3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

Akt/PKB ELISA

ProductInformation

Product Number **CS0160**
Storage Temperature 2-8 °C

TECHNICAL BULLETIN

Product Description

Akt/PKB ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for quantitative determination of Akt/PKB protein in cell lysates. A monoclonal antibody specific for Akt/PKB (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate strips provided. Akt/PKB standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the Akt/PKB antigen binds to the immobilized (capture) antibody. After incubation, the excess reagents are washed away and a biotin-conjugated monoclonal antibody specific for Total Akt/PKB, is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized Akt/PKB. After removal of excess detection antibody, horseradish peroxidase-labeled streptavidin (SAV-HRP) is added. This binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove excess SAV-HRP, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Akt/PKB present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of Akt/PKB.

The Akt/PKB ELISA was validated using human cell lines,however, it does cross react with mouse and rat Akt/PKB. This assay can be used to normalize the Akt/PKB content of the samples when using Sigma Phospho-Akt/PKB (pThr^{308}) ELISA (Product No. CS0110) and Phospho-Akt/PKB (pSer^{473}) ELISA (Prod. No. CS0120).

Akt, also known as protein kinase B-a?(PKB-a) or RAC-PKa was initially identified as one of the downstream targets of PI-3 Kinase (PI3-K). Akt is now known to consist of three highly conserved isoforms, which are designated in humans as Akt1, Akt2, and Akt3. Each isoform consists of an N-terminus pleckstrin homology (PH) domain, which mediates lipid-protein or protein-protein interactions, and a C-terminus kinase catalytic domain. Although each kinase is expressed differentially in a tissue-specific manner, they respond in a similar fashion to various stimuli. Akt can be activated by a diverse array of growth factors and physiologic stimuli in a PI3-K-dependent manner. Activation of Akt kinase is a multi-step process involving both membrane translocation and phosphorylation. Activated PI3-K generates 3' phosphoinositide products, 3,4,5-triphosphates (PI-3,4,5-P3) and PI-3,4-P2.

Akt is recruited from the cytosol to the plasma membrane through the interaction of its PH domain with these phosphoinositides. Upon membrane localization, Akt undergoes a conformational change, which makes it accessible to phosphorylation at threonine-308 and serine-473 in the kinase domain by PDK-1 and related kinases. Activated Akt then acts as a key mediator of signals for cell survival, proliferation, angiogenesis, and a number of metabolic effects of insulin. The effects of Akt activation may be mediated by modulation of expression or activity of various molecules including Bcl-2, BAD, caspase-9, endothelial nitric oxide synthase (eNOS), glycogen synthase, and transcription factors (NF-kB, Forkhead, CREB, Mdm2).

Because of its growth-promoting effects, Akt is also emerging as a central player in tumorigenesis. A number of oncogenes and tumor suppressor genes act upstream of Akt to influence cancer progression. Deletion of PTEN, a tumor suppressor gene that encodes a phosphatase, correlates with increased Akt activity in several cancers. Similarly, over expression of active Ras, Her/Neu, or Akt genes causes hyperactivation of Akt in many cancers including pancreatic, gastric, breast, ovarian and prostate adenocarcinomas. Small-molecule therapeutics that block PI3K signaling might inhibit cancer cells by blocking many aspects of the tumor-cell phenotype.[1-6]

Reagents

- **Akt/PKB Standard Human, 2 vials, Product No. A 0478**- Lyophilized from purified full length recombinant human Akt, expressed in SF21 cells. Refer to vial label for quantity and reconstitution volume.

- **Standard Diluent Buffer**, **25 mL**, **Product No. S 3943**- containing BSA and sodium azide as a preservative.
- **Monoclonal Anti- Akt/PKB-coated 96 well plate, 1 plate, Product No. H 9287** - A plate using break-apart strips coated with monoclonal antibody specific for full length Akt/PKB (regardless of phosphorylation state).
- **Monoclonal Anti-Akt/PKB-Biotin Conjugate, 11 mL, Product No. A 0353**– A detection antibody, which recognizes a full-length Akt/PKB (regardless of phosphorylation state). Ready to use.
- **Streptavidin-HRP Concentrate (100x), 1 vial, 0.125 mL, Product No. S 4318 -** contains 3.3 mM thymol and 50% glycerol, viscous. *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Product No H 8912** – contains thymol and BSA. Ready to use.
- **Wash Buffer Concentrate 25x, 100 mL, Product No. W 2639** - *See Reagent Preparation for handling, dilution and storage instructions.*
- **Stabilized Chromogen (TMB), 25 mL, Product No. S 3318** – Light sensitive. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P 4870**

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm
- Calibrated adjustable precision pipettes for volumes between 5 µl and 1,000 µL.
- Cell extraction buffer (see recommended extraction procedure)
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Graph paper: linear, log-log, or semi-log, as desired
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard
- Absorbent paper towels to blot the plate
- Calibrated beakers and graduated cylinders in various sizes
- Vortex mixer

Precautions and Disclaimer

The kit 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

Sample Preparation

- Samples of choice – extracts of cell lysates
- Samples should be frozen if not analyzed shortly after collection.
- Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.

