



Non-Radioisotopic Kit for Measuring AKT/PKB kinase Activity

# CycLex AKT/PKB kinase Assay/Inhibitor Screening Kit

Cat# CY-1168

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## Intended Use

The CycLex 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.



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## Introduction

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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.



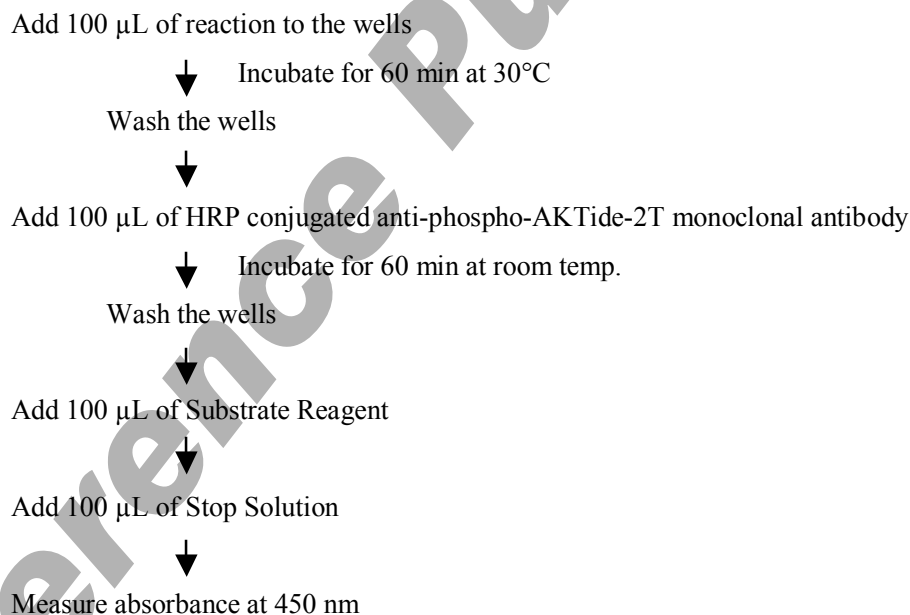
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## Principle of the Assay

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The CycLex 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





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## Materials Provided

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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 2 %Tween<sup>®</sup>-20

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

**20X ATP:** Lyophilized ATP Na<sub>2</sub> salt. Reconstitute contents of vial with **0.8 mL** of ddH<sub>2</sub>O. (See section "Preparation of Working Solution" page 6.)

**HRP conjugated Detection Antibody:** One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-phospho-AKTide-2T (AT-3E2) monoclonal antibody. 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<sub>2</sub>SO<sub>4</sub>. Ready to use.

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## Materials Required but not Provided

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- **AKT positive control:** Available from CycLex (AKT1 positive control: Cat# CY-E1168-1, AKT2 positive control: Cat# CY-E1168-2. One vial containing 5 units/100 µL AKT enzyme. Positive control should be added to the first well at 25 m units/well. For instance, diluted positive control 1:20 with enzyme dilution buffer, use 10 µL for 1 assay. (Unused AKT enzyme should be stored in aliquots at -70°C.) *The AKT1 Positive control should be diluted with an enzyme dilution buffer 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 control.
- **10X Staurosporine (10 µM):** Staurosporine is 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 200-1000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Wash bottle or multichannel dispenser** for plate washing.
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **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.
- **500 or 1000 mL graduated cylinder**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**



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## Precautions and Recommendations

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- Store the AKT enzyme at -70°C and the ATP at -20°C when not in use. Store all other components at 4°C. Do not expose reagents to excessive light. Avoid freeze/thaw cycles.
- 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 used in this kit contain either sodium Kathon-CG as preservatives. 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 Substrate Solution which contains hydrogen peroxide.
- Avoid contact with Stop Solution which contains Sulfuric Acid.
- 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 the appropriate AKT1 positive control (Cat# CY-E1168-1) or AKT2 positive control (Cat# CY-E1168-2), separately available from CycLex, 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 Solution

1. Prepare a working solution of **Wash Buffer** by adding 100 mL of the **10X Wash Buffer** (provided) to 900 mL of ddH<sub>2</sub>O. 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<sub>2</sub>O 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 (ATP plus)** by mixing following reagents.

