Non-Radioisotopic Kit for Measuring AKT/PKB Activity

CycLex AKT/PKB Kinase Assay/Inhibitor Screening Kit

Cat# CY-1168

Intended Use.. 1
Storage... 1
Introduction 2
Principle of the Assay................................. 3
Materials Provided 4
Materials Required but not Provided 4
Precautions and Recommendations............ 5
Detailed Protocol... 6-9
Evaluation of Results 10
Assay Characteristics 10
Troubleshooting 10
Reagent Stability 10
Example of Test Results.............................. 11-12
References.. 13

Intended Use

The MBL Research Product **AKT/PKB kinase Assay/Inhibitor Screening Kit** is designed to measure the activities of purified AKTs for the rapid and sensitive evaluation of inhibitors or activators. The phospho-serine specific monoclonal antibody used in this assay kit has been demonstrated to recognize the phospho-serine residue in AKTide-2T, which is efficiently phosphorylated by AKTs.

Applications of this kit include:
1) Screening inhibitors or activators of AKT.
2) Detecting the effects of pharmacological agents on AKT activity.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at 4°C.
- Don't expose reagents to excessive light.

Introduction

The AKT oncogene was isolated from the directly transforming murine retrovirus AKT8, which was isolated from an AKR mouse thymoma cell line. Staal cloned the human cellular homolog, AKT1 (1). He found a 20-fold amplification of the AKT1 gene in 1 of 5 gastric adenocarcinomas tested.

Phosphoinositide 3-kinases (PI3Ks) generate specific inositol lipids that have been implicated in the regulation of cell growth, proliferation, survival, differentiation and cytoskeletal changes. One of the best characterized targets of PI3K lipid products is the protein kinase AKT or protein kinase B (PKB). In quiescent cells, AKT resides in the cytosol in a low-activity conformation. Upon cellular stimulation, AKT is activated through recruitment to cellular membranes by PI3K lipid products and phosphorylation by 3'-phosphoinositide-dependent kinase-1 (PDK1).

Mammals have three closely related AKT genes, encoding the isoforms AKT1, AKT2 and. AKT3 AKT2 and. AKT3 show 81 and 83% amino acid identity with AKT1 respectively. All AKT isoforms show a broad tissue distribution and consist of an N-terminal PH domain, a kinase domain and a C-terminal regulatory tail Two specific sites, one in the kinase domain (Thr308 in AKT1) and the other in the C-terminal regulatory region (Ser473 in AKT1), need to be phosphorylated for full activation of these kinases. AKT was among the first proteins known to contain a PH domain, a few years before the function of this domain came to light. The PH domain of AKT specifically binds PI3K lipid products, and a firm link between PI3K and AKT signalling has now been established. AKT is cytosolic in unstimulated cells, and some of it translocates to the plasma membrane upon activation of PI3K, where it becomes activated (2, 3). Active AKT then appears to detach from the plasma membrane and to translocate through the cytosol to the nucleus. The mechanism of this translocation is unclear.

AKT1 was found to mediate insulin- and insulin-like growth factor (IGF-1)-induced cellular responses, such as the inhibition of glycogen synthase kinase-3 (4), the stimulation of glucose uptake (5) and the promotion of cell survival by inhibiting apoptosis (6). Overexpression of AKT1 or AKT2 is associated with some human ovarian, pancreatic, and breast carcinomas (7-9).