Cell Extraction Buffer

10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM Na4P2O7
2 mM Na3VO4
1% Triton X-100
10% glycerol
0.1% SDS
0.5% deoxycholate
1 mM PMSF (stock is 0.3 M in DMSO)

PMSF is very unstable and must be added prior to use, even if added previously.

Protease inhibitor cocktail (Sigma Product No. P 2714)

Add 250 µL of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

Stable for 2-3 weeks at 4 ºC or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at –20 ºC.

Thaw on ice. *Add the protease inhibitors just before use.*

Procedure for Extraction of Proteins from Cells

The recommended Cell Extraction Buffer and procedure are optimized to achieve effective protein phosphorylation. Researchers may use the procedures that work best in their hands. They will have to assay their lysates for the satisfactory extraction and/or phosphorylation.

Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at –80 ºC and lysed at a later date).

4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10-minute intervals. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of p38 MAPK. For example, 10^8 Jurkat cells grown in RPMI plus 10% FBS can be extracted in 1 mL of Extraction Buffer.
5. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4 ºC.
6. Aliquot the clear lysate to clean microcentrifuge tubes.

Before assay: extracted cell lysate samples containing Akt/PKB protein should be diluted with Standard Diluent Buffer at least 1:10. This dilution is necessary to reduce the matrix effect of the cell lysate buffer. Example: 0.1-1 µl of the clarified cell extract diluted to a volume of 100 µl/well in Standard Diluent Buffer is sufficient for the detection of Akt/PKB.

Reagent Preparation

Akt/PKB Standard
1. Reconstitute one vial of Standard with Standard Diluent Buffer according to label directions. Mix gently and wait 10 minutes to ensure complete reconstitution. Label as 20 ng/mL Akt/PKB.
2. Prepare serial standard dilutions as follows:

Tube#	Standard Buffer	Standard from tube #:	Final Akt/PKB ng/mL
1	Reconstitute according to label instructions		20 ng/mL
2	0.25 mL	0.25 mL (1)	10 ng/mL
3	0.25 mL	0.25 mL (2)	5 ng/mL
4	0.25 mL	0.25 mL (3)	2.5 ng/mL
5	0.25 mL	0.25 mL (4)	1.25 ng/mL
6	0.25 mL	0.25 mL (5)	0.62 ng/mL
7	0.25 mL	0.25 mL (6)	0.31 ng/mL
8	0.5 mL	-	0 ng/mL

   Mix thoroughly between steps.
3. Use within 1 hour of reconstitution.

Streptavidin-HRP Concentrate (SAV-HRP) (100x)
Contains 50% glycerol and is very viscous. To ensure accurate dilution follow instructions below:
1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Mix: 10 µl IgG-SAV-HRP concentrate +1 mL HRP Diluent (sufficient for one 8-well strip, prepare more if needed)
   Label as SAV-HRP Working Solution.
4. Return the unused SAV-HRP concentrate to the refrigerator.

Wash Buffer
1. Equilibrate to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25x + 24 volumes of deionized water
3. Label as Working Wash Buffer.
4. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days.

Storage/Stability

All components of this kit are stable at 2 to 8 ºC. Any unused reconstituted standard should be discarded or frozen at -70 ºC. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit expiration date. To obtain C of A go to www.sigma-aldrich.com

Procedure

Precautions
- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 ºC).
- Use only the coated 96 well capture plate provided with the kit
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2 - 8°C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.

- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 2 hours of assay completion.

Washing directions

- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle four times, blotting as dry as possible after the 4th wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

1) **Incubate 100 µL of Standards and Samples (diluted >1:10) for 2 hours at RT. (Optional: Incubate overnight at 4°C)**

   aspirate and wash 4x

2) **Incubate 100 µL of Detection Antibody for 1 hour at RT.**

   aspirate and wash 4x

3) **Incubate 100 µL of SAV-HRP for 30 minutes at RT.**

   aspirate and wash 4x

4) **Incubate 100 µL of stabilized Chromogen for 30 minutes at RT *(in the dark)*.**

5) **Add 100 µL of Stop Solution and read at 450nm.**

Total Time 4 hours

Akt/PKB ELISA Assay Summary

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilutions wells and 2 wells for each sample to be assayed.
- Remove appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch.