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

*\*You will need 80-90 µL of Kinase Reaction Buffer (ATP plus) per assay well. Mix well. Discard any unused Kinase Reaction Buffer (ATP plus) 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 **sample** to the well of the assay plate on ice. Duplicate wells containing 20 units/10 µL AKT1 (Cat # CY-E1168-1) or AKT2 (Cat # CY-E1168-2) should be included in each assay as a positive control for phosphorylation.
4. Begin the kinase reaction by addition of **90 µL 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 AT-3E2** 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  $\mu$ L** of **Substrate Reagent** to each well and incubate **at room temperature (ca.25°C) for 5–15 minutes.**
9. Add **100  $\mu$ L** 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.

**Note-1:** Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

**Note-2:** Reliable signals are obtained when either O.D. values do not exceed 0.25 units for the blank (no enzyme control), or 2.5 units for the AKT positive control.

**Note-3:** If the microplate reader is not capable of reading absorbance greater than the absorbance of the Weel positive control, perform a second reading at 405 nm. A new O.D. values, measured at 405 nm, is used to determine AKT activity of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

## 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  $\mu$ L** of diluted **sample** to the well of the assay plate on ice. Duplicate wells containing 20 m units/10  $\mu$ L AKT1 (Cat # CY-E1168-1) or AKT2 (Cat # CY-E1168-2) should be included in each assay as a positive control for phosphorylation.
4. Begin kinase reaction by addition of **90  $\mu$ L 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  $\mu$ L 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  $\mu$ L** of **HRP conjugated Detection Antibody AT-3E2** 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  $\mu$ L** of **Substrate Reagent** to each well and incubate **at room temperature (ca.25°C) for 10-15 minutes.**
- 10 add **100  $\mu$ L** 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 $\mu$ L	80 $\mu$ L	80 $\mu$ L
10X Inhibitor or equivalent	10 $\mu$ L	-	-
Solvent for Inhibitor	-	10 $\mu$ L	-
10X Staurosporine* (10 $\mu$ M)	-	-	10 $\mu$ L
AKT positive control (2.5 m unit/ $\mu$ L)** or your enzyme fraction	10 $\mu$ L	10 $\mu$ L	10 $\mu$ L

\* Cat# S-4400: See Page 4, section "Materials Required but not Provided"

\*\* AKT positive control: Cat# CY-E1168: See Page 4, section "Materials Required but not Provided"

1. Following the above table, add the Reagents to each well of the microplate. Finally, initiate reaction by adding 10  $\mu$ L of diluted "**CycLex AKT positive control**" 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 **Standard Assay** steps 5-10, page 6-7.



**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 $\mu$ L	80 $\mu$ L	-	90 $\mu$ L	90 $\mu$ L
Kinase Buffer (ATP minus)	-	-	90 $\mu$ L	-	-
10X Staurosporine (10 $\mu$ M)*	-	10 $\mu$ L	-	-	-
Your enzyme fraction	10 $\mu$ L	10 $\mu$ L	10 $\mu$ L	-	-
AKT positive control (2.5 m unit/ $\mu$ L)**	-	-	-	10 $\mu$ L	-
Buffer	-	-	-	-	10 $\mu$ L

\* Cat# S-4400: See Page 4, section “Materials Required but not Provided”

\*\* AKT positive control: Cat# CY-E1168: See Page 4, section “Materials Required but not Provided”

- Following the above table, add the Reagents to each well of the microplate. Finally, initiate the reaction by adding 10  $\mu$ L of “Your enzyme fraction” 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**.
- Follow the **Standard Assay** steps 5-10, page 6-7.

