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# GSK-3β EIA kit

## Catalog No. ADI-900-144

## 96 Well Kit

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## Description

The GSK-3β EIA kit is a complete kit for the quantitative determination of pan or total GSK-3β in cell lysates. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to GSK-3β immobilized on a microtiter plate to bind the GSK-3β in the standards or sample. A recombinant GSK-3β Standard is provided in the kit. After a short incubation, the excess sample or standard is washed out and a rabbit polyclonal antibody to GSK-3β is added. This antibody binds to GSK-3β captured on the plate. The polyclonal antibody detects a non-phosphorylated region of GSK-3β and therefore detects GSK-3β regardless of phosphorylation status. After a short incubation, the excess antibody is washed out and goat anti-rabbit IgG conjugated to horseradish peroxidase is added, which binds to the polyclonal GSK-3β antibody. Excess conjugate is washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of GSK-3β in either standards or samples. To measure phospho GSK-3β, please refer to the [pSer$^{9}$]GSK-3β EIA kit, Catalog No. ADI-900-123A. For further explanation of the principles and practices of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

## Introduction

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 9, which prevents GSK-3β from phosphorylating glycogen synthase[3]. 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[4]. 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[5]. 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 Wnt proteins, GSK-3β is unable to phosphorylate β-catenin, which leads to stabilization of β-catenin[6]. 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[7]. 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[8].

## Precautions

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1. Stop Solution 2 is a 1N hydrochloric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of buffers, however it is possible that high levels of interfering substances may cause variation in assay results.
4. The GSK-3β Standard provided, Catalog No. 80-1362, should be handled with care because of the known and unknown effects of GSK-3β.
5. The GSK-3β Standard should be stored at or below -20 °C. Do not repeatedly freeze-thaw.

## Materials Supplied

**1. GSK-3β Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-1096**
A plate using break-apart strips coated with a mouse monoclonal antibody specific to GSK-3β.

**2. Total GSK-3β EIA Antibody, 10 mL, Catalog No. 80-1357**
A yellow solution of rabbit polyclonal antibody to GSK-3β.

**3. Assay Buffer 21, 100 mL, Catalog No. 80-1519**
Tris buffered saline containing proteins, detergents and phosphatase inhibitor.

**4. Total GSK-3β EIA Conjugate, 10 mL, Catalog No. 80-1366**
A blue solution of goat anti-rabbit IgG conjugated to Horseradish peroxidase.

**5. Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**
Tris buffered saline containing detergents.

**6. GSK-3β Standard, 0.10 mL, Catalog No. 80-1362**
One vial containing 100,000 pg/mL of recombinant GSK-3β.

**7. TMB Substrate, 10 mL, Catalog No. 80-0350**
A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. **Protect from prolonged exposure to light.**

**8. Stop Solution 2, 10 mL, Catalog No. 80-0377**
A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: **Caustic.**

**9. RIPA Cell Lysis Buffer 2, 100 mL, Catalog No. 80-1284**
50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS.

**10. Total GSK-3β Assay Layout Sheet, 1 each, Catalog No. 30-0222**

**11. Plate Sealer, 3 each, Catalog No. 30-0012**

## Storage

All components of this kit, **except the GSK-3β Standard**, are stable at 4 °C until the kit's expiration date. The GSK-3β Standard **must** be stored at or below -20 °C.

## Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Phenylmethylsulfonyl fluoride (PMSF), Sigma #P7626 or equivalent.
3. Protease inhibitor cocktail (PIC), Sigma #P8340 or equivalent.
4. Precision pipets for volumes between 100 µL and 1,000 µL.
5. Repeater pipet for dispensing 100 µL.
6. Disposable beakers for diluting buffer concentrates.
7. Graduated cylinders.
8. A microplate shaker.
9. Adsorbent paper for blotting.
10. Microplate reader capable of reading at 450 nm, preferably with correction between 570 nm and 590 nm.
11. Graph paper for plotting the standard curve.
12. Polypropylene tubes