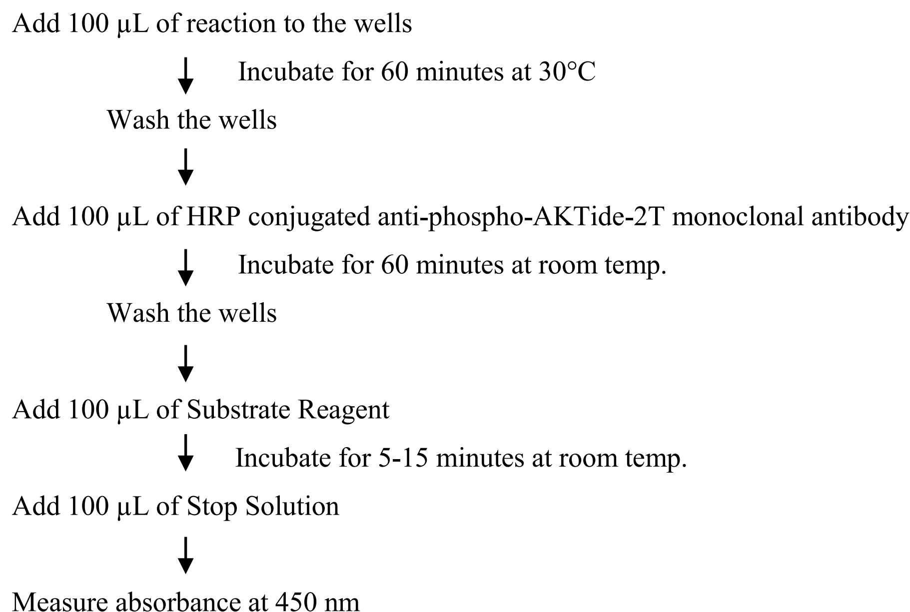
Measurement of AKT activity

The protocol generally regarded as most sensitive for the quantitative measurement of AKT activity involves incubation of the AKT sample with substrate, either a natural or synthetic polypeptide (such as AKTide-2T), in the presence of $Mg^{2+}$and ^{32}P-labeled ATP. The reaction is terminated by "spotting" a sample onto a phospho-cellulose P81 filter paper disc, followed by washing extensively to remove unincorporated radiolabel and the radioactivity counted. While sensitive, this method is labor-intensive, generates hazardous radioactive waste and depends on a radioisotope of short half-life. It is particularly unsuitable when kinase assays are only performed on an infrequent basis. The **CycLex AKT/PKB kinase Assay/Inhibitor Screening Kit** uses peroxidase coupled anti-phospho-AKTide-2T monoclonal antibody as a reporter molecule in a 96-well ELISA format. This assay provides a non-isotopic, sensitive and specific method to measure the activities of AKT/PKB kinase.

Principle of the Assay

The MBL Research Product **CycLex AKT/PKB kinase Assay/Inhibitor Screening Kit** is a single-site, semi-quantitative immunoassay for AKT activity. Plates are pre-coated with "AKTide-2T" (10), which can be efficiently phosphorylated by AKT1, 2 and 3 on a microtiter plate. The detector antibody is AT-3E2, an antibody that specifically detects only the phosphorylated "AKTide-2T". The **CycLex AKT/PKB kinase Assay/Inhibitor Screening Kit** can be used to study the kinetics of a purified or partially purified AKT as well as to screening AKT inhibitor or activator. To perform the test, the sample is diluted in Kinase Buffer, pipetted into the wells and allowed to phosphorylate the bound substrate following the addition of Mg^{2+} and ATP. The amount of phosphorylated substrate is measured by binding it with a horseradish peroxidase conjugate of AT-3E2, a anti-phospho-AKTide-2T monoclonal antibody, which then catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stopping reagent). The color is quantitated by spectrophotometry and reflects the relative amount of AKT activity in the sample. For kinetic analysis, the AKT containing sample is added to the wells in a similar fashion and at varying times the reaction is stopped by the addition of the chelator, sodium ethylenediaminetetraacetate (EDTA) and the amount of phosphorylated substrate determined as before.

Summary of Procedure



Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microtiter plate kit.

Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with AKTide-2T as an AKT substrate.

10X Wash Buffer: One bottle containing 100 mL of 10X buffer containing Tween®-20

Kinase Buffer: One bottle containing 20 mL of 1X buffer; used for Kinase Reaction Buffer and sample dilution.

20X ATP: One vial of lyophilized ATP Na_2 salt.

HRP conjugated Detection Antibody: One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-phospho-AKTide-2T monoclonal antibody (AT-3E2). Ready to use.

Substrate Reagent: One bottle containing 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle containing 20 mL of 1 N H_2SO_4. Ready to use.

