Non-Radioisotopic Kit for Measuring Chk1, Chk2 and C-TAK1 Activities

CycLex Checkpoint Kinase Assay/Inhibitor Screening Kit-1

Cat# CY-1162

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Intended Use

The CycLex Research Product **CycLex Checkpoint Kinase Assay / Inhibitor Screening Kit-1** is designed to measure the activities of purified checkpoint kinase enzyme such as Chk1, Chk2 and C-TAK1 for the rapid and sensitive evaluation of checkpoint kinase inhibitors using recombinant checkpoint kinases. The phospho-specific monoclonal antibody used in this assay kit has been demonstrated to recognize the phospho-serine 216 residue in Cdc25C, which is phosphorylated by checkpoint kinases.

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

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

Three different human Cdc25 family members exist with Cdc25A regulating the G1/S transition and Cdc25B and Cdc25C involved in G2/M progression. Evidence suggests that two critical amino acids, threonine 14 and tyrosine 15, located within the cyclin-dependent kinases represent the major target for the Cdc25 family of protein phosphatases. Dephosphorylation of these two critical amino acid residues is essential for proper cell cycle progression and the subsequent association of cyclin-dependent kinases with their associated cyclins (1).

Given their crucial role in cell cycle progression and checkpoint control, the regulation of the activity of the various Cdc25 family members has been the subject of numerous investigations. For the case of Cdc25C, enzymatic activity has been demonstrated to be low during interphase, in part because the phosphatase is phosphorylated on serine 216. In response to DNA damage, various intracellular kinases including Chk1, Chk2 and C-TAK1 appear to phosphorylate Cdc25C on this residue (2–6).

One of the important functional consequences of phosphorylation of Ser-216 is to create a consensus binding site for 14-3-3 protein binding (4). A variety of evidence suggests that in human cells, the binding of 14-3-3 increases the cytoplasmic localization of the protein (7–9). In addition to 14-3-3 binding, Cdc25C is also actively transported from the nucleus through a leptomycin B-sensitive pathway that requires an N-terminal nuclear export sequence (9).

Measurement of checkpoint kinases (Chk1, Chk2 and C-TAK1) activity

The protocol generally regarded as most sensitive for the quantitative measurement of checkpoint kinase activity involves incubation of the checkpoint kinase sample with substrate, either a natural or synthetic polypeptide (such as Chktide substrate peptide), in the presence of $Mg^{2+}$and ^{32}P-labeled ATP. The reaction is terminated by "spotting" a sample onto a filter paper disc, followed by immersion in acid to precipitate the radiolabeled product. The filter papers are then washed 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 Checkpoint Kinase Assay / Inhibitor Screening Kit-1** uses a peroxidase coupled anti-phospho-Cdc25C S216 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 checkpoint kinases.

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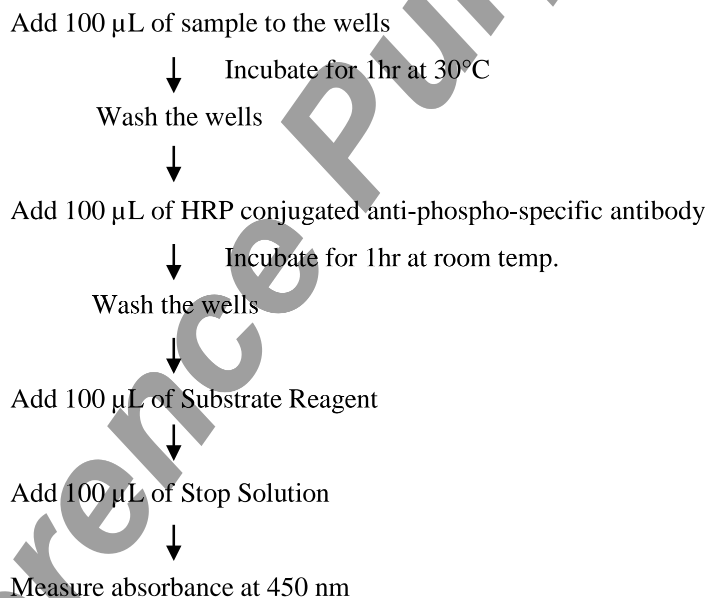
Principle of the Assay

The CycLex Research Products **CycLex Checkpoint Kinase Assay / Inhibitor Screening Kit-1** is a single-site, semi-quantitative immunoassay for checkpoint kinase activity. Plates are pre-coated with a substrate corresponding to recombinant Cdc25C, which contains serine residues that can be phosphorylated by checkpoint kinases, including Chk1, Chk2 and C-TAK1.

