

ab139436

Akt Kinase Activity Assay Kit

Instructions for Use

For screening inhibitors or activators of Akt and for quantitative measurement of Akt activity in purified or partially purified enzyme preparations.

This product is for research use only and is not intended for diagnostic use.

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1. Background

Akt, also known as Protein Kinase B (PKB) is a 57 kDa serine/threonine kinase belonging to a subfamily termed the AGC protein kinases that include PKB isoforms, the cyclic-AMP-dependent PKA, SGK and p90RSK. There are three widely expressed isoforms of PKB (PKB α , PKB β and PKB γ , also known as Akt1, Akt2 and Akt3, respectively) that mediate many of the downstream events controlled by PI3-kinase. Each isoform is composed of an N-terminal PI(3,4,5)P₃- and PI(3,4)P₂-binding Pleckstrin Homology (PH) domain and a C-terminal kinase catalytic domain.

Activation of Akt involves a complex series of events. First, PI3-kinase-generated lipid products, PI(3,4,5)P₃ and PI(3,4)P₂, recruit PKB to the plasma membrane through their affinity for the PH domain of PKB. At the plasma membrane, PKB is thought to undergo a conformational change and becomes activated by the phosphorylation of two residues, Thr-308 within the P-loop of the protein kinase domain and Ser-473.

Akt has been the subject of intense study due to its role in transducing signals from PI3-kinase that regulates cell survival and intermediary metabolism. Akt plays a key role in cancer progression by stimulating cell proliferation and inhibiting apoptosis and is suggested to be a key mediator of insulin signaling. These findings

indicate that Akt is likely to be a hot drug target for the treatment of cancer, diabetes and stroke.

Abcam Akt Kinase Activity Assay Kit (ab139436) provides a safe, rapid and reliable method for screening inhibitors or activators of Akt and for quantitating the activity of Akt in purified or partially purified enzyme preparations.

The kit is based on a solid phase enzyme-linked immuno-absorbent assay (ELISA) that utilizes a specific synthetic peptide as a substrate for Akt and a polyclonal antibody that recognizes the phosphorylated form of the substrate. The assay is designed for analysis of Akt activity in the solution phase.

2. Assay Summary

Prepare all the reagents, samples, and standards as instructed.



Soak wells with Kinase Assay Dilution buffer for 10 minutes at room temperature.



Aspirate wells and add samples to appropriate wells.



Add ATP to all wells to initiate the reaction. Incubate for 90 minutes at 30°C



Empty wells to terminate the kinase reaction.



Add Phosphospecific Substrate Antibody to each well. Incubate for 60 minutes at room temperature.



Add Anti-Rabbit IgG: HRP Conjugate and incubate for 30 minutes at room temperature.



Aspirate and wash each well. Add TMB Substrate Solution to each well. Incubate for 30-60 mins at room temperature



Add Stop Solution 2 and measure absorbance immediately.

3. Precautions

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Store Active Akt separately at -80°C.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9 Reagent Preparation.

5. Materials Supplied

Item	Amount	Storage Temperature
Akt Substrate Microtiter Plate	1 unit	+4°C
Akt Phosphospecific Substrate Antibody (1 µg/mL solution)	5 mL	+4°C
Anti-Rabbit IgG: HRP Conjugate (1 mg/mL solution)	20 µL	+4°C
Antibody Dilution Buffer	10 mL	+4°C
Kinase Assay Dilution Buffer	10 mL	+4°C
ATP	2 mg	+4°C
Active Akt	30 µL	-80°C
20X Wash Buffer	30 mL	+4°C
TMB Substrate	10 mL	+4°C
Stop Solution 2	10 mL	+4°C

6. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Deionized or distilled water
- Disposable pipette tips
- Precision pipettes capable of accurately delivering volumes between 1 μL and 1000 μL
- Repeater pipettes
- Squirt bottle, manifold dispenser, or automated microtiter plate washer
- Graduated cylinders
- Adsorbent paper for blotting
- Microtiter plate reader capable of measuring absorbance at 450 nm
- Adhesive plate sealers
- Mono Q anion exchange columns

7. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

8. Technical Hints

- For statistical results, it is recommended that assays be run in triplicate.
- To avoid cross contamination, change disposable pipette tips between the addition of each standard, sample, and reagent and add to the side of the wells. Use separate troughs/reservoirs for each reagent.
- This assay requires pipetting of small volumes. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated and pipette tips are pre-rinsed with the reagent.
- Mix all reagents and samples gently, yet thoroughly, prior to use. Avoid foaming of reagents.
- Consistent, thorough washing of each well is critical. If using an automatic washer, check washing head before use. If washing manually, ensure all wells are completely filled at each wash. Air bubbles should be avoided.
- When aspirating, tilt plate slightly to carefully remove liquid from the well.
- Absorbance is a function of the incubation time. Therefore, prior to starting the assay it is recommended that all reagents are ready to use and all required strip-wells secured in the microtiter frame. This will ensure equal elapsed time for each pipetting step, without interruption.

- For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 15 minutes.
- Before performing the kinase assay, it is strongly recommended that an initial experiment be performed to determine an appropriate dilution of the purified sample and reaction time to carry out subsequent studies.
 - Perform a time course using various kinase concentrations, including a no-enzyme blank, to confirm a linear response of the kinase with respect to phosphorylation.
 - Select a reaction time and kinase concentration from the results obtained in the previous step. This will provide a sufficient window of phosphorylation to yield statistically reliable results.

9. Reagent Preparation

The following kit components should be brought to room temperature (18-25°C) prior to use: Akt Substrate Microtiter Plate, Antibody Dilution Buffer, Kinase Assay Dilution Buffer, 20X Wash Buffer, TMB Substrate and Stop Solution 2.

9.1 **Active Akt**

The Active Akt is intended to be used as a positive control and can be serially diluted in Kinase Assay Dilution Buffer to a final volume of 30 µL. Keep preparations on ice. 30 µL of Kinase Assay Dilution Buffer (without kinase) can be used as the assay blank. If assaying on separate occasions, once thawed, the purified Active Akt may be aliquotted into smaller portions, stored at -80°C and subsequently thawed only once. Refrozen aliquots may result in a reduction in kinase activity.

9.2 **ATP**

Centrifuge the vial before removing the cap to ensure maximum product recovery. Reconstitute the ATP with 2 mL of Kinase Assay Dilution Buffer. Mix gently by inversion. Any remaining diluted ATP can be stored at -20°C for up to 6 months or until the kit's expiry date whichever is earlier.

9.3 **Anti-Rabbit IgG: HRP Conjugate**

Centrifuge the vial before removing the cap to ensure maximum product recovery. Dilute the Anti-Rabbit IgG: HRP Conjugate to 1 µg/mL (1:1000) with Antibody Dilution Buffer in a polypropylene tube. A minimum of 4 mL of working solution is required for 96-wells (40 µL/well). If only using a portion of the plate, dilute only what is needed for the number of wells used. Mix gently by inversion. Do not re-use or store any remaining diluted Anti-Rabbit IgG: HRP Conjugate.

9.4 **20X Wash Buffer**

Bring the 20X Wash Buffer to room temperature and swirl gently to dissolve any crystals that may have formed during storage. Dilute the 30 mL of 20X Wash Buffer with 570 mL of deionized or distilled water. Once diluted, the 1X Wash Buffer is stable at room temperature for up to 4 weeks. For longer term storage, the Wash Buffer should be stored at +4°C.

10. Sample Collection and Storage

10.1 Cell Lysates (Adherent Cells) –

- 10.1.1 Treat cells according to desired protocol (i.e. agonist/inhibitor).

Note: Desired confluence of plate is determined by individual researcher. Recommended: 90% confluency/100 mm dish.

- 10.1.2 Remove media from plate using suction filtration.
- 10.1.3 Wash plate 1X with ice-cold PBS (1 M, pH 7.4).
- 10.1.4 Add 1 mL of lysis buffer, pH 7.2 [20 mM MOPS, 50 mM β -glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM phenylmethane-sulphonylfluoride (PMSF) and 10 μ g/mL leupeptin and aprotinin] to 100 mm tissue culture plate and allow to stand for 10 minutes on ice.