1st incubation

a. Add 100 µL Standard Diluent to zero wells.
b. Add 100 µL standards, samples or controls to the appropriate wells. *Samples in Cell Extraction Buffer must be diluted at least 1:10 (1:50 or 1:100 may be necessary) in Standard Diluent Buffer. The dilutions should be optimized for each assay.*
c. Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature. *Alternatively, plate may be incubated overnight at 2 to 8 °C.*
d. Wash wells 4 times following washing instructions.
e. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer

2nd incubation

a. Add 100 µL Monoclonal Anti-Akt/PKB-Biotin Conjugate (detection) antibody to all wells (except chromogen blanks).
b. Tap gently on the plate to mix, cover with Plate Cover and incubate 1 hour at room temperature.
c. Wash wells for a total of 4 times following washing instructions.
d. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

3rd incubation

a. Add 100 µL SAV-HRP Working Solution to each well except the chromogen blanks.
b. Cover with Plate Cover and incubate 30 minutes at room temperature.
c. Wash wells for a total of 4 times following washing instructions.
d. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation

a. Add 100 µL of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*
b. Do not cover the plate
c. Incubate approximately 30 minutes at room temperature in the dark (*place plate in a drawer or cabinet).*

Note: If your multiwell plate reader does not register optical density (OD) above 2.0, incubate only 20-25 minutes.

Stop step

a. Add 100 µL of Stop Solution to each well. This stops the reaction
b. Tap gently to mix. *The solution will turn yellow.*

Absorbance reading

a. Any commercially available multiwellplate reader capable of reading at OD 450 nm may be used.
b. Blank the plate reader against the Chromogen Blank wells (contain Chromogen and Stop Solution).
c. Read the absorbance of the entire plate at 450 nm within 2 hours after addition of Stop Solution.

Results

The results may be calculated using any immunoassay software package. The four-parameter algorithm provides the best curve fit. If the software program is not readily available, the concentrations of Akt/PKB may be calculated as follows:

1. Calculate the Average Net OD for each standard dilution and samples as follows:
   Average Net OD=
   Average Bound OD – Average Chromogen Blank OD
2. On graph paper plot the Average Net OD (nm) of standard dilution (nm) against the concentration (ng/mL) of Akt/PKB for the standards. Draw the best curve through these points to construct the standard curve.
3. The Akt/PKB concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
4. Multiply the values obtained for the samples by dilution factor of each sample.
5. Samples producing signals higher than the 20 ng/mL standard should be further diluted and assayed.

Product Profile

Typical Results

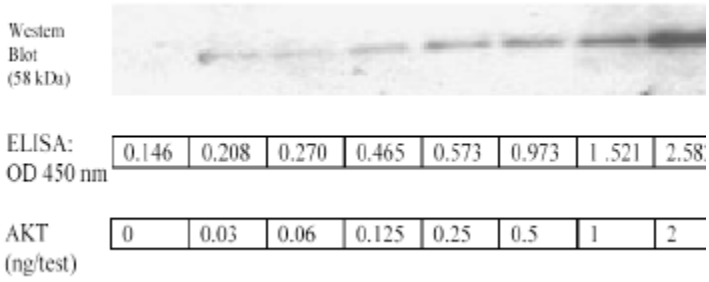
The standard curve below is for illustration only and **should not be used** to calculate results in your assay. Run standard curve in each assay.

OD 450 nm	Akt/PKB Standard (ng/mL)
0.240	0
0.299	0.3
0.313	0.6
0.463	1.25
0.556	2.50
1.257	5
1.835	10
3.376	20

Limitations:

- Do not extrapolate the standard curve beyond the 20 ng/mL standard point.
- The dose response is non-linear in this region and accuracy will be compromised.
- Other buffers and matrices have not been investigated.

Performance Characteristics



Specificity

The Akt/PKB ELISA is specific for measurement of human Akt/PKB independent of phosphorylation state. The assay can also quantitate mouse and rat Akt protein.

Jurkat were treated with wortmannin, a PI3-K specific inhibitor, at concentrations range 0-500 nM for 3 hours, lysed, and assayed in parallel by both Akt/PKB ELISA and Phospho-Akt/PKB (pSer^{473})

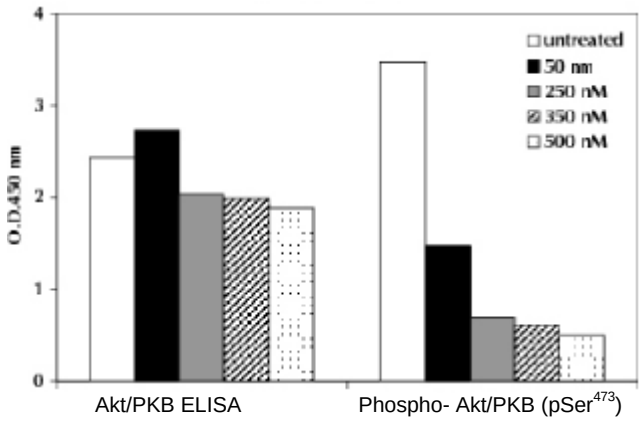


Fig. 1
Akt/PKB ELISA and Phospho-Akt/PKB (pSer^{473}) on wortmannin-treated Jurkat cells