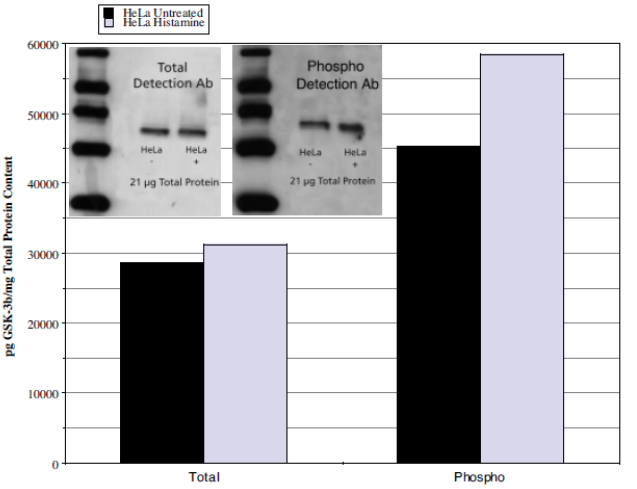
## Sample Handling

The GSK-3β EIA kit is compatible with GSK-3β samples in cell lysates. Samples diluted sufficiently into Assay Buffer 21 plus Inhibitors (see Reagent Preparation, page 6, #2) can be read directly from a standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. It is recommended that all samples be lysed with the provided RIPA Cell Lysis Buffer 2 modified by the addition of PMSF and PIC (see Reagent Preparation, page 6, #4) immediately prior to use. Samples lysed in RIPA Cell Lysis Buffer 2 plus Inhibitors must be diluted at least 1:6 with Assay Buffer 21 plus Inhibitors prior to running in the assay and should contain no more than 70 µg/mL Total Cellular Protein (TCP). Note that this dilution is based on the lysis of 2.0 million Jurkat cells per mL containing 0.4 mg/mL of TCP. The 1:6 dilution contained approximately 333,000 cells per mL with a calculated recovery of 108%.

**If the end user chooses to use another lysis buffer or a greater number of cells, it is up to the end user to determine the appropriate dilution of samples and assay validation.** Only standard curves generated in Assay Buffer 21 plus Inhibitors should be used to calculate the concentration of GSK-3β. Samples must be stored frozen at or below -70 °C to avoid loss of bioactive GSK-3β. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen samples should be brought to 4 °C slowly and gently mixed.

**Note: If you use the same cell lysates in our phospho GSK-3β Enzyme Immunoassay Kit (900-123), you must add phosphatase inhibitors to your RIPA Cell Lysis Buffer 2 in addition to the recommended protease inhibitors (PIC and PMSF). Add sodium orthovanadate, such as Sigma #S6508, to a final concentration of 2 mM and sodium pyrophosphate, such as Sigma #S6422, to a final concentratin of 20 mM.**

## HeLa Cell Treatment with Histamine




This experiment was adapted from a protocol outlined in reference #9. Two million HeLa cells per mL were used in this experiment. They were treated with 100 µM histamine, an inhibitor of GSK-3β activation, or left untreated (for a negative control) for 60 minutes at 37 °C. Cells were harvested with trypsin and centrifuged briefly at 800 x g and the supernatant was discarded. The cell pellet was resuspended and washed with PBS. Cells were pelleted at 1,000 x g for 5 minutes and the supernatant was discarded. The cell pellet was resuspended with RIPA Cell Lysis Buffer 2, vortexed and incubated on ice for 60 minutes. The lysate was vortexed and centrifuged at 16,000 x g for 20 minutes at 4 °C. The lysates were split for the Western Blot and the EIA to generate the data illustrated. Note that the samples were normalized to 21 µg of protein per lane in the Western Blot. Fractions were run in the assay and the resulting picogram determinations were divided by the protein concentration. The resulting values are expressed as pg GSK-3β/mg of total protein from each fraction.

## Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. **Standards must be made up in plastic tubes. Do not use glass tubes.**
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag provided. The wells should be used in the frame provided.
8. **Prior to addition of antibody, conjugate and substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

## Reagent Preparation

1. **Wash Buffer**
   Prepare the Wash Buffer by diluting 100 mL of the supplied concentrate with 1,900 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. **Assay Buffer 21 plus Inhibitors**
   Immediately prior to use in the assay, PMSF and PIC must be added to the buffer. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5 µL/mL or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1 mM.