Note: Add PMSF, leupeptin and aprotinin to tissue culture plate prior to preparation of lysate.

- 10.1.5 After 10 minutes incubation period, scrape cells using a rubber policeman/cell scraper and collect cell lysate in a pre-chilled 1.5 mL microcentrifuge

tube. Keep on ice. Optional: Sonicate lysate, 3 x 20 second intervals.

10.1.6 Centrifuge at 13,000 rpm for 15 minutes.

10.1.7 Transfer clear supernatant to a pre-chilled 1.5mL microcentrifuge tube. This is the cytosolic fraction.

Note: samples may be stored at -80°C. For optimal results, it is recommended that fresh lysates be used for each experiment as the kinase activity decreases with each subsequent freeze thaw.

10.1.8 Determine protein concentration using BCA method.

10.2 Cell Lysates (Suspension Cells) –

10.2.1 Treat cells according to desired protocol (i.e. agonist/inhibitor).

10.2.2 Transfer cells to 15 ml conical tube.

10.2.3 Spin cells at 1200 rpm for 5-10 minutes to pellet. Optional: Wash cells with 5 mL of 1X with ice-cold PBS (1 M, pH 7.4).

10.2.4 Add 1 mL of lysis buffer, pH 7.2 [20 mM MOPS, 50 mM β -glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM phenylmethane-

sulphonylfluoride (PMSF) and 10 µg/mL leupeptin and aprotinin] to 100 mm tissue culture plate and allow to stand for 10 minutes on ice.

Note: Add PMSF, leupeptin and aprotinin to tissue culture plate prior to preparation of lysate.

10.2.5 After 10 min incubation period, scrape cells using a rubber policeman/cell scraper and collect cell lysate in a pre-chilled 1.5 mL microcentrifuge tube. Keep on ice. Optional: Sonicate lysate, 3 x 20 second intervals.

10.2.6 Centrifuge at 13,000 rpm for 15 minutes.

10.2.7 Transfer clear supernatant to a pre-chilled 1.5 mL microcentrifuge tube and store at -80°C. This is the cytosolic fraction.

Note: samples may be stored at -80°C. For optimal results, it is recommended that fresh lysates be used for each experiment as the kinase activity decreases with each subsequent freeze thaw.

10.2.8 Determine protein concentration using BCA method.

10.3 Tissue Extracts (Protocol 1) –

- 10.3.1 Weigh ~ 1 g of tissue, place in a Petri dish on ice and slice tissue into tiny pieces.
- 10.3.2 Add 5 mL of lysis buffer, pH 7.2 [20 mM MOPS, 50 mM β -glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM phenylmethane-sulphonylfluoride (PMSF) and 10 μ g/mL leupeptin and aprotinin].

Note: Add PMSF, leupeptin and aprotinin prior to preparation of lysate.

- 10.3.3 Transfer sample in lysis buffer to a pre-chilled 15 mL conical tube and process the tissue using a polytron at setting of 10,000 rpm (3 x 20 second bursts).
- 10.3.4 Allow to stand on ice for 10 minutes
- 10.3.5 Centrifuge at 150,000 g for 30 minutes at 4°C.
- 10.3.6 Transfer clear supernatant to pre-chilled 1.5 mL microcentrifuge tube. This is the cytosolic fraction.

Note: samples may be stored at -80°C. For optimal results, it is recommended that fresh lysates be used for each experiment as the kinase

activity decreases with each subsequent freeze thaw.

10.3.7 Determine protein concentration using BCA method.

10.4 **Tissue Extracts (Protocol 2) –**

10.4.1 Slice tissue into thin sections using a cryostat (3-5 micron sections).

10.4.2 Place sections into a pre-chilled microcentrifuge tube containing 1 mL of lysis buffer, pH 7.2 [20 mM MOPS, 50 mM β -glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM phenylmethane-sulphonylfluoride (PMSF) and 10 μ g/mL leupeptin and aprotinin].