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**Evaluation of Results**

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- 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.
- 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.
- 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.

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**Assay Characteristics**

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The CycLex 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.



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## Troubleshooting

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1. The **CycLex AKT positive control** (Cat# CY-E1168-1, -2) should be run in duplicate, when a standard assay is being performed, 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.

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## Reagent Stability

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All of the reagents included in the CycLex 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.

**For research use only, not for use in diagnostic or therapeutic procedures**

## Example of Test Results

Fig.1 Dose dependency of recombinant AKT enzyme reaction

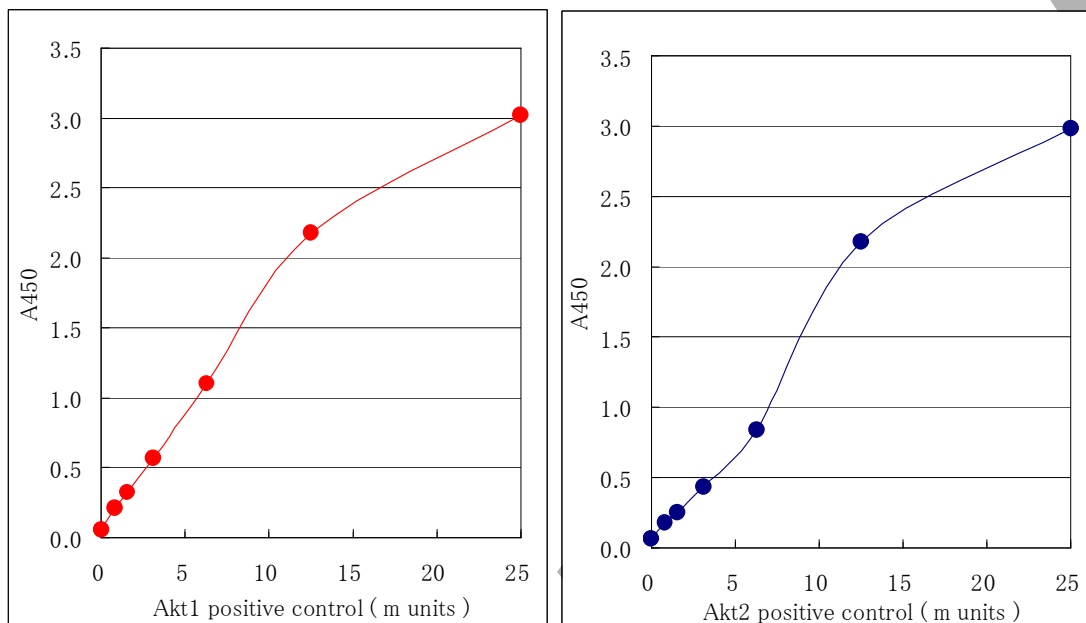


Fig.2 Time course of recombinant AKT enzyme reaction

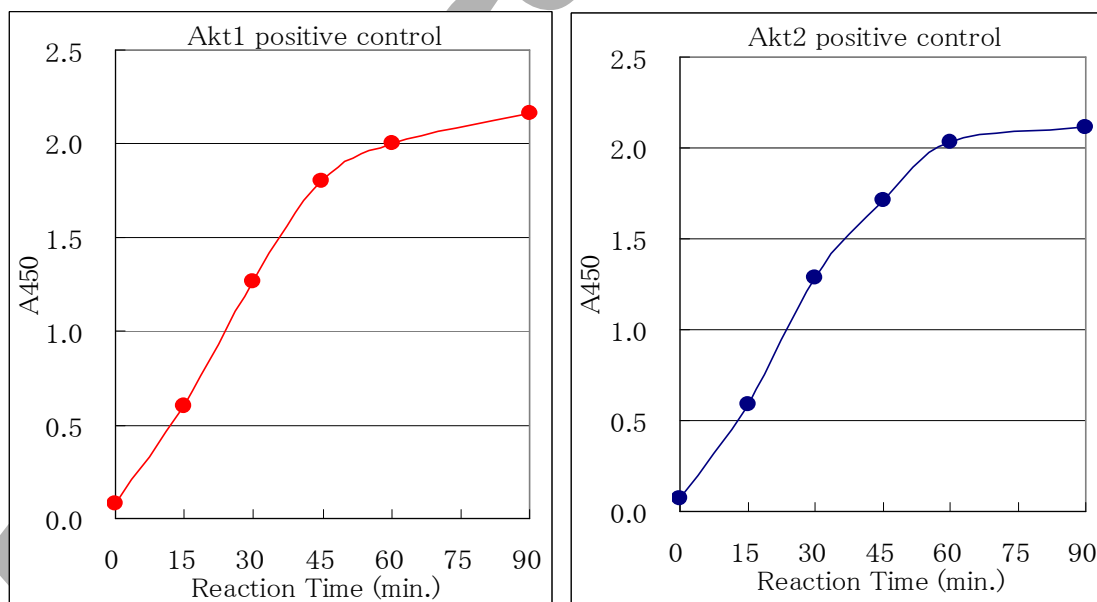
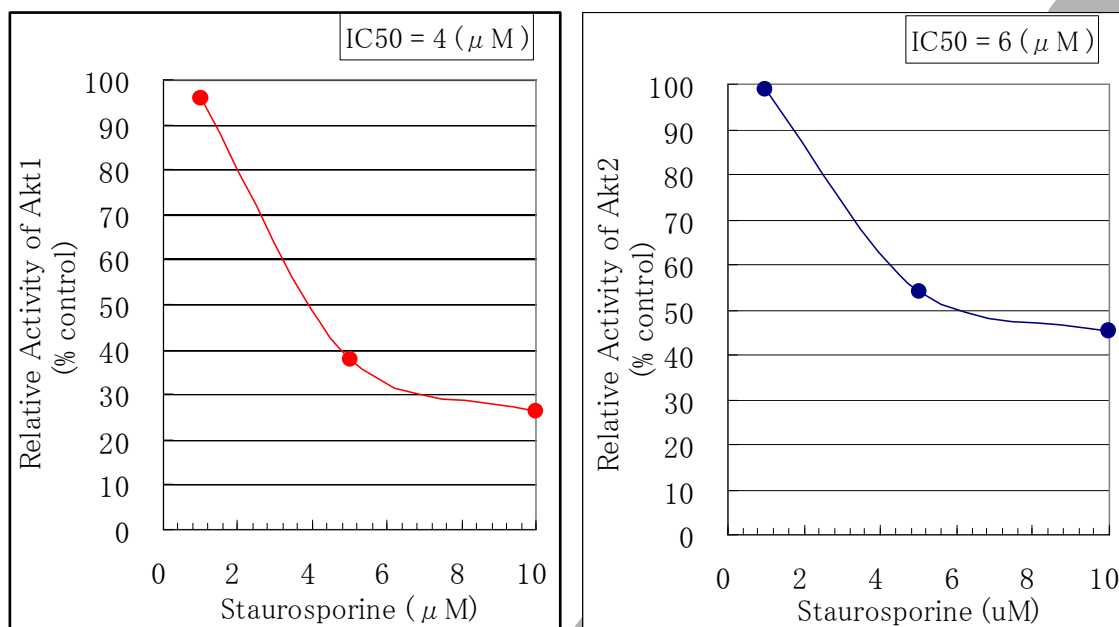




Fig.3 Effect of broad-spectrum kinase inhibitor staurosporine on AKT activity





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## Related Products

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- \* AKT1 Positive control: Cat# CY-E118-1
- \* AKT2 Positive control: Cat# CY-E118-2
- \* Anti-phospho-AKTide-2T monoclonal antibody (Clone AT-3E2): Cat# CY-M1025

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