by adding 6 mL 10X Blocking Buffer to 54 mL 1X Wash Buffer. Unused 1X Incubation Buffer may be stored at -20°C for 6 months after performing the ELISA.
- 7.5 Prepare the 1X GSK3 beta Detector Antibody by diluting the 10X GSK3 beta Detector Antibody 10-fold with 1X Incubation Buffer immediately prior to use. Prepare 0.5 mL for each 8 well strip used.
- 7.6 Prepare the 1X HRP Label by diluting the stock 10X HRP Label 10-fold with 1X Incubation Buffer immediately before use. Prepare 0.5 mL for each 8 well strip used.

8. Sample Preparation

Note: Extraction buffer can be supplemented with phosphatase inhibitors, PMSF and protease inhibitor cocktail prior to use. Supplements should be used according to manufacturer's instructions. After protein extraction, sample must be kept cold at all times. Non- captured GSK3 beta protein is highly sensitive to degradation at room temperature.

8.1 Preparation of cell lysate

- 8.1.1 Collect non adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. If the adherent cells are detaching, as typical for apoptotic cells, collect both floating cells and remaining adherent cells. Typical centrifugation conditions for cells are 500 x g for 10 min at 4°C.
- 8.1.2 Rinse cells twice with PBS.
- 8.1.3 Solubilize cell pellet at 2×10^7 /mL in 1X Extraction Buffer.
- 8.1.4 Incubate on ice for 20 minutes. Centrifuge at 16,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C

ab123454 GSK3 beta ELISA Kit

for no more than 48 hours. Do not freeze/thaw the sample extract. The sample protein concentration in the extract may be quantified using a protein assay. The extract buffer absorbs at 280nm, therefore the A280 method for protein measurement should not be used.

8.2 Preparation of dilution series of the GSK3 beta standard protein not included in this kit but available as ab63193

Note: It is strongly recommended to prepare a dilution series of a protein standard available from the abcam catalogue (ab63193). The relative levels of GSK3 beta in other experimental samples can be interpolated from within the protein standard.

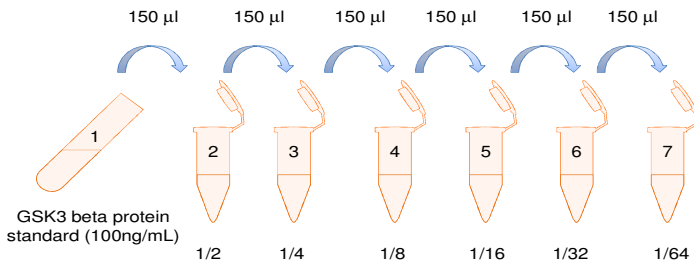
- 8.2.1 To prepare serially diluted positive control sample, label six tubes #1-7.
- 8.2.2 Dilute the protein standard to 5µg/mL with the storage buffer as shown in the protein datasheet (25% glycerol, 150mM Imidazole, 50mM sodium phosphate, 300mM sodium chloride, 0.2mM DTT, 0.1mM PMSF, pH 7), aliquot and store at -80C.
- 8.2.3 Prepare the plate standard by diluting the protein standard in 1X Incubation buffer to 100ng/mL. Label

ab123454 GSK3 beta ELISA Kit

this tube #1. It is recommended to always prepare fresh and keep cold at all times.

8.2.4 Label tubes #2 – 7. Add 150 μ L of 1X incubation buffer to each of #2 through #7. Keep tubes cold at all times.

8.2.5 Transfer 150 μ L from tube #1 to tube #2. Mix thoroughly. With a fresh pipette tip transfer 150 μ L from #2 to #3. Mix thoroughly. Repeat for Tubes #4 through #7. Use 1X incubation buffer as the zero standard tube labeled #8. Use fresh control sample dilutions for each assay.



9. Assay Procedure

Equilibrate all reagents to room temperature before use. Keep samples on ice at all times before loading onto the plate. It is recommended to work with fresh extracts. If working with frozen extracts, load as soon as the extracts are thawed. It is also recommended all samples and controls be assayed in duplicate.

- 9.1 Prepare all reagents, working dilutions of controls and samples as directed in the previous sections.
- 9.2 Remove unused microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and seal.
- 9.3 Add 50 μ L of each sample per well. It is recommended to include a dilution series of the protein standard (section 7.2). Also include a no material control (1X incubation buffer) as a zero standard.
- 9.4 Cover/seal the plate and incubate for 2 hours at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
- 9.5 Aspirate each well and wash, repeat this once more for a total of **three** washes. Wash by aspirating or decanting from

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wells then dispensing 300 μ L 1X Wash Buffer into each well as described above. Complete removal of liquid at each step is essential for good performance. After the last wash, remove the remaining buffer by aspiration or decanting. Invert the plate and blot it against clean paper towels to remove excess liquid.

9.6 Immediately before use prepare sufficient (0.5 mL/strip used) 1X GSK3 beta protein Detector Antibody (step 6.5) in 1X Incubation Buffer. Add 50 μ L 1X Detector antibody to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.

9.7 Repeat the aspirate/wash procedure above.

9.8 Immediately before use prepare sufficient (0.5 mL/strip used) 1X HRP Label in 1X Incubation Buffer (step 6.6). Add 50 μ L 1X HRP Label to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.