The detector antibody specifically detects only the phosphorylated form of serine 216 residue on Cdc25C. The **CycLex Checkpoint Kinase Assay / Inhibitor Screening Kit-1** may be used to study the kinetics of a purified or partially purified individual checkpoint kinase (Chk1, Chk2 and C-TAK1) as well as to screening individual checkpoint kinase inhibitor. 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 1F1, a anti-phospho-Cdc25C serine 216 specific 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 checkpoint kinases activity in the sample. For kinetic analysis, the checkpoint kinase 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 recombinant Cdc25C as Checkpoint kinases substrate.

10X Wash Buffer: One bottle containing 100 mL of 10X buffer containing 2 %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 20 mL of HRP (horseradish peroxidase) conjugated anti-phospho-Cdc25C S216 (1F1) 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_2SO_4. Ready to use.

Materials Required but not Provided

- **Recombinant Chk1, Chk2 and C-TAK1 positive control:** Available from CycLex (Chk1 positive control: Cat# CY-E1162-1, Chk2 positive control: Cat# CY-E1162-2 and C-TAK1: Cat# CY-E1162-3); One vial containing 2 Units checkpoint kinase enzyme. Positive control should be added to the first well at 10 m units/well. For instance, diluted positive control 1 m unit/µL, use 10 µL for 1 assay. (Unused checkpoint kinase enzyme should be stored in aliquots at below -70°C.)
- **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

- Store the checkpoint kinase enzyme at below -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 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 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.**

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Detailed Protocol

The **CycLex Checkpoint Kinase Assay / Inhibitor Screening Kit-1** 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 checkpoint kinase positive control (Cat# CY-E1162-1-3), 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_2O. Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Prepare **20X ATP Solution** by adding **1.6 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 **1.25 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) **20X ATP Solution**	**9.5 mL** **0.5 mL**	**950 µL** **50 µL**	**95 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).

3. Duplicate wells containing **10 µL** of checkpoint kinase positive control (10 m units) 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 or lid, 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 plate sealer or lid, and incubate **at room temperature (ca.25°C) for 60 minutes**. Discard any unused conjugate after use.

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

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 Assays

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).

3. Duplicate wells containing **10 µL** of checkpoint kinase positive control (10 m units) 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** in duplicate per well in timed intervals (suggested interval is 5 minutes but should be individually determined for each system). After the final addition, cover with plate sealer or lid, and 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 plate sealer or lid, and incubate **at room temperature (ca.25°C) for 60 minutes**. Discard any unused conjugate.

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

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

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Stop Solution.

Recommendations

Special considerations when screening activators and inhibitors

In order to estimate the inhibitory effect on individual checkpoint kinase 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 individual checkpoint kinase 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**
CycLex checkpoint kinase (1 m unit/µL) ** **or your enzyme fraction**	**10 µL**	**10 µL**	**10 µL**

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

** Chk1 positive control: Cat# CY-E1162-1, Chk2 positive control: Cat# CY-E1162-2 and C-TAK1: Cat# CY-E1162-3: 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 µL of **"CycLex checkpoint kinase"** 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.

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Special considerations when measuring precise Checkpoint kinase activity

In order to measure the activity of checkpoint kinase family 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 checkpoint kinase family 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 fraction	**10 µL**	**10 µL**	**10 µL**	**-**	**-**
CycLex checkpoint kinase (1 m unit/µL) **	**-**	**-**	**-**	**10 µL**	**-**
Buffer	**-**	**-**	**-**	**-**	**10 µL**

* Cat# S-4400: See Page 4, section "Materials Required but not Provided"
** Chk1 positive control: Cat# CY-E1162-1, Chk2 positive control: Cat# CY-E1162-2 and C-TAK1: Cat# CY-E1162-3: 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 the reaction by adding 10 µL 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**.

2. Follow the **Standard Assay** steps 5-10, page 6-7.

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Evaluation of Results

1. Average the absorbance values for the checkpoint kinase sample duplicates (positive control) and all experimental sample duplicate values (when applicable). When checkpoint kinase positive control (10 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 0.15.

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 individual checkpoint kinase.

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 CycLex Research Product **Checkpoint Kinase Assay / Inhibitor Screening Kit-1** has been shown to detect the activity of indicated checkpoint kinases in the column fractions of mammalian cell lysates. The assay may be used to follow the purification of individual checkpoint kinase.