Materials Required but not Provided

- **AKT Positive Controls:** Available from MBL (AKT1: Cat# CY-E1168-1, AKT2: Cat# CY-E1168-2). *The AKT1 Positive Controls should be diluted with Enzyme dilution buffer (See below) to avoid inactivating the enzyme activity in low protein concentration condition.*
- **Enzyme dilution buffer**: Mix 9-parts of Kinase buffer and 1-part of 10X BSA (100 µg/mL x 0.25 mL), which is supplied with AKT Positive Controls (AKT1: Cat# CY-E1168-1, AKT2: Cat# CY-E1168-2).
- **(Optional) 10X Staurosporine (10 µM):** A broad spectrum protein kinase Inhibitor, available from Sigma, Cat# S-4400. 1 mM stock solution (DMSO) diluted 1:100 in Kinase Buffer.
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Wash bottle or multichannel dispenser** for plate washing.
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **(Optional) Microplate washer:** Manual washing is possible but not preferable.
- **Plate reader:** capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1,000 mL graduated cylinder**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**
- Allow all the components to come to room temperature before use.
- All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents in this kit may contain preservatives or other chemicals. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of human origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**
- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

Detailed Protocol

The **CycLex AKT/PKB kinase Assay /Inhibitor Screening Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since conditions may vary, running an aliquot of AKT1 Positive Control or AKT2 Positive Control (See the section "Materials Required but not Provided" above) should be included in each assay. Disposable pipette tips and reagent troughs should be used for all transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solutions

1. Prepare a working solution of **Wash Buffer** by adding 100 mL of the **10X Wash Buffer** (provided) to 900 mL of deionized (distilled) water (ddH_2O). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Prepare **20X ATP Solution** by adding **0.8 mL** of ddH_2O to the vial of **20X ATP** (provided, lyophilized). Mix gently until dissolved. The final concentration of the **20X ATP Solution** should be **2.5 mM**. Store the solution in small aliquots (e.g. 100 µL) at -20°C.

3. Prepare **Kinase Reaction Buffer** by mixing following reagents.

	96 assays	**10 assays**	**1 assay**
Kinase Buffer (provided)	**9.5 mL**	**950 µL**	**95 µL**
20X ATP Solution	**0.5 mL**	**50 µL**	**5 µL**
Total	**10 mL**	**1000 µL**	**100 µL**

**You will need 80-90 µL of Kinase Reaction Buffer per assay well. Mix well. Discard any unused Kinase Reaction Buffer after use.*

Standard Assay

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.

2. Prepare all samples (diluted with Kinase Buffer as needed). All samples should be assayed in duplicate.

3. Add **10 µL** of diluted enzyme sample to the well of the assay plate on ice. Duplicate wells containing 10 µL of AKT1 or AKT2 Positive Control (2 m units/µL) should be included in each assay as a positive control for phosphorylation.

4. Begin the kinase reaction by addition of **90 µL** of **Kinase Reaction buffer** per well, cover with plate sealer, and incubate **at 30°C for 60 minutes**.

5. Wash wells five times with Wash Buffer making sure each well is filled completely. Remove residual Wash Buffer by gentle tapping or aspiration.

6. Pipette **100 µL** of **HRP conjugated Detection Antibody** into each well, cover with a plate sealer and incubate **at room temperature (ca.25°C) for 60 minutes**. Discard any unused conjugate.

7. Wash wells five times as same as in step5.

8. Add **100 µL** of **Substrate Reagent** to each well and incubate **at room temperature (ca.25°C) for 5–15 minutes**.

9. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

10. Measure absorbance in each well using a spectrophotometric plate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the plate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Kinetic Assay

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.

2. Prepare all samples (diluted with Kinase Buffer as needed). All samples should be assayed in duplicate.

3. Add **10 µL** of diluted enzyme sample to the well of the assay plate on ice. Duplicate wells containing 10 µL of AKT1 or AKT2 Positive Control (2.5 m units/µL) should be included in each assay as a positive control for phosphorylation.

