AKT1 [pS473] Ultrasensitive ELISA Kit

Catalog Number KHO0541 (96 tests)

Pub. No. MAN0014653 **Rev.** 3.0 (30)

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 AKT1 [pS473] Ultrasensitive ELISA Kit is a solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA) designed to detect and quantify the level of AKT protein that is phosphorylated at serine residue 473 in cell lysates.

Although performance characterization of the kit was done primarily with human cell lines, cross-reactivity with mouse and rat cells was observed.

For normalizing the AKT1 content of the samples, an AKT1 (Total) ELISA Kit (Cat. No. KHO0531) is available for detection of 2 vials of standard AKT content independent of phosphorylation status.

Contents and storage

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

Contents	Cat. No. KHO0541 (96 tests)
AKT1 [pS473] Standard, lyophilized; contains 0.1% sodium azide.	2 vials
Standard Diluent Buffer; contains 0.1% sodium azide, red dye[1]	25 mL
Antibody Coated Wells; 96-well plate	1 plate
AKT1 [pS473] Detection Antibody; contains 0.1% sodium azide, blue dye[1]	6 mL
Anti-Rabbit IgG HRP (100X)	0.125 mL
HRP Diluent; contains 3.3 mM thymol, yellow dye[1]	25 mL
Wash Buffer Concentrate (25X)	100 mL
Stabilized Chromogen, Tetramethylbenzidine (TMB)	25 mL
Stop Solution	25 mL
Adhesive Plate Covers	3

[1] To avoid pipetting mistakes, colored Standard Diluent Buffer, Detection Antibody, and HRP Diluent are provided to monitor the addition of solution to each well. Dyes do not interfere with test results.

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; beakers, flask and cylinders for preparation of reagents
- 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 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 PMSF (0.3 M stock in DMSO) to 1 mM and 50 µL protease inhibitor cocktail (e.g., Sigma Cat. No. P-2714) for each 1 mL of Cell Extraction Buffer.

Prepare cell lysate

1. Collect cells by centrifugation (non-adherent cells) or scraping from culture flasks (adherent cells), then wash cells twice with cold PBS.
2. 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.
3. 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 AKT1 [pS473]. Researchers must optimize the extraction procedures for their own applications.
4. Transfer the lysate into microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4°C.
5. Transfer the supernatant into clean microcentrifuge tubes. Samples can be stored at −80°C (avoid multiple freeze-thaw cycles).

Pre-dilute samples

Sample concentrations should be within the range of the standard curve. Because conditions may vary, each investigator should determine the optimal dilution for each application.

- Perform sample dilutions with Standard Diluent Buffer.
- Dilute samples prepared in Cell Extraction Buffer 1:5 or greater in Standard Diluent Buffer (e.g., 10 µL sample into 40 µL buffer).

  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 1:5 sample dilution has been found to be satisfactory, higher dilutions such as 1:10 or 1:20 may be optimal.

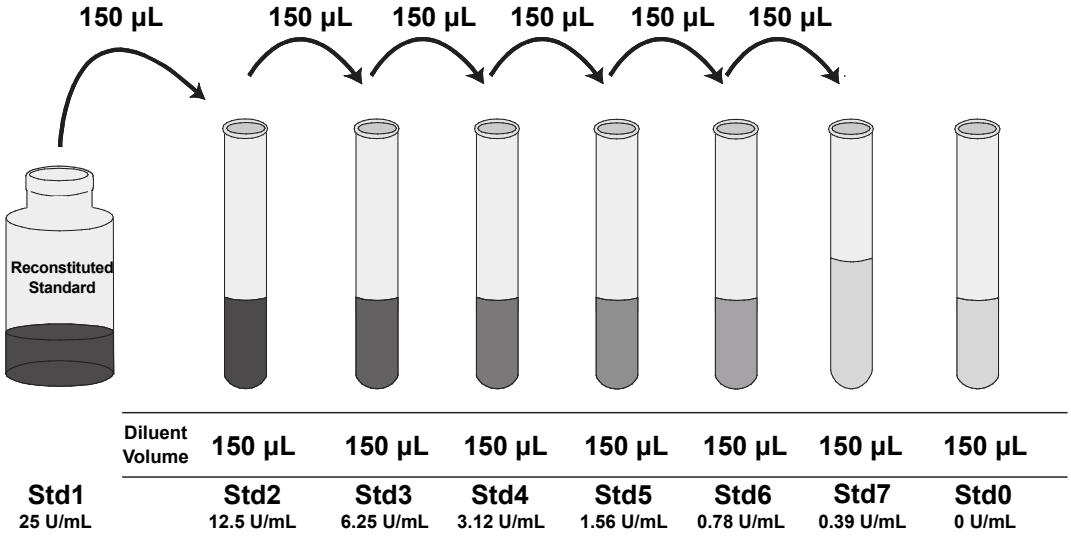
  For 4×10^7 Jurkat cells, use 0.1–5 µL of the clarified lysate diluted to 100 µL in Standard Diluent Buffer for each well.

Dilute standards

Note: Use glass or plastic tubes for diluting standards.

Note: The AKT1 [pS473] Standard is prepared using purified, full length, recombinant, human AKT1 expressed in Sf21 cells. One Unit of standard is defined as the amount of AKT1 [pS473] derived from 100 pg of AKT, which was phosphorylated by MAPKAPK2 and PDK1.

1. Reconstitute AKT1 p[S473] Standard to 25 Units/mL with Standard Dilution 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 25 Units/mL AKT1 [pS473]. **Use the standard within 10 minutes of reconstitution.**
2. Add 150 µL Standard Diluent Buffer to each of 7 tubes labeled as follows: 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, and 0 Units/mL AKT1 [pS473].
3. Make serial dilutions of the standard as shown in the following dilution diagram. Mix thoroughly between steps.
4. Discard remaining reconstituted standard or freeze in aliquots at −80°C. Standard can be frozen and thawed one time only without loss of activity. Return Standard Diluent Buffer to the refrigerator.