Troubleshooting

1. The **CycLex checkpoint kinase** 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. <u>Do not allow the plate to dry out</u>. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the CycLex Research Product **CycLex Checkpoint Kinase Assay / Inhibitor Screening Kit-1** 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 and checkpoint kinase component must be stored at below -70°C. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

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Example of Test Results

Fig.1-1 Dose dependency of recombinant Chk1 enzyme reaction

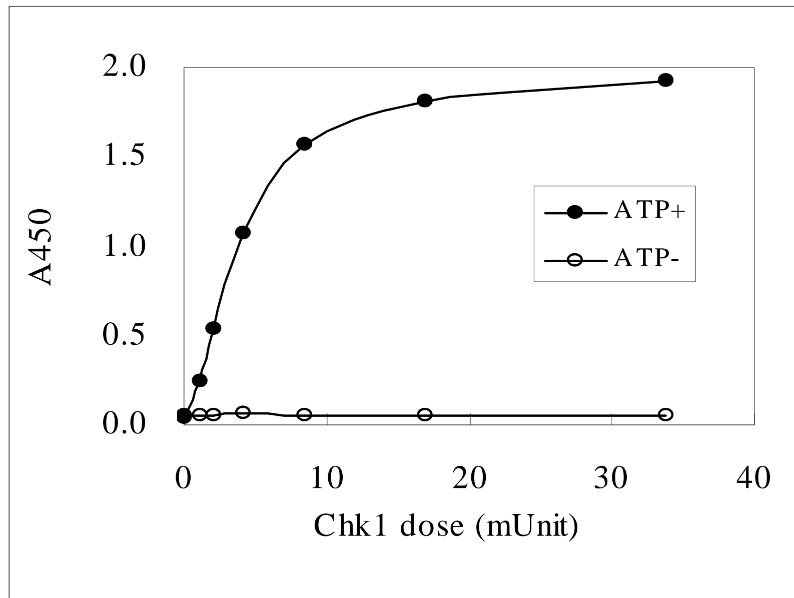

Fig.1-2 Dose dependency of recombinant Chk2 enzyme reaction

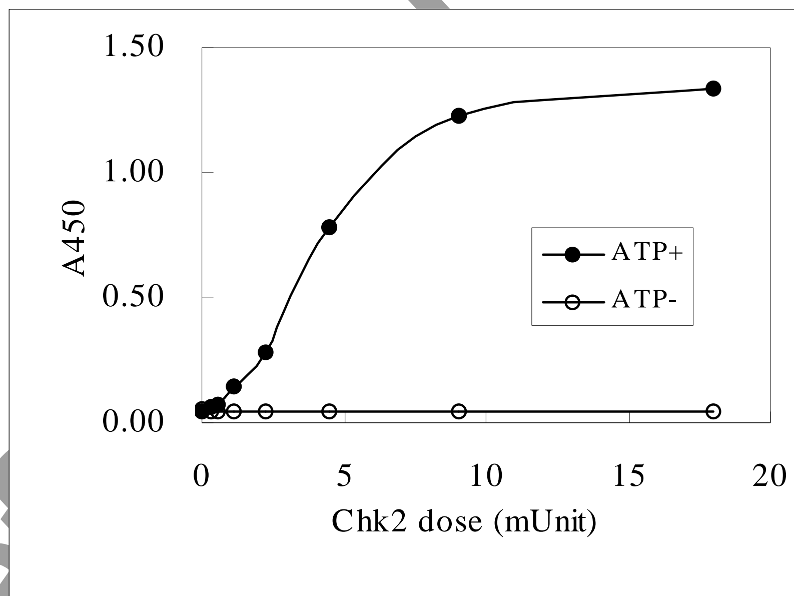

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Fig.1-3 Dose dependency of recombinant C-TAK1 enzyme reaction

