Product Manual

[pSer$^{473/474}$]Akt1/2 ELISA kit

Catalog # ADI-900-162

96 well enzyme-linked immunoassay kit
For use with cell lysates

All reagents, except standard, should be stored at 4°C. Store standard at -20°C.

Check our website for additional protocols, technical notes, MSDS and FAQs.

For proper performance, use the insert provided with each individual kit received.

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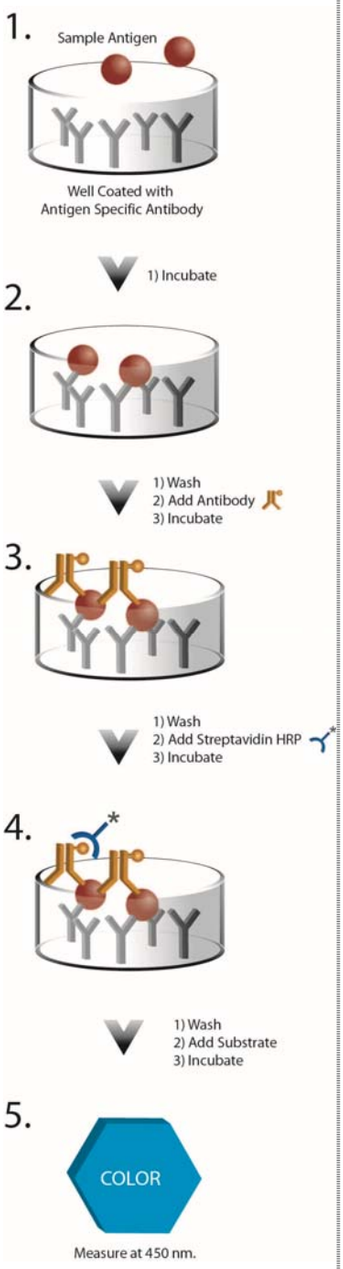


INTRODUCTION

The [pSer$^{473/474}$]Akt1/2 ELISA kit is a complete kit for the quantitative determination of Akt1 and Akt2 from human, mouse, and rat cell lysates. The kit recognizes Akt3 with low affinity; see Specificity in the Performance Characteristics section for more information. Please read the entire kit insert before performing this assay.

The Akt (PKB) family of protein kinases are serine/threonine kinases, with three mammalian family members identified to date (Akt1, Akt2, Akt3). Akt is a well-characterized member of PI3 kinase-mediated signaling pathways, regulating cell growth, apoptosis, glycogen synthesis, and other cellular responses through its phosphorylation of downstream substrates[1]. Its activation is triggered by binding of phospholipid and phosphorylation at two key residues: Thr308 by PDK1, and Ser473/474 by PDK2, now identified as mTOR[2,3,4]. Deregulation of Akt signaling has been associated with cancer, diabetes, and schizophrenia[1]. Akt1 is the cellular homologue of the murine thymoma retroviral oncogene v-akt[5], and its role in anti-apoptotic and pro-mitotic pathways has made Akt a molecular target for anti-cancer therapeutic intervention[6]. Activation of Akt has been shown to inhibit apoptosis by phosphorylating the Bcl-2 related protein Bad, and increases p53 degradation by phosphorylating Mdm2[7,8]. Mitotic substrates of Akt include GSK3α, p21^{CIP1}, and p27^{KIP1}, cell cycle inhibitors negatively regulated by Akt phosphorylation[9,10,11]. The enzyme has been shown to mediate angiogenesis through regulation of thrombospondins, which may cooperate with pro-mitotic and anti-apoptotic functions of Akt to promote tumorigenesis[12].

PRINCIPLE

1. Samples and standards are added to wells coated with a monoclonal antibody specific for the amino terminus of Akt. The plate is then incubated.
2. The plate is washed, leaving only bound Akt on the plate. A yellow solution of antibody, specific for Akt phosphorylated at Ser473/474, is then added. This binds the Akt1, 2 (phospho-Ser473/474) captured on the plate. The plate is then incubated.
3. The plate is washed to remove excess antibody. A blue solution of streptavidin-HRP conjugate is added to each well, binding to the biotinylated antibody, which is attached to the Akt1, 2 (phospho-Ser473/474). The plate is again incubated.
4. The plate is washed to remove excess streptavidin-HRP conjugate. TMB Substrate solution is added. The substrate generates a blue color when catalyzed by the HRP.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is directly proportional to the level of Akt1, 2 (phospho-Ser473/474) in the sample.