Prepare 1X Anti-Rabbit IgG HRP solution

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

1. For each 8-well strip used in the assay, pipet 10 µL Anti-Rabbit IgG HRP (100X) 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 and add detector

a. Add 50 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. Add 50 µL of AKT1 p[S473] Detection Antibody solution into each well except the chromogen blanks.
c. Tap the side of the plate to mix. Cover the plate with a plate cover and incubate 3 hours at room temperature.
d. Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.

2 Add IgG HRP

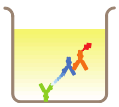
a. Add 100 µL Anti-Rabbit IgG HRP into each well except the chromogen blanks.
b. Cover the plate with plate cover and incubate for 30 minutesw at room temperature.
c. Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.

3 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.

4 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 the upper limit of the standard curve in Standard Diluent Buffer and reanalyze. Multiply the concentration by the appropriate dilution factor.

Performance characteristics

Standard curve example

The following data was obtained for the various standards over the range of 0 to 25 Units/mL AKT1 [pS473].

Standard AKT1 p[S473] (Units/mL)	Optical Density (450 nm)
25	3.07
12.5	1.68
6.25	0.97
3.12	0.60
1.56	0.37
0.78	0.27
0.39	0.20
0	0.12

Recovery

Unstimulated Jurkat cell lysates were prepared in Cell Extraction Buffer (200 µg/mL total protein). Recombinant AKT1 [pS473] was spiked into the lysates at two levels and percent recovery calculated over endogenous levels. On average, 93% recovery was observed.

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)	19.9	9.9	3.0
Standard Deviation	1.1	0.6	0.1
% Coefficient of Variation	5.4	5.8	4.3

Intra-assay precision

Samples of known AKT1 [pS473] concentration were assayed in replicates of 16 to determine precision within an assay.

Parameters	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	20.4	10.0	3.0
Standard Deviation	1.1	0.5	0.1
% Coefficient of Variation	5.5	4.5	4.2

High-dose hook effect

Samples spiked with AKT1 p[S473] Standard up to 125 Units/mL give responses higher than that obtained from the highest standard point.

Sensitivity

The analytical sensitivity of the assay is <0.2 Units/mL AKT1 [pS473]. 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 AKT1 [pS473] extracted from 1,000 Jurkat cells cultured in complete medium.

In addition, the sensitivity of the ELISA is ~8-fold greater than that of western blot when tested against known quantities of AKT1 [pS473].

ELISA: OD 450 nm	3.067	1.678	0.969	0.604	0.371	0.269	0.204	0.117
AKT1 (Units/test)	2.5	1.25	0.625	0.313	0.156	0.078	0.039	0

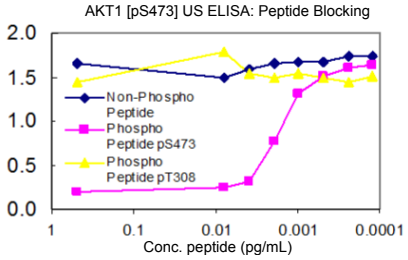
Linearity of dilution

Cell Extraction Buffer was spiked with Jurkat cell lysate (200 µg/mL) 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.

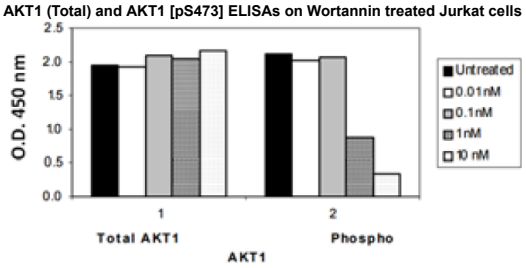
Dilution	Cell Lysate		
	Measured (Units/mL)	Expected	
		(Units/mL)	%
Neat	18.87	18.87	100
1/2	9.27	9.44	98.2
1/4	5.39	4.72	114.3
1/8	2.80	2.36	118.7
1/16	1.34	1.18	113.3

Specificity

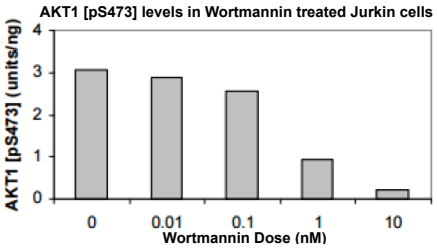
The AKT1 [pS473] US ELISA Kit did not show cross-reactivity with AKT2 and AKT3 recombinant proteins tested at 100 ng/mL. It was confirmed by peptide competition. Only the phosphopeptide containing the phosphorylated serine blocks the ELISA signal. The same sequence with a non-phosphorylated serine at position 473, or a phosphorylated threonine at position 308, was not blocked.



In the relationship between AKT1 phosphorylation and PI-3 kinase activity, Jurkat cells were treated with wortmannin (a PI-3 kinase inhibitor) at varying concentrations (0–10 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.

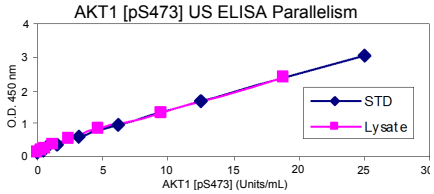


There is normalization of AKT [pS473] by the AKT1 (Total) ELISA Kit on wortmannin treated Jurkat cells.



Parallelism

Natural AKT1 [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 AKT1 [pS473] standard curve. The standard accurately reflects the AKT1 [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: Bender MedSystems GmbH | Campus Vienna Biocenter 2 | 1030 Vienna, Austria

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