4. Begin kinase reaction by addition of **90 µL** of **Kinase Reaction Buffer** in duplicate per well in timed intervals (suggested interval is 5 minutes but should be individually determined for each system). After the final addition, incubate **at 30°C for 20 minutes**.

5. Stop the reaction by flicking out the contents. (Alternatively, the reaction may be terminated by the addition of 150 µL 0.1 M Na EDTA, pH 8.0 to each well).

6. Wash wells five times with Wash Buffer making sure each well is filled completely. Remove residual Wash Buffer by gentle tapping or aspiration.

7. Pipette **100 µL** of **HRP conjugated Detection Antibody** into each well, cover with a plate sealer and incubate **at room temperature (ca.25°C) for 60 minutes**. Discard any unused conjugate.

8. Wash wells as same as in Step 6.

9. Add **100 µL** of **Substrate Reagent** to each well and incubate **at room temperature (ca.25°C) for 10-15 minutes**.

10 add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

11. Measure absorbance in each well using a spectrophotometric plate reader at dual wavelengths of

450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the plate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Recommendations

Special considerations when screening activators or inhibitors

In order to estimate the inhibitory effect on individual AKT activity in the test chemicals correctly, it is necessary to conduct the control experiment of "Solvent control" at least once for every experiment and "Inhibitor control" at least once for the first experiment, in addition to "Test sample", as indicated in the following table. When test chemicals cause an inhibitory effect on AKT activity, the level of A450 is weakened as compared with "Solvent control". The high level of A450 is not observed in "Inhibitor control" (usually A450<0.3).

Assay reagents	Test sample	Solvent control	Inhibitor control
Kinase Reaction buffer	80 µL	80 µL	80 µL
10X Inhibitor or equivalent	10 µL	-	-
Solvent for Inhibitor	-	10 µL	-
10X Staurosporine (10 µM)*	-	-	10 µL
AKT Positive Control (2.5 m unit/µL)* or Your enzyme samples	10 µL	10 µL	10 µL

* See the section "Materials Required but not Provided" above.

1. Following the above table, add the Reagents to each well of the microplate. Finally, initiate reaction by adding 10 µL of diluted "AKT Positive Control" or "Your enzyme samples" to each well and mixing thoroughly at room temperature. Cover with plate sealer or lid, and incubate **at 30°C for 60 minutes**.

2. Follow the step 5 to 10 of "**Standard Assay**" above.

Special considerations when measuring precise AKT activity

In order to measure the activity of AKT correctly, it is necessary to conduct the control experiment of "Inhibitor control" at least once for every experiment and "ATP minus control" at least once for the first experiment, in addition to "No enzyme control" as indicated in the following table. Although the level of A450 increases in "Test sample" when AKT enzyme activity is in the sample, the high level of A450 is not observed in "Inhibitor control", "ATP minus control" and "No enzyme control".

Assay reagents	Test Sample	Inhibitor control	ATP minus control	Positive control	No enzyme control
Kinase Reaction buffer	90 µL	80 µL	-	90 µL	90 µL
Kinase Buffer (provided)	-	-	90 µL	-	-
10X Staurosporine (10 µM)*	-	10 µL	-	-	-
Your enzyme samples	10 µL	10 µL	10 µL	-	-
AKT Positive Control (2.5 m unit/µL)*	-	-	-	10 µL	-
Buffer	-	-	-	-	10 µL

* See the section "Materials Required but not Provided" above.

1. Following the above table, add the Reagents to each well of the microplate. Finally, initiate the reaction by adding 10 µL of "Your enzyme samples" or "AKT Positive Control" or "Buffer" to each well and mixing thoroughly at room temperature. Cover with plate sealer or lid, and incubate **at 30°C for 60 minutes**.

2. Follow the step 5 to 10 of "**Standard Assay**" above.

Evaluation of Results

1. Average the absorbance values for the AKT sample duplicates (positive control) and all experimental sample duplicate values (when applicable). When AKT Positive Control (25 m units/assay) is included as an internal control for the phosphorylation reaction, the absorbance value should be greater than 1.0 with a background less than 0.2.