MATERIALS SUPPLIED

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

1. Assay Buffer 32
   100ml, Catalog No. 80-1632
   Tris buffered saline containing BSA and detergents.
2. Akt1 (phospho-Ser473) Standard
   560pg, Catalog No. 80-1660
   Two vials, each containing 560 pg of lyophilized recombinant human phospho Akt1.
3. Cell Lysis Buffer 5
   100ml, Catalog No. 80-1631
   20mM Tris, pH 7.4, 500mM NaCl, 10mM EDTA, 1% Triton X-100, 20mM NaF, 20mM β-glycerophosphate
4. Akt Clear Microtiter Plate
   One Plate of 96 Wells, Catalog No. 80-1630
   A plate of break-apart strips coated with a mouse monoclonal antibody raised against a synthetic peptide derived from the N-terminus of human Akt1.
5. Wash Buffer Concentrate
   100ml, Catalog No. 80-1287
   Tris buffered saline containing detergents.
6. Akt1, 2 (phospho-Ser473/474) Antibody
   10ml, Catalog No. 80-1635
   A yellow solution of biotinylated monoclonal antibody raised against a synthetic peptide homologous to Akt, phosphorylated at Ser473/474.
7. Akt1, 2 (phospho-Ser473/474) Conjugate
   10ml, Catalog No. 80-1653
   A blue solution of streptavidin conjugated to horseradish peroxidase.
8. TMB Substrate
   10ml, Catalog No. 80-0350
   A solution of 3,3’,5,5’ tetramethylbenzidine (TMB) and hydrogen peroxide
9. Stop Solution 2
   10ml, Catalog No. 80-0377
   A 1N solution of hydrochloric acid in water
10. Akt1, 2 (phospho-Ser473/474) Assay Layout Sheet
    1 each, Catalog No. 30-0242
11. Plate Sealer, 3 each, Catalog No. 30-0012

Protect substrate from prolonged exposure to light.

Stop solution is caustic. Keep tightly capped.

Reagents require separate storage conditions.

STORAGE

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

OTHER MATERIALS NEEDED

1. Deionized or distilled water.
2. Phenylmethylsulfonyl fluoride (PMSF), Sigma #P7626 or equivalent.
3. Protease inhibitor cocktail (PIC), Sigma #P8340 or equivalent.
4. Phosphatase inhibitor cocktail (PhIC), Sigma #P2850 or equivalent.
5. Precision pipets for volumes between 5µl and 1,000µl.
6. Repeater pipet for dispensing 100µl.
7. Disposable beakers for diluting buffer concentrates.
8. Graduated cylinders.
9. A microplate shaker.
10. Lint-free paper for blotting.
11. Microplate reader capable of reading at 450 nm.

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

If inhibitors other than those recommended are use, the end user is responsible for assay validation. In some cases, certain protease inhibitor cocktails may cause performance differences.

Plastic tubes must be used for standard preparation.

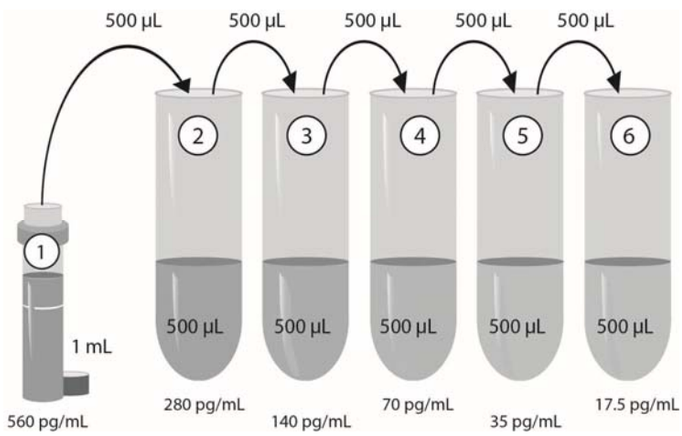
REAGENT PREPARATION

1. **Wash Buffer**
   Prepare the wash buffer by diluting 50ml of the supplied Wash Buffer Concentrate with 950ml of deionized water. This can be stored at room temperature until the kit's expiration, or for 3 months, whichever is earlier.