The data indicate that the total Akt/PKB ELISA detects both phosphorylated and non-phosphorylated Akt/PKB in Jurkat cells, whereas the Phospho-Akt/PKB (pSer^{473}) ELISA detects decreasing levels of phosphorylated Akt/PKB (pSer^{473}) proportional to the increasing doses of wortmannin. The amount of total Akt/PKB remained comparable in both assays

Sensitivity

Sensitivity of this assay is <0.1 ng/mL of human Akt/PKB. . Sensitivity was calculated by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. In Jurkat cells cultured in complete medium, this sensitivity corresponded to the Akt/PKB protein extractable from 1500 cells/well. The sensitivity of this ELISA was compared to immunoblotting using known quantities of Akt/PKB. The results show that ELISA is approximately 10 times more sensitive in detecting Akt/PKB than immunoblotting.

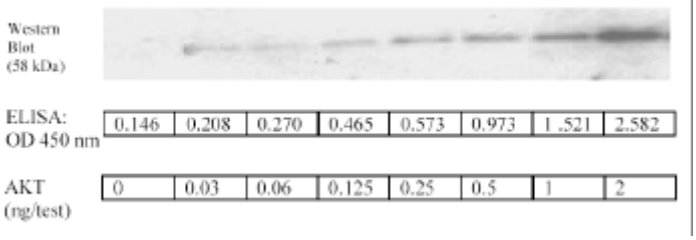


Fig. 2 Comparison of Akt/PKB ELISA vs. immunoblot

1. Intra-Assay Precision
Samples of known Akt/PKB concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	10	3.7	1.4
Standard Deviation (SD)	0.7	0.3	0.09
% Coefficient of Variation	6.9	9.9	6.3

2. Inter-Assay Precision
Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	10.9	3.5	1.8
Standard Deviation (SD)	1.07	0.3	0.1
% Coefficient of Variation	9.7	9.6	8.7

Sample Recovery

To evaluate recovery, extract buffer was diluted 1:10 with Standard Diluent Buffer to bring the SDS concentration <0.01%. Recombinant Akt/PKB was spiked into the extract at 3 levels and the percent recovery over endogenous levels calculated. On average, 101% recovery was observed.

Parallelism

Natural Akt/PKB from Jurkat cells and 3T3-L1 mouse cell extracts was serially diluted in Standard Diluent Buffer. The optical density of each dilution was plotted against the Akt/PKB standard curve. Parallelism was demonstrated by the figure below that indicated that the standard accurately reflects Akt/PKB content in samples.

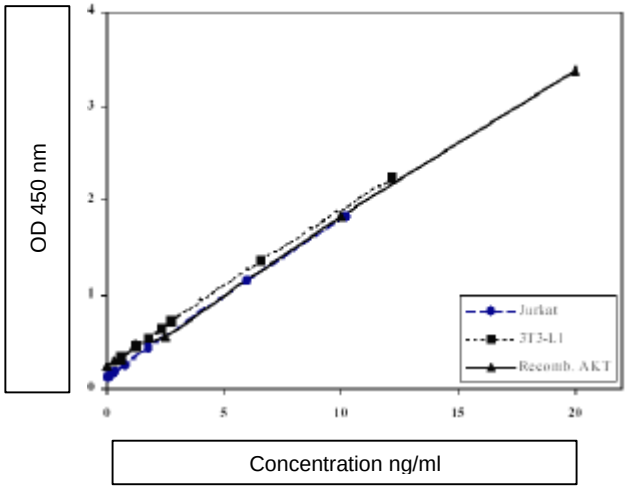


Fig. 3 Akt/PKB ELISA: Parallelism

Linearity of Dilution

Jurkat cells were grown in tissue culture medium containing 10% fetal calf serum and lysed with Cell Extraction Buffer. This lysate was diluted in Standard Diluent Buffer over the range of the assay and measured for Akt/PKB content. Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99 in both cases

Cell Lysate			
Dilution	**Measured (ng/mL)**	**Expected (ng/mL)**	**% Expected**
Neat	13.9	13.9	100
1:2	6.2	6.9	90
1:4	3.5	3.5	100
1:8	2.1	1.8	116

References

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AH/JK 11/2003