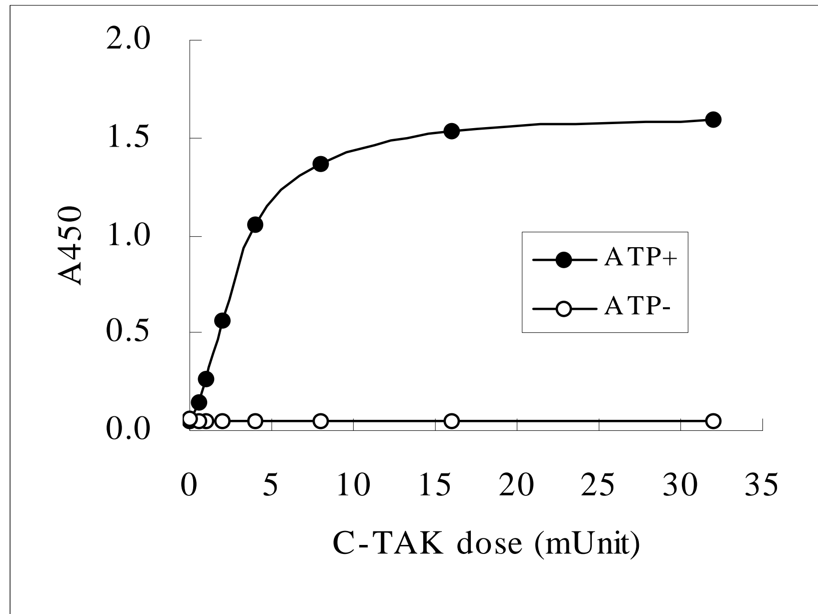

Fig.2-1 Time course of recombinant Chk1 enzyme reaction

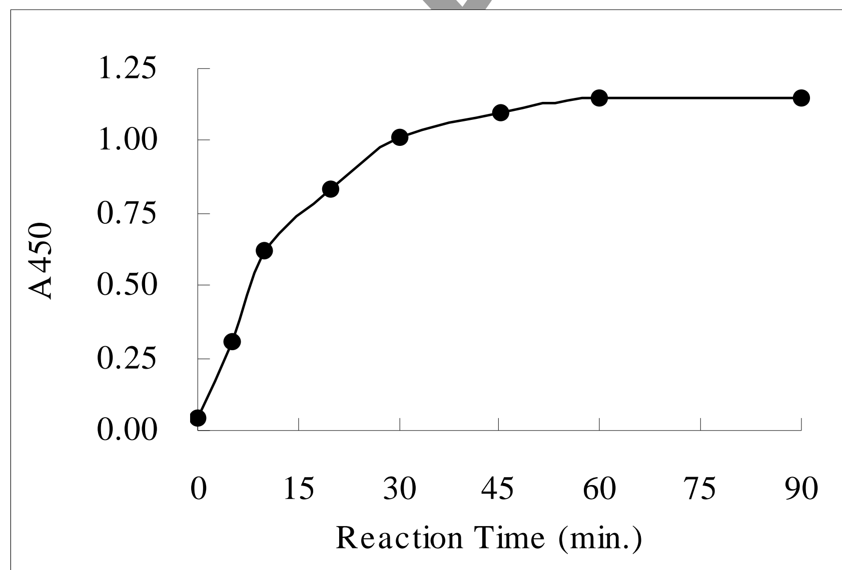

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Fig.2-2 Time course of recombinant Chk2 enzyme reaction