2. **Addition of Inhibitor to Cell Lysis Buffer 5**
   Immediately prior to use, PIC, PhIC, and PMSF must be added to Cell Lysis Buffer 5. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5µl/ml PIC, or equivalent concentration according to alternate vendor's specification sheet. If using Sigma Phosphatase Inhibitor Cocktail #P2850, add 10µl/ml PhIC, or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1mM.

   Note: Inhibitors must be freshly added to Cell Lysis Buffer 5 to ensure optimal integrity of the samples. Buffers treated with inhibitors should be used within 1 hour of preparation.

Preparation of Akt1 (phospho-Ser473) Standard Curve



Reconstitute one vial of Akt1 (phospho-Ser473) Standard with 1ml Assay Buffer 32. Mix thoroughly without foaming. Label vial of reconstituted Akt1 (phospho-Ser473) Standard #1. Label five 12x75mm polypropylene tubes #2 through #6. Pipet 500µl of Assay Buffer 32 into tubes #2 through #6. Add 500µl of Standard #1 to tube #2. Vortex thoroughly. Add 500µl of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 though #6.

Diluted standards should be used within 1 hour of preparation. The concentrations of the standards are labeled above.

SAMPLE HANDLING

If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

This assay is suitable for measuring Akt phosphorylated at Ser473/474 in a wide range of cell lysates. Prior to assay, frozen samples should be slowly brought to 4°C and centrifuged, if necessary, to isolate residual cell debris.

A minimum 1:8 dilution of the samples is required to remove matrix interference of Cell Lysis Buffer 5. Due to differences in cell types, number of cells, or total cellular protein concentration, lysates may require greater dilution with the assay buffer to remove interference or to be read within the standard range. Below are examples of lysates that have been run in this assay.

Samples must be stored at or below -20°C to avoid loss of bioactive analyte. Avoid repeated freeze/ thaw cycles.

Note: Based on amino acid sequence comparison, rat Akt3 is not expected to read in this kit.

Sample	# cells per ml of lysis buffer	Total cellular protein (mg/ml)	% Recovery	Recom-om-mended Dilution
Jurkat cells (human)	2.6 million	2.09	97%	1:160
3T3 cells (mouse)	-----	1.61	90%	1:160
C6 cells (rat)	-----	2.58	100%	1:160

Add inhibitors to buffers prior to preparing samples.

Protocol for Cell Lysis

1. Harvest cells and centrifuge at 250 x g (~1534 rpm) for 7 minutes at 4°C. Discard supernatant.
2. Resuspend pellet and wash with Hank's Balanced Salt Solution (without phenol red) or PBS.
3. Centrifuge at 250 x g (~1534 rpm) for 7 minutes at 4°C. Discard supernatant.
4. Resuspend pellet with Cell Lysis Buffer 5 plus protease and phosphatase inhibitors (see recommended inhibitors on pg. 4).
5. Vortex and incubate on ice for 30 minutes.
6. Centrifuge at 16,000 x g (~12,274 rpm) for 20 minutes at 4°C.
7. Place the supernatants into a clean tube.
8. The supernatants may be aliquoted and stored at or below -20°C or used immediately in the assay.

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

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

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

Pipet the reagents to the sides of the wells to avoid possible contamination.

Prior to the addition of the antibody, conjugate and substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.

ASSAY PROCEDURE

Refer to the Assay Layout Sheet to determine the number of wells to be used.

Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

1. Pipet 100µl of the assay buffer into the S0 (0 pg/ml standard) wells.
2. Pipet 100µl of Standards #1 through #6 to the bottoms of the appropriate wells.
3. Pipet 100µl of the samples to the bottoms of the appropriate wells.
4. Seal the plate. Incubate for 1 hour shaking* at room temperature.
5. Empty the contents of the wells and wash by adding 400µl of wash buffer to every well. Repeat 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.
6. Pipet 100µl of yellow antibody into each well except the blank.
7. Seal the plate. Incubate for 1 hour shaking* at room temperature.
8. Wash as above (Step 5).
9. Add 100µl of blue conjugate to each well except the blank.
10. Seal the plate. Incubate for 30 minutes on a plate shaker (~500 rpm) at room temperature.
11. Wash as above (Step 5).
12. Pipet 100µl of substrate solution into each well.
13. Incubate for 30 minutes shaking* at room temperature.
14. Pipet 100µl of stop solution into each well.
15. After blanking the plate reader against the substrate blank, read optical density at 450 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

*Shaking is preferably carried out on a suitable plate or orbital shaker set at a speed to ensure adequate mixing of the contents of the wells. The optimal speed for each shaker will vary and may range from 120-700 rpm.