   **This modified Assay Buffer 21 must be used for all sample and standard dilutions to ensure optimal integrity of GSK-3β. Fresh Assay Buffer 21 plus Inhibitors must be made for each assay.**

3. **GSK-3β Standards**
   Allow the 100,000 pg/mL GSK-3β standard solution to warm to room temperature. Label seven polypropylene 12x75 mm tubes #1 through #7. Pipet 475 µl of Assay Buffer 21 plus Inhibitors into tube #1. Pipet 250 µL of Assay Buffer 21 plus Inhibitors into tubes #2 though #7. Add 25 µl of the 100,000 pg/mL Standard to tube #1. Vortex thoroughly. Add 250 µL of standard #1 into tube #2 and vortex. Add 250 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #7.

   **The concentration of GSK-3β in tubes #1 through #7 will be 5,000, 2,500, 1,250, 625, 312.5, 156.3 and 78.1 pg/mL respectively. See Total GSK-3β Assay Layout Sheet for dilution details. Diluted standards should be used within 60 minutes of preparation.**

4. **RIPA Cell Lysis Buffer 2 plus Inhibitors**
   Allow buffer to come to room temperature. Ensure it is completely in solution prior to use. Immediately prior to use in cell lysis, PMSF and PIC must be added to the buffer. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5 µL/mL or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1mM.

   **Fresh RIPA Cell Lysis Buffer 2 plus Inhibitors must be made each time the cells are lysed.**

## Assay Procedure

**Bring all reagents to room temperature for at least 30 minutes prior to opening.**

**All standards, controls and samples should be run in duplicate.**

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.
2. Pipet 100 µL of Assay Buffer 21 plus Inhibitors into the S0 (0 pg/mL standard) wells.
3. Pipet 100 µL of Standards #1 through #7 into the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.
7. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 4 more times for a total of **5 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 100 µL of yellow Antibody into each well, except the Blank.
9. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.
10. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 4 more times for a total of **5 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 100 µL of blue Conjugate to each well, except the Blank.
12. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm.
13. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 4 more times for a total of **5 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
14. Pipet 100 µL of Substrate Solution into each well.
15. Incubate for 30 minutes at room temperature on a plate shaker at ~500 rpm.
16. Pipet 100 µL Stop Solution 2 to each well.
17. Blank the plate reader against the Blank wells, read the optical density at 450 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all the readings.

## Calculation of Results

Several options are available for the calculation of the concentration of GSK-3β in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of GSK-3β can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

2. Using linear graph paper, plot the Average Net OD for each standard versus GSK-3β concentration in each standard. Approximate a straight line through the points. The concentration of GSK-3β in the unknowns can be determined by interpolation.

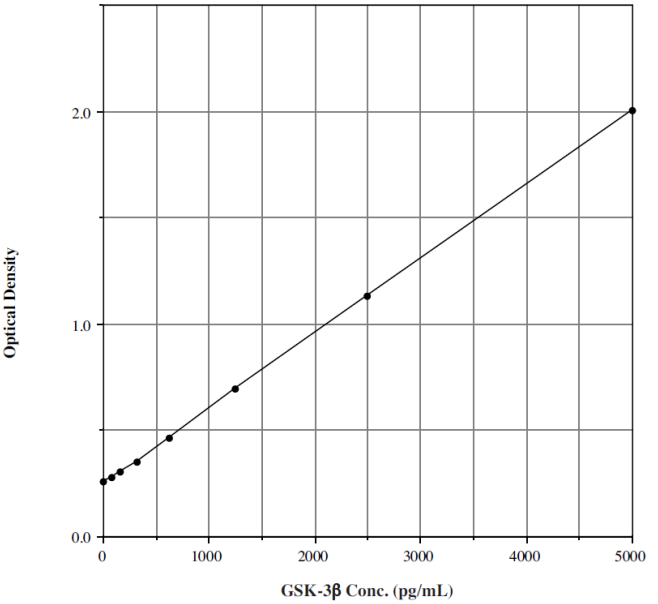
## Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

