AKT [pS473] ELISA Kit

Catalog Number KHO0111 (96 tests)

Pub. No. MAN0014862 **Rev.** 2.0

CAUTION! This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state, and local regulations for disposal.

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *ELISA Technical Guide* (Pub. no. MAN0006706). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Product description

The Invitrogen™ AKT [pS473] ELISA Kit is a solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA). This assay is designed to detect and quantify the level of AKT protein that is phosphorylated at serine residue 473 in cell lysates. The assay will recognize both natural and recombinant AKT [pS473].

AKT, also known as the protein kinase B-α (PKB-α) or RAC-PKα, 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. Although each kinase is expressed differentially in a tissue-specific manner, they respond in a similar fashion to various stimuli.

For normalizing the AKT content of the samples, an AKT (Total) ELISA Kit (Cat. No. KHO0101) is available for detection of AKT content independent of phosphorylation status.

Contents and storage

Upon receipt, store the kit at 2°C to 8°C.

Contents	Cat. No. KHO0111 (96 tests)
AKT [pS473] Standard, lyophilized; contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume	2 vials
Standard Diluent Buffer; contains 0.1% sodium azide	25 mL
Antibody Coated Wells, 96 Well Plate	1 plate
AKT [pS473] Detection Antibody; contains 0.1% sodium azide	11 mL
Anti-Rabbit IgG HRP (100X); contains 3.3 mM thymol	0.125 mL
HRP Diluent; contains 3.3 mM thymol	25 mL
Wash Buffer Concentrate (25X)	100 mL
Stabilized Chromogen, Tetramethylbenzidine (TMB)	25 mL
Stop Solution	25 mL
Plate Covers, adhesive strips	3

Required materials not supplied

- Distilled or deionized water
- Microtiter plate reader with software capable of measurement at or near 450 nm
- Plate washer–automated or manual (squirt bottle, manifold dispenser, or equivalent)
- Calibrated adjustable precision pipettes and glass or plastic tubes for diluting solutions
- Cell Extraction Buffer (Cat. No. FNN0011, or see "Prepare Cell Extraction Buffer")

Before you begin

IMPORTANT! Reagents are lot-specific. Do not mix or interchange different reagent lots from various kit lots.

- Review the **Procedural guidelines** and **Plate washing directions** in the *ELISA Technical Guide* available at **thermofisher.com**.
- Allow reagents to reach room temperature before use. Mix to redissolve any precipitated salts.

Prepare 1X Wash Buffer

1. Dilute 16 mL of Wash Buffer Concentrate (25X) with 384 mL of deionized or distilled water. Label as 1X Wash Buffer.
2. Store the concentrate and 1X Wash Buffer in the refrigerator. Use the diluted buffer within 14 days.

Prepare Cell Extraction Buffer

Note: See the *ELISA Technical Guide* for detailed information on preparing Cell Extraction Buffer.

1. Prepare 5 mL of Cell Extraction Buffer.

   Cell Extraction Buffer consists of 10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate.
2. Immediately before use, add 1 mM PMSF (0.3 M stock in DMSO) and 250 µL protease inhibitor cocktail (e.g., Sigma Cat. No. P-2714).

Prepare cell lysate

1. Collect cells in PBS by centrifugation (non-adherent cells) or scraping from culture flasks (adherent cells).
2. Wash cells twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. The pellet can be stored at −80°C and lysed at a later date if desired.
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes, on ice. Vortex at 10 minute intervals.

   Note: The volume of Cell Extraction Buffer used depends on the number of cells in the cell pellet, and expression levels of AKT [pS473]. For example, 4×10^6 Jurkat cells can be extracted in 1 mL of Cell Extraction Buffer. Researchers must optimize the extraction procedures for their own applications.
5. Transfer the lysate into microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4°C.
6. Transfer the supernatant into clean microfuge tubes. Samples can be stored at −80°C (avoid multiple freeze-thaw cycles).

Pre-dilute samples

Because conditions may vary, we recommend that each investigator determine the optimal dilution for each application.

- Dilute samples prepared in Cell Extraction Buffer 1:10 or greater in Standard Diluent Buffer (e.g., 10 µL sample into 90 µL buffer).