9.9 Repeat the aspirate/wash procedure above, however, performing a total of **four** washes.

9.10 Add 50 μ L TMB Development Solution to each empty well and immediately record the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode:	Kinetic
Wavelength:	600 nM
Time:	up to 15 min.
Interval:	20 sec. - 1 min.
Shaking:	Shake between readings

Alternative– In place of a kinetic reading, at a **user defined**, time record the endpoint OD data at (i) 600 nm or (ii) stop the reaction by adding 50 μ L stop solution (1N HCl) to each well and record the OD at 450 nm.

9. Data Analysis

Average the duplicate protein standard readings and plot against their concentrations after subtracting the zero standard reading. Draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Read relative GSK3 beta

ab123454 GSK3 beta ELISA Kit

concentrations for unknown samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

TYPICAL STANDARD CURVE - *For demonstration only.*

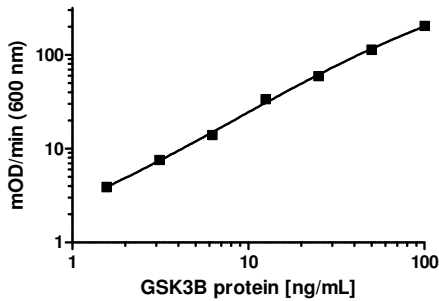


Figure 1. Example standard curve. A dilution series of recombinant GSK3 beta protein (ab63193) in the working range of the assay (0.7 – 100ng/mL)

TYPICAL EXPERIMENTAL RESULTS - For demonstration only.

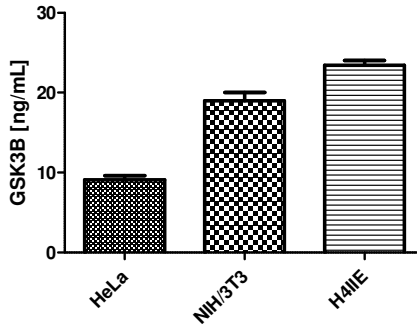


Figure 2. HeLa (Human), NIH-3T3 (Mouse) and H4IIE (Rat) were loaded at 0.5 mg/mL in triplicate and raw intensity was interpolated from the standard curve using GSK3 beta recombinant protein (ab63193)

Typical working ranges for cell extracts	
Sample Type	Range
HeLa cell extract	0.05 – 0.5 mg/mL
H4IIE cell extract	0.2 – 1 mg/mL

SENSITIVITY

Determined minimum detectable dose of GSK3 beta (zero dose n=16 + 2 standard deviations) = 0.7 ng/mL

LINEARITY OF DILUTION

Linearity of dilution determined by comparing dilution series of HeLa cell extract (starting extract concentration is 0.5 mg/mL) and H4IIE cell extract (starting extract concentration is 1 mg/mL).

Sample Type	% Expected Value on HeLa cells	% Expected Value on H4IIE cells
1:1	100	100
1:2	78	72
1:4	63	80
1:8	96	92
1:16	116	165

REPRODUCIBILITY

Parameter	CV%
Intra (n=8)	4.6
Inter (n=3)	15

RECOVERY

If assay is to be performed on serum samples or samples with very low protein concentration, it is recommended to add serum or extraction buffer to the standard protein for accurate results.

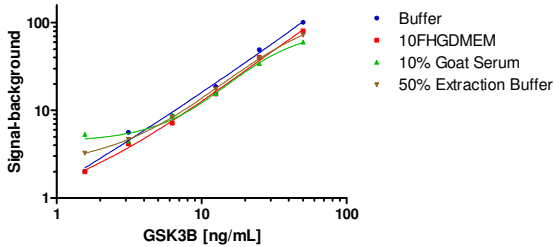


Figure 3. Sample recovery under 3 different conditions compared with the wash buffer from the kit. 50% extraction buffer and 10% goat serum decrease the dynamic range of the assay

10. Specificity

Species– Human, mouse and rat reactive. Others untested.

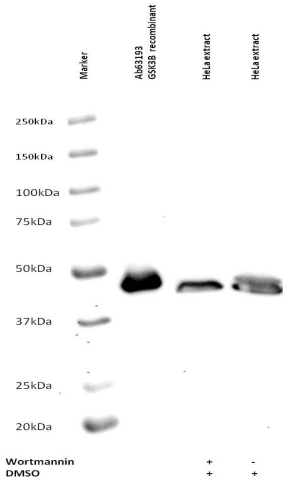


Figure 4. Demonstration of assay specificity. Western blot using the GSK3 beta detector antibody (ab32391).

HeLa cells treated with DMSO show a doublet band due to endogenous phosphorylation of the protein in this cell line. Wortmannin, a PI3-K inhibitor, dephosphorylates AKT and in-turn dephosphorylates GSK3 beta.

Dephosphorylation removes the doublet band observed with vehicle treatment only.

11. Troubleshooting

Problem	Cause	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Low GSK3 beta concentration in sample	Use appropriate positive control
	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Sample degraded	Work with freshly extracted samples. Keep samples cold before loading on the plate.
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Make fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80 °C, all other assay components 4 °C. Keep substrate solution protected from light

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