| Sample | Average OD | Net OD | **GSK-3β (pg/mL)** |
|---|---|---|---|
| Blank | (0.067) | | |
| S0 | 0.322 | 0.255 | 0 |
| S1 | 2.070 | 2.003 | 5,000 |
| S2 | 1.201 | 1.134 | 2,500 |
| S3 | 0.763 | 0.696 | 1,250 |
| S4 | 0.533 | 0.466 | 625 |
| S5 | 0.418 | 0.351 | 312.5 |
| S6 | 0.371 | 0.304 | 156.3 |
| S7 | 0.344 | 0.277 | 78.1 |
| Unknown 1 | 0.949 | 0.882 | 1,786 |
| Unknown 2 | 0.345 | 0.278 | 82.3 |

## Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate GSK-3β concentrations; each user must run a standard curve for each assay.




## Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[10].

### Sensitivity

Sensitivity was calculated by determining the average optical density bound for twenty-four (24) wells run with 0 pg/mL Standard, and comparing to the average optical density for twenty-four (24) wells run with Standard #7. The detection limit was determined as the concentration of GSK-3β measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

| | |
|---|---|
| Mean OD for S0 = | 0.205 ± 0.010 (5.0%) |
| Mean OD for Standard #7 = | 0.226 ± 0.007 (3.0%) |
| Delta Optical Density (78.1 - 0 pg/mL) = 0.226 - 0.205 = | 0.021 |
| 2 SD's of 0 pg/mL Standard = 2 x 0.010 = | 0.020 |
| Sensitivity = $\frac{0.020}{0.021}$ x 78.1 pg/mL = | **74.4 pg/mL** |

## Linearity

A sample containing 2,876 pg/mL GSK-3β was serially diluted 5 times 1:2 in the Assay Buffer 21 plus Inhibitors supplied in the kit and measured in the assay. The data was plotted graphically as actual GSK-3β concentration versus measured GSK-3β concentration.

The line obtained had a slope of 0.96 with a correlation coefficient of 0.99.

## Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of GSK-3β and running these samples multiple times (n=24) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of GSK-3β in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of GSK-3β determined in these assays as calculated by a 4 parameter logistic curve fitting program.

| | GSK-3β (pg/mL) | Intra-assay % CV | Inter-assay % CV |
|---|---|---|---|
| Low | 128 | 12.8 | |
| Medium | 416 | 4.0 | |
| High | 1,846 | 2.5 | |
| Low | 111 | | 18.7 |
| Medium | 449 | | 6.6 |
| High | 1,823 | | 4.0 |

## Cross Reactivities

The cross reactivities for a number of related compounds were determined by dissolving the cross reactants in the kit assay buffer at concentrations of either 50,000 pg/ml or 500,000 pg/ml. These samples were then measured in the GSK-3β assay

| Compound | Cross Reactivity |
|---|---|
| GSK-3β | 100% |
| GSK-3α | <0.01% |
| ATP Citrate Lyase | <0.02% |
| MEK 1 | <0.16% |
| AKT | <0.16% |
| phospho JNK | <0.16% |
| ERK | <0.16% |
| phospho ERK | <0.16% |

## Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

GSK-3β concentrations were measured in RIPA Cell Lysis Buffer 2. GSK-3β was spiked into the undiluted sample of this matrix which was then diluted with the kit assay buffer and assayed in the kit. The following results were obtained:

| Sample | % Recovery* | Recommended Dilution* |
|---|---|---|
| RIPA Cell Lysis Buffer 2 | 96.7% | ≥1:4 |
| 2 Million Jurkat cells/mL | 108% | ≥1:6 |

**WARNING: If the end user chooses to not use the provided RIPA Cell Lysis Buffer 2 or a greater number of cells than recommended for the final dilution, it is up to the end user to determine the appropriate dilution of samples and assay validation for their chosen cell lysis buffer.**

* See Sample Handling instructions on page 4 for details.

## References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## Use of Product

This product contains research chemicals. As such, they should be used and handled only by or under the supervision of technically qualified individuals. This product is not intended for diagnostic or human use.

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