CALCULATION OF RESULTS

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

Several options are available for the calculation of the concentration of Akt1, 2 (phospho-Ser473/474) 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 concentrations can be calculated as follows:

1. Calculate the average Net OD 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

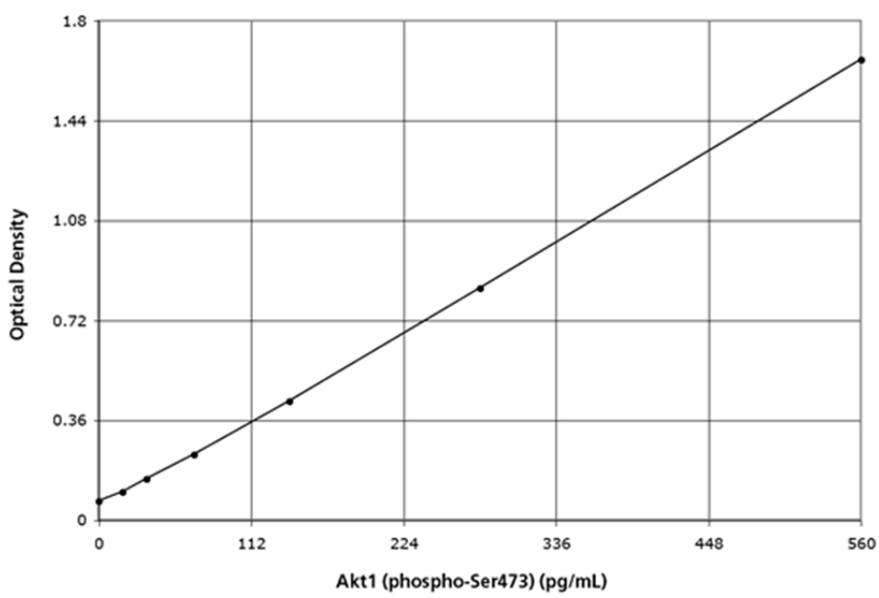
2. Using linear graph paper, plot the average Net OD for each standard versus Akt1 (phospho-Ser473) concentration in each standard. Approximate a straight line through the points. The concentration of the unknowns can be determined by interpolation.

   Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

TYPICAL RESULTS

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

Sample	Net OD	Akt1 (phospho-Ser473) (pg/ml)
S0	0.069	**0**
S1	1.660	**560**
S2	0.836	**280**
S3	0.429	**140**
S4	0.237	**70**
S5	0.149	**35**
S6	0.102	**17.5**
Unknown 1	0.576	**193.4**
Unknown 2	0.113	**31.3**



PERFORMANCE CHARACTERISTICS

Specificity

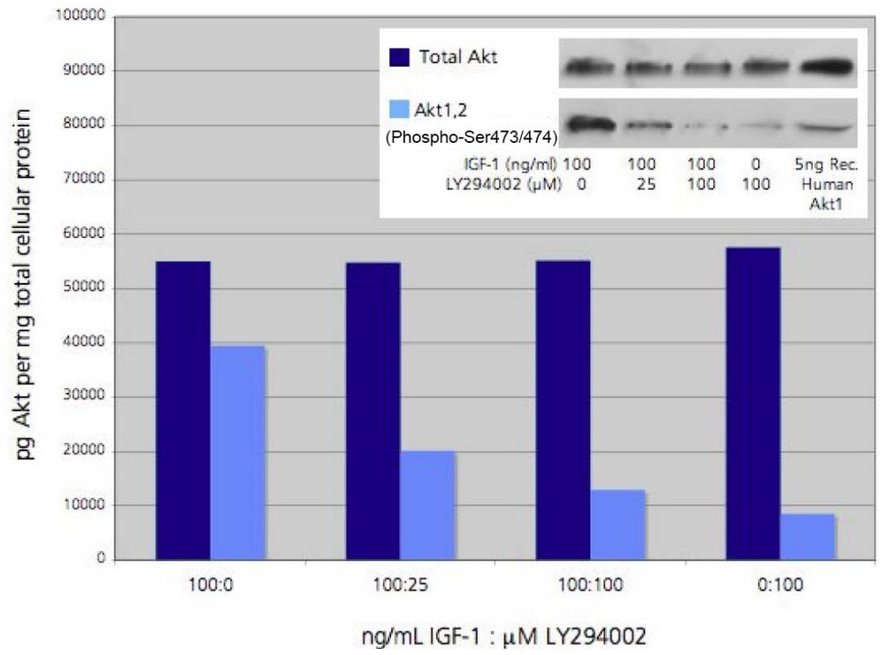
The cross reactivities for a number of related compounds were determined by diluting cross reactants in the assay buffer at several concentrations. These samples were then measured in the assay.