Note: Add PMSF, leupeptin and aprotinin prior to preparation of lysate.

10.4.3 Using a handheld homogenizer, perform 3 x 20 second bursts.

10.4.4 Allow to stand on ice for 10 minutes.

10.4.5 Sonicate lysate 3 x 20 second intervals.

10.4.6 Allow to stand on ice for 10 minutes.

10.4.7 Centrifuge at 13,000 rpm for 15 minutes.

10.4.8 Transfer clear supernatant to a pre-chilled 1.5 mL microcentrifuge tube and store at -80°C . This is the cytosolic fraction. Transfer clear supernatant to a pre-chilled 1.5 mL microcentrifuge tube. This is the cytosolic fraction.

Note: samples may be stored at -80°C . For optimal results, it is recommended that fresh lysates be used for each experiment as the kinase activity decreases with each subsequent freeze thaw.

10.4.9 Determine protein concentration using BCA method. Note: There are several acceptable methods for preparing tissue lysates that have been published in the literature. The preceding protocols are provided as examples of suitable methods.

11. Sample Preparation

General Sample information:

Sample preparations have not been used directly with this assay. It is suggested that purified or partially purified kinase preparations be used.

11.1 Partially Purified Fractions –

- 11.1.1 Prepare cell lysates or tissue extracts according to desired protocol.
- 11.1.2 Evaluate total protein concentration.
- 11.1.3 Equilibrate Mono Q anion exchange column (1 mL column) with Buffer A (containing 10mM MOPS, pH 7.2, 25mM β -glycerolphosphate, 5mM EGTA, 2mM EDTA, 2mM sodium orthovanadate and 2nM DTT).
- 11.1.4 Load 1-2 mg of protein onto Mono Q anion-exchange column and run at a flow-rate of 0.5 mL/min using a 12 mL linear NaCl gradient (0 – 0.8 M NaCl).
- 11.1.5 Collect between 0.25 – 0.5 mL fractions.
- 11.1.6 Assay fractions as outlined in manual.

11.2 For Inhibitor Or Activator Screening –

- 11.2.1 Dilute the inhibitor appropriately. It is recommended that the inhibitor diluent by itself be used as a negative control.
- 11.2.2 Incubate the kinase in the presence of the inhibitor prior to initiating the kinase reaction (Section 13)

12. Plate Preparation

- Determine the number of wells to be used. If less than 96 pre-coated microtiter wells are needed, remove the excess wells from the frame and return them to the foil pouch. Reseal the pouch containing the unused wells with the desiccant and store at 4°C.
- Soak each well of the Akt Substrate Microtiter Plate with 50 µL of Kinase Assay Dilution Buffer at room temperature for 10 minutes.
- Carefully aspirate liquid from all wells.

13. Assay Procedure

- **Equilibrate Akt Substrate Microtiter Plate, Antibody Dilution Buffer, Kinase Assay Dilution Buffer, 20X Wash Buffer, TMB Substrate and Stop Solution 2 to room temperature prior to use.**
- **For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 15 minutes.**
- **It is recommended to assay all wells in triplicate.**

13.1. Add 30 μ L of each of the following to appropriate wells:

- Purified Active Akt control (diluted)
- Samples
- Blank (Kinase Assay Dilution Buffer with no kinase)
- Negative Control (Inhibitor Diluent with no inhibitor)
(use for inhibitor screening studies)

13.2. Initiate reaction by adding 10 μ L of diluted ATP to each well, except blank. To avoid cross contamination, change pipette tips for each well.