2. For screening of purification/chromatography fractions, on graph paper, plot the mean absorbance values for each of the samples on the Y-axis versus the fraction number on the X-axis to determine the location of the eluted, purified AKT.

3. For kinetic analysis, on graph paper, plot the mean absorbance values for each of the time points on the Y-axis versus the time of each reaction (minutes) on the X-axis.

Assay Characteristics

The MBL Research Product **CycLex AKT/PKB kinase Assay/Inhibitor Screening Kit** has been shown to detect the activity of AKT in column fractions of human or animal cell extracts. The assay shows good linearity of sample response. The assay may be used to follow the purification of AKT.

Troubleshooting

1. All samples and standards should be assayed in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.

2. The reaction curve is nearly a straight line if the kinetics of the assay is of the first order. Variations in the protocol can lead to non-linearity of the curve, as can assay kinetics of other than first order. For a non-linear curve, point to point or quadratic curve fit methods should be used.

3. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for washer maintenance.

4. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the MBL Research Product **CycLex AKT/PKB kinase Assay/Inhibitor Screening Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt kit reagents should be stored at 4°C, except the ATP must be stored at -20°C. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

Example of Test Results

Fig.1 Dose dependency of recombinant AKT enzyme reaction

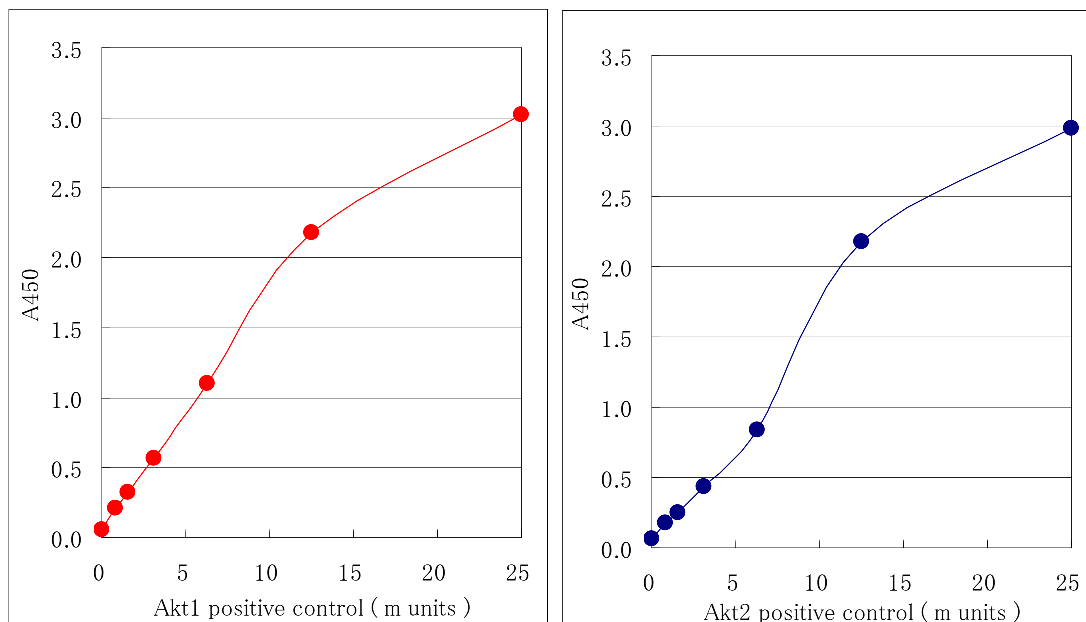


Fig.2 Time course of recombinant AKT enzyme reaction

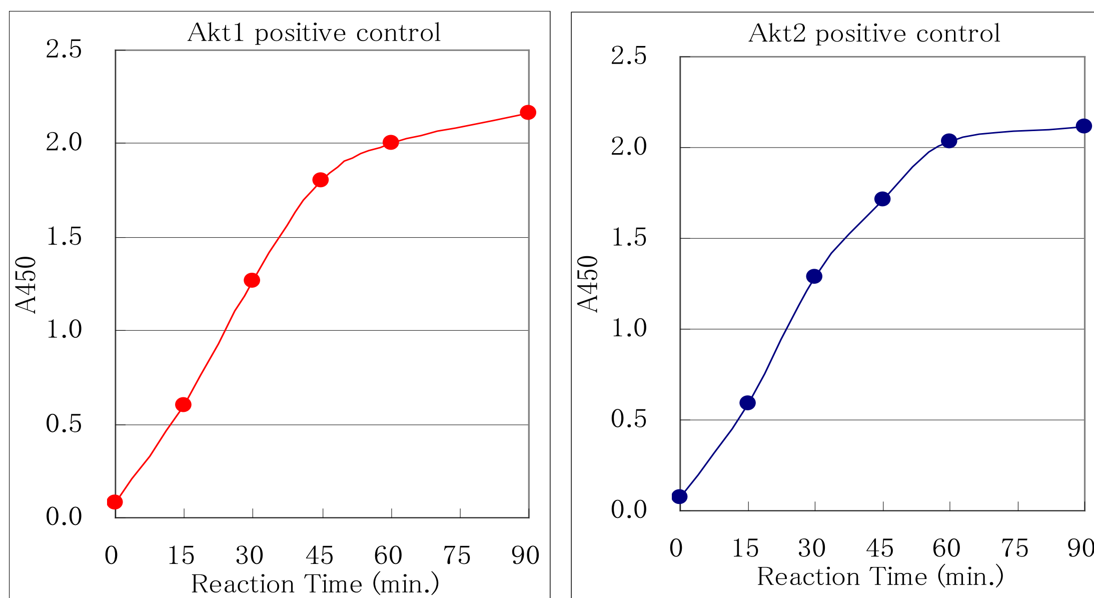
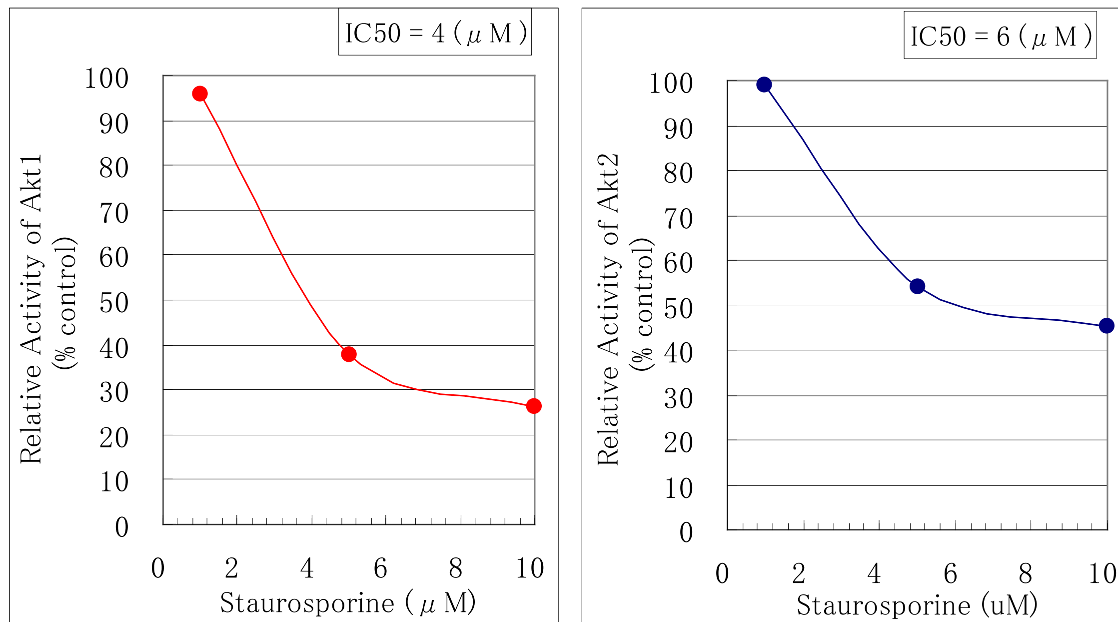


Fig.3 Effect of broad-spectrum kinase inhibitor staurosporine on AKT activity



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