Compound	Cross Reactivity
Inactive Akt1	0.05%
Inactive Akt3	<0.03%
p38α, inactive	0.17%
ERK1, inactive	< 0.03%
ERK2, inactive	< 0.03%
JNK1, inactive	< 0.03%

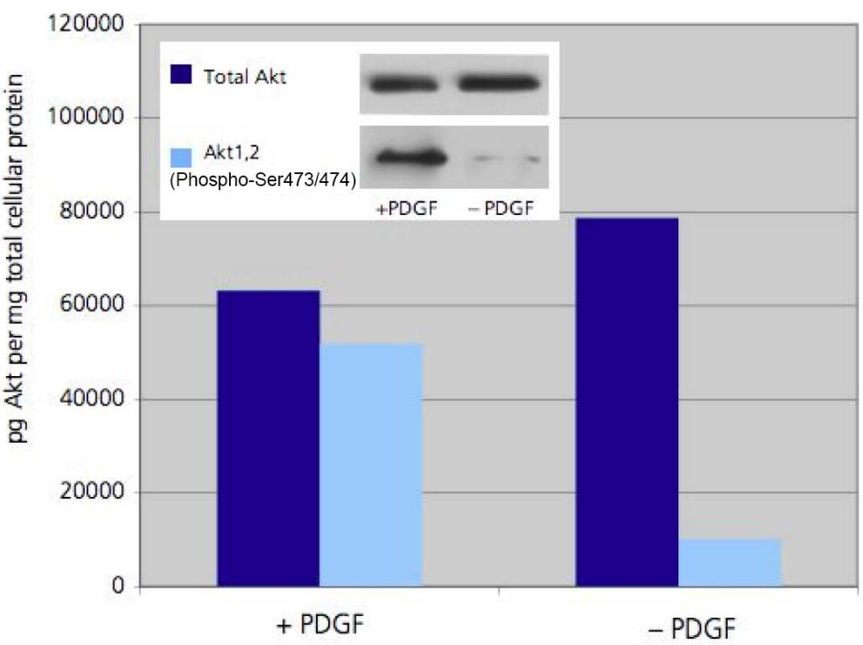
Akt1 and Akt2 have been shown to cross react at ≥100%, while Akt3 has demonstrated lower levels of cross reactivity in the assay. Rat Akt3 is not expected to react in the assay at all due to poor sequence homology. The percent cross reactivity calculation is relative to the Akt1 (phospho-Ser473) standard used in the assay. The percent cross reactivity varies depending on the percent phosphorylation and protein concentration of the cross reactant used and has been shown to vary between vendors as well as between lots from the same vendor.