13.3. Cover wells with an adhesive plate sealer and incubate at 30°C for up to 90 minutes, preferably with gentle, thorough shaking every 20 minutes by hand or a shaker with rotate angle at 60 rpm. Thorough mixing is recommended to yield optimal results

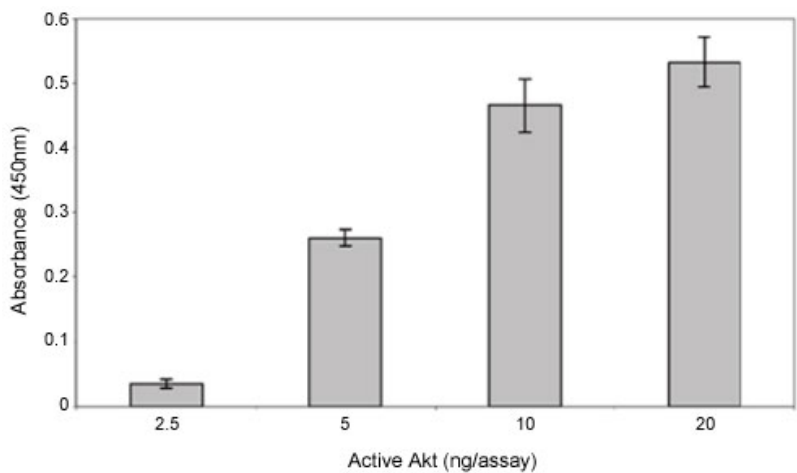
Note: It is recommended that the experiment uses the predetermined time point generated as outlined in Section 8.

- 13.4. Stop reaction by emptying contents of each well. Invert the plate and carefully pat dry on clean paper towels.
- 13.5. Add 40 μ L of the Phosphospecific Substrate Antibody to each well, except blank.
- 13.6. Cover wells with a fresh adhesive plate sealer and incubate at room temperature for 60 minutes, preferably with gentle, thorough shaking every 20 minutes
- 13.7. Aspirate liquid from all wells.
- 13.8. Add 100 μ L of 1X Wash Buffer to all wells, using a multi-channel pipette, manifold dispenser, automated microplate washer, or a squirt bottle. (To reduce background, it may be necessary to wait 1-2 minutes between each wash).
- 13.9. Repeat the aspirating and washing 3 more times with 1X Wash Buffer for a total of 4 washes.
- 13.10. After the 4th wash, aspirate the liquid from all wells. Invert the plate and carefully pat dry on clean paper towels.
- 13.11. Add 40 μ L of the diluted Anti-Rabbit IgG: HRP Conjugate to each well, except blank.
- 13.12. Incubate at room temperature for 30 minutes, preferably with gentle, thorough shaking every 10 minutes.
- 13.13. Wash plate as described above.

- 13.14. Add 60 μL of the TMB Substrate to each well.
- 13.15. Incubate at room temperature for 30-60 minutes (incubation time should be monitored by the investigator according to color development).
- 13.16. Add 20 μL of the Stop Solution 2 to each well in the same order that the TMB Substrate was added.
- 13.17. Set up the microplate reader according to the manufacturer's instructions. Set wavelength at 450 nm and measure the absorbance.

14. Typical Data

The graph below illustrates results using purified active Akt included with this kit. Varying quantities of purified active Akt were added the Akt Substrate Mictotiter Plate and incubated for 60 minutes at 30°C. Activity was detected as described in the Assay Procedure. The data represented below is an example only and should not be used to calculate actual assay results.



15. Data Analysis

Calculating Kinase Activity in Column Fractions

Relative kinase activity =

$$\frac{(\text{Average Absorbance}_{(\text{sample})} - \text{Average Absorbance}_{(\text{blank})})}{\text{Volume used in assay}} \times \begin{matrix} \text{Dilution} \\ \text{Factor} \end{matrix}$$

Calculating Kinase Activity of Purified Kinase

Relative kinase activity =

$$\frac{(\text{Average Absorbance}_{(\text{sample})} - \text{Average Absorbance}_{(\text{blank})})}{\text{Quantity of purified kinase used per assay}}$$

16. Typical Sample Values

Precision

	Intra-Assay	Inter-Assay
%CV	<10%	<12%

17. Interferences

The activity of the Anti-Rabbit IgG: HRP Conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.

18. Troubleshooting

Problem	Cause	Solution
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Inaccurate pipetting	Check pipettes
High background	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the Active Kinase at -80°C, all other assay components 4°C. Keep substrate solution protected from light.

Technical Support

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