  For 4×10^6 Jurkat cells, use 0.1–5 µL of the clarified lysate diluted to 100 µL in Standard Diluent Buffer for each well.

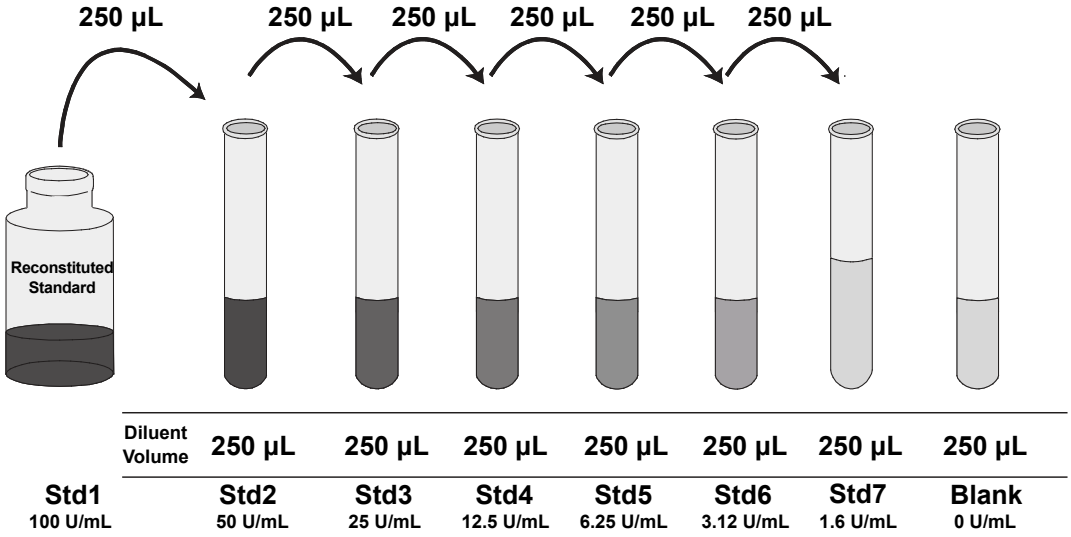
  This dilution is necessary to reduce the matrix effect of the Cell Extraction Buffer. SDS concentration should be less than 0.01% before adding to the plate. While a 1:10 sample dilution has been found to be satisfactory, higher dilutions such as 1:25 or 1:50 may be optimal.
- Dilute samples >100 Units/mL with Standard Diluent Buffer.

Dilute standards

Note: Use glass or plastic tubes for diluting standards.

Note: This AKT [pS473] Standard is prepared using purified, full length, human recombinant AKT expressed in S*f*21 cells. One Unit of standard is defined as the amount of AKT [pS473] derived from 100 pg of AKT, which was phosphorylated by MAPKAP2 and PDK1. Subsequent lots of standard are normalized to this lot of material to allow consistency of AKT [pS473] quantitation.

1. Reconstitute AKT [pS473] Standard to 100 Units/mL Standard Diluent Buffer. Refer to the standard vial label for instructions. Swirl or mix gently and allow the contents to sit for 10 minutes to ensure complete reconstitution. Label as 100 Units/mL AKT [pS473]. **Use the standard within 1 hour of reconstitution.**
2. Add 250 µL Standard Diluent Buffer to each of 7 tubes labeled as follows: 50, 25, 12.5, 6.25, 3.12, 1.6, and 0 Units/mL AKT [pS473].
3. Make serial dilutions of the standard as shown in the following dilution diagram. Mix thoroughly between steps.
4. Remaining reconstituted standard should be discarded or frozen in aliquots at –80°C for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.



Prepare 1X Anti-Rabbit IgG HRP solution

Note: Prepare 1X Anti-Rabbit IgG HRP solution within 15 minutes of usage.

The Anti-Rabbit IgG HRP (100X) is in 50% glycerol, which is viscous. To ensure accurate dilution:

1. For each 8-well strip used in the assay, pipet 10 µL Anti-Rabbit IgG HRP (100X) solution, wipe the pipette tip with clean absorbent paper to remove any excess solution, and dispense the solution into a tube containing 1 mL of HRP Diluent. Mix thoroughly.
2. Return the unused Anti-Rabbit IgG HRP (100X) solution to the refrigerator.