Stimulation Experiments

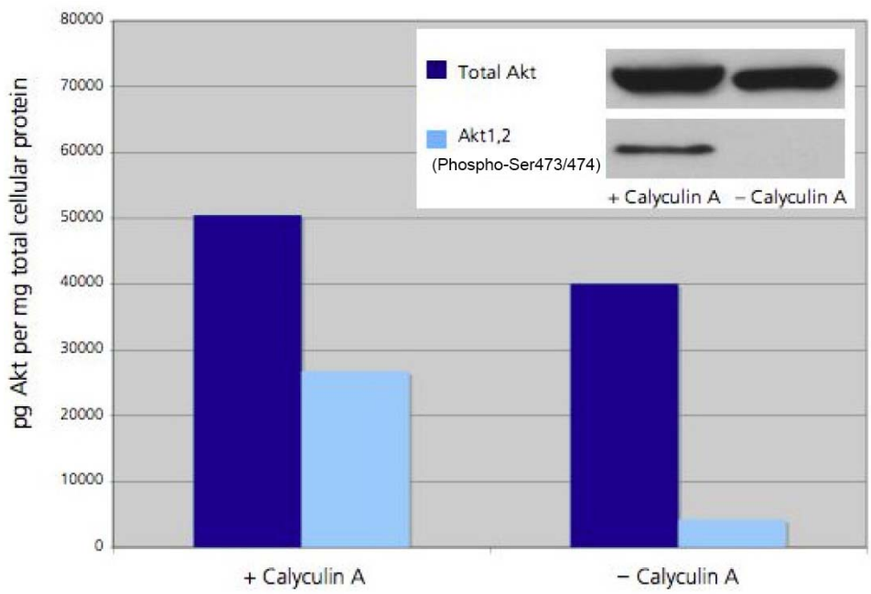
This experiment was adapted from a protocol outlined in reference 13. Human Jurkat cells (2.08×10^6) were treated with increasing concentrations of LY294002, a cell permeable inhibitor of PI3-Kinase, for 20 minutes at 37°C, followed by treatment with 100ng/ml IGF-1, an inducer of Akt phosphorylation, for 30 minutes at 37°C. Cells were washed 3 times in HBSS and lysed in Cell Lysis Buffer 5 treated with inhibitors. Total cellular protein for each sample was determined using a BCA protein assay. Approximately 30µg of total cellular protein or 5ng of recombinant phospho Akt1 (phospho-Ser473) was run on an 8-16% Tris-glycine gradient gel. Proteins were then transferred to a nitrocellulose membrane and probed with antibodies against total Akt and Akt1, 2 (phospho-Ser473/474). The same lysates were also diluted in the assay buffer and run in this kit, as well as a kit specific for total Akt, independent of phosphorylation.



This experiment was adapted from a protocol outlined in reference 2. Mouse 3T3 cells were grown to 75% confluence. The cells were serum starved overnight, then treated with or without 100µg/ml PDGF, an inducer of Akt phosphorylation, at 37°C for 12 minutes. Cells were washed 3 times in HBSS and lysed in Cell Lysis Buffer 5 treated with inhibitors. Total cellular protein for each sample was determined using a BCA protein assay. Approximately 30µg of total cellular protein or 5ng of recombinant Akt1 (phospho-Ser473) was run on an 8-16% Tris-glycine gradient gel. Proteins were then transferred to a nitrocellulose membrane and probed with antibodies against total Akt and Akt1, 2 (phospho-Ser473/474). The same lysates were also diluted in the assay buffer and run in this kit, as well as a kit specific for total Akt, independent of phosphorylation.



Rat C6 cells were grown to 90% confluence. They were treated with or without 25ng/ml Calyculin A, an inducer of Akt phosphorylation, at 37°C for 30 minutes. Cells were washed 3 times in HBSS and lysed in Cell Lysis Buffer 5 treated with inhibitors. Total cellular protein for each sample was determined using a BCA protein assay. Approximately 30µg of total cellular protein or 5ng of recombinant Akt1 (phospho-Ser473) was run on an 8-16% Tris-glycine gradient gel. Proteins were then transferred to a nitrocellulose membrane and probed with antibodies against total Akt and Akt1, 2 (phospho-Ser473/474). The same lysates were also diluted in the assay buffer and run in this kit, as well as a kit specific for total Akt, independent of phosphorylation.



Sensitivity

Sensitivity was calculated as the ratio of the mean OD plus 2 standard deviations of twenty-four replicates of the 0 pg/ml standard to the mean of twenty-four replicates of the lowest standard, multiplied by the concentration of that standard (17.5pg/ml). This value was determined to be 5.55pg/ml.

Linearity

A buffer sample containing Akt1 (phospho-Ser473) was serially diluted 1:2 in assay buffer and measured in the assay. The results are shown in the table below.

Dilution	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
Neat	----------	455.5 pg/ml	---------
1:2	227.8 pg/ml	227.3 pg/ml	99.8 %
1:4	113.9 pg/ml	116.6 pg/ml	102.4 %
1:8	56.9 pg/ml	58.2 pg/ml	102.3 %
1:16	28.5 pg/ml	32.4 pg/ml	113.7 %
1:32	14.2 pg/ml	18.5 pg/ml	130.3 %

Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing Akt1 (phospho-Ser473) in a single assay.

pg/ml	%CV
18	15.9
46	9.7
181	2.5

Inter-assay precision was determined by measuring buffer controls of varying Akt1 (phospho-Ser473) concentrations in multiple assays over several days.

pg/ml	%CV
25	15.4
48	10.5
185	3.3

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