Perform ELISA (Total assay time: 4 hours)

IMPORTANT! Perform a standard curve with each assay.

- Allow all components to reach room temperature before use. Mix all liquid reagents prior to use.
- Determine the number of 8-well strips required for the assay. Insert the strips in the frames for use. Re-bag any unused strips and frames, and store at 2°C to 8°C for future use.

1 Bind antigen

a. Add 100 µL of standards, controls, or samples (see "Pre-dilute samples"on page 2) to the appropriate wells. Leave the wells for chromogen blanks empty.
b. Cover the plate with a plate cover and incubate 2 hours at room temperature.
c. Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.

2 Add detector antibody

a. Add 100 µL of AKT [pS473] Detection Antibody solution into each well except the chromogen blanks.
b. Cover the plate with a plate cover and incubate 1 hour at room temperature.
c. Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.

3 Add IgG HRP

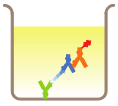
a. Add 100 µL 1X Anti-Rabbit IgG HRP solution into each well except the chromogen blanks.
b. Cover the plate with plate cover and incubate for 30 minutes at room temperature.
c. Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.

4 Add Stabilized Chromogen

a. Add 100 µL Stabilized Chromogen to each well. The substrate solution begins to turn blue.
b. Incubate for 30 minutes at room temperature in the dark.

Note: TMB should not touch aluminum foil or other metals.

5 Add Stop Solution



Add 100 µL Stop Solution to each well. Tap the side of the plate to mix. The solution in the wells changes from blue to yellow.

Read the plate and generate the standard curve

1. Read the absorbance at 450 nm. Read the plate within 2 hours after adding the Stop Solution.
2. Use curve-fitting software to generate the standard curve. A 4 parameter algorithm provides the best standard curve fit. Optimally, the background absorbance may be subtracted from all data points, including standards, unknowns and controls, prior to plotting.
3. Read the concentrations for unknown samples and controls from the standard curve. Multiply value(s) obtained for sample(s) by the appropriate factor to correct for the sample dilution.

   Note: Dilute samples producing signals greater than that of the highest standard in Standard Diluent Buffer and reanalyze. Multiply the concentration by the appropriate dilution factor.

Performance characteristics

Standard curve example

The following data were obtained for the various standards over the range of 0 to 100 Units/mL AKT [pS473].

Standard AKT [pS473] (Units/mL)	Optical Density (450 nm)
100	2.58
50	1.30
25	0.78
12.5	0.54
6.25	0.30
3.12	0.24
1.6	0.22
0	0.17

Inter-assay precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

Parameters	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	42.0	17.4	8.4
Standard Deviation	3.6	1.4	0.8
% Coefficient of Variation	7.0	8.0	9.8

Intra-assay precision

Samples of known AKT [pS473] concentration were assayed in replicates of 16 to determine precision within an assay.

Parameters	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	42.9	17.2	7.6
Standard Deviation	3.3	1.26	0.4
% Coefficient of Variation	7.8	7.3	5.5

Cross-reactivity

Characterization of this ELISA kit was done primarily on human cells, but cross-reactivity with mouse and rat cells was observed.

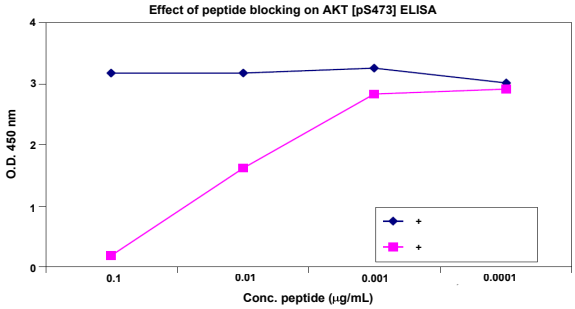
The following recombinant phosphoproteins had no cross-reactivity when tested at 100 ng/mL: p38 MAPK, p44 ERK1, p42 ERK2, JNK1, human insulin receptor, rat insulin receptor, human HGFR (c-met).

Recovery

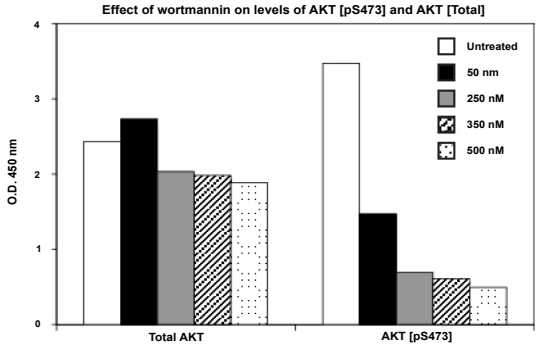
Unstimulated mouse cell lysates were prepared in Cell Extraction Buffer (200 µg/mL total protein). Recombinant AKT [pS473] was spiked into the lysates at 3 concentrations, and percent recovery calculated over endogenous levels. On average, 93% recovery was observed.

Specificity

AKT [pS473] specificity was confirmed by peptide competition. The data shows that only the phosphopeptide containing the phosphorylated serine blocks the ELISA signal. The same sequence with a non-phosphorylated serine at position 473 was not blocked.



To demonstrate the relationship between AKT phosphorylation and PI-3 kinase activity, Jurkat cells were treated with wortmannin (a PI-3 kinase inhibitor) at varying concentrations from 0–500 nM for 3 hours. Parallel assays for AKT [Total] and AKT [pS473] showed that the amount of total AKT remained comparable, but levels of phosphorylation at serine 473 decreased in a dose dependent manner with increasing doses of wortmannin.



Linearity of dilution

Cell Extraction Buffer was spiked with AKT [pS473] and serially diluted in Standard Diluent Buffer over the range of the assay. Linear regression analysis of sample values versus the expected concentration yielded a correlation coefficient of 0.99.

Dilution	Measured (Units/mL)	Expected	
		(Units/mL)	%
Neat	48.9	48.9	100
1/2	23.6	24.4	97
1/4	11.1	12.2	91
1/8	5.7	6.1	93
1/16	2.8	3.0	93

Sensitivity

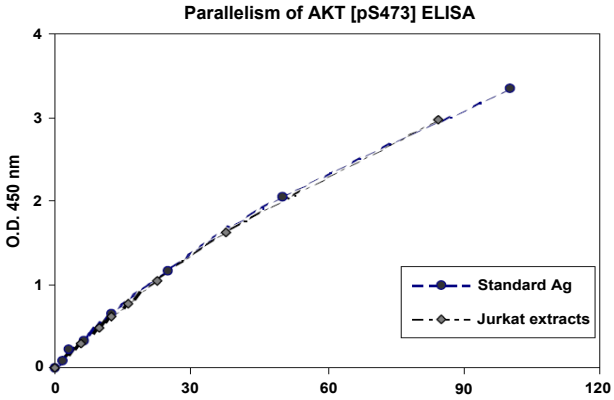
The minimum detectable dose of AKT [pS473] is <0.8 Units/mL. This was determined by adding two standard deviations to the mean O.D. obtained from 30 assays of the zero standard. The value corresponds to the amount of AKT [pS473] extracted from 4000 Jurkat cells cultured under optimal condition.

In addition, the sensitivity of the ELISA is ~2-fold greater than that of western blot when tested against known quantities of AKT [pS473].

ELISA: OD 450 nm	0.173	0.597	0.697	0.725	0.960	1.316	1.987	3.183
AKT (Units/test)	0	0.156	0.312	0.625	1.25	2.5	5	10

Parallelism

Endogenous AKT [pS473] from extracts of Jurkat cells cultured in RPMI + 10% FCS were serially diluted in Standard Diluent Buffer. The optical density of each dilution was plotted against the AKT [pS473] standard curve. The standard accurately reflects the full AKT [pS473] content in samples.



Limited product warranty

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Product label explanation of symbols and warnings

REF	Catalog Number	LOT	Batch code		Temperature limitation		Use by		Manufacturer		Consult instructions for use		Caution, consult accompanying documents

Manufacturer's address: Life Technologies Corporation | 7335 Executive Way | Frederick, MD 21704 | USA

The information in this guide is subject to change without notice.