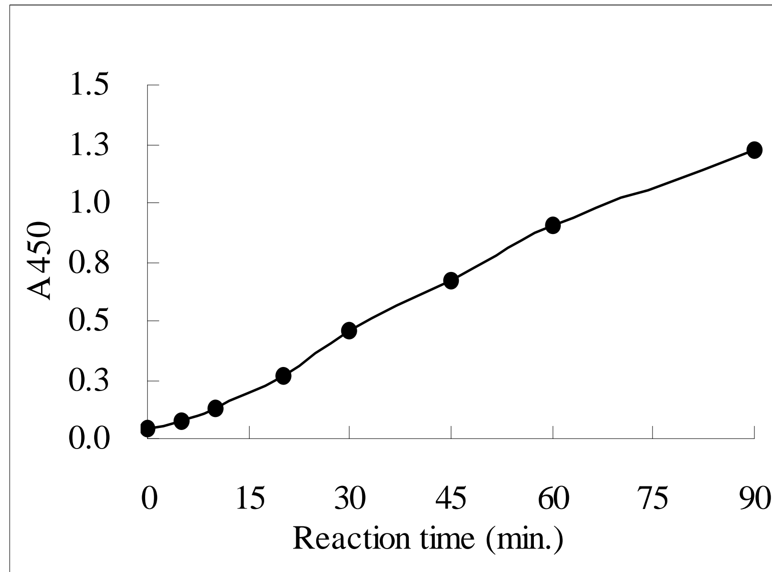

Fig.2-3 Time course of recombinant C-TAK1 enzyme reaction

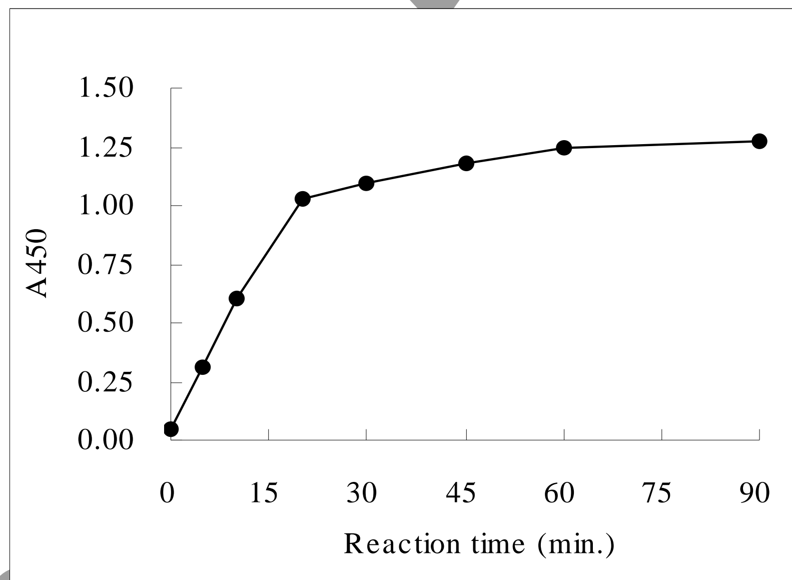

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Fig.3-1 Km for ATP (recombinant Chk1)

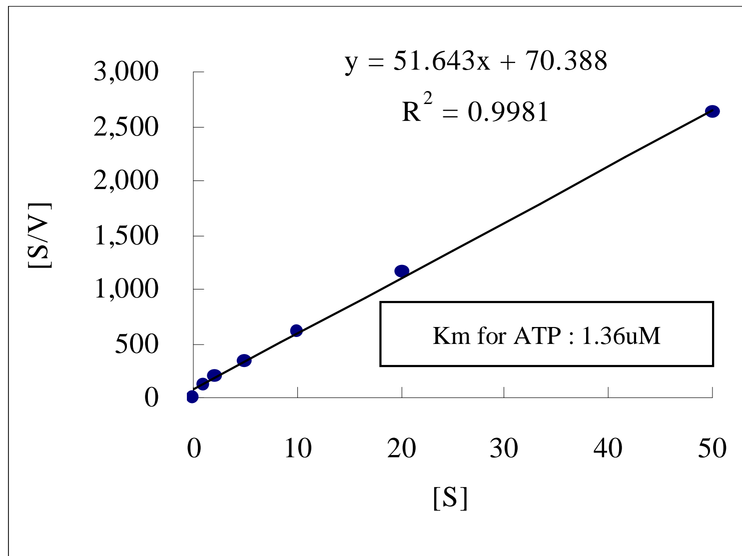

Fig.3-2 Km for ATP (recombinant Chk2)

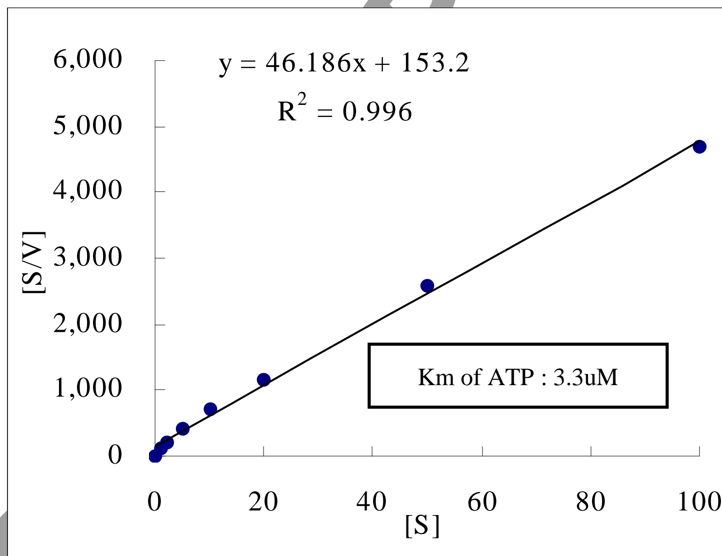

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Fig.3-3 Km for ATP (recombinant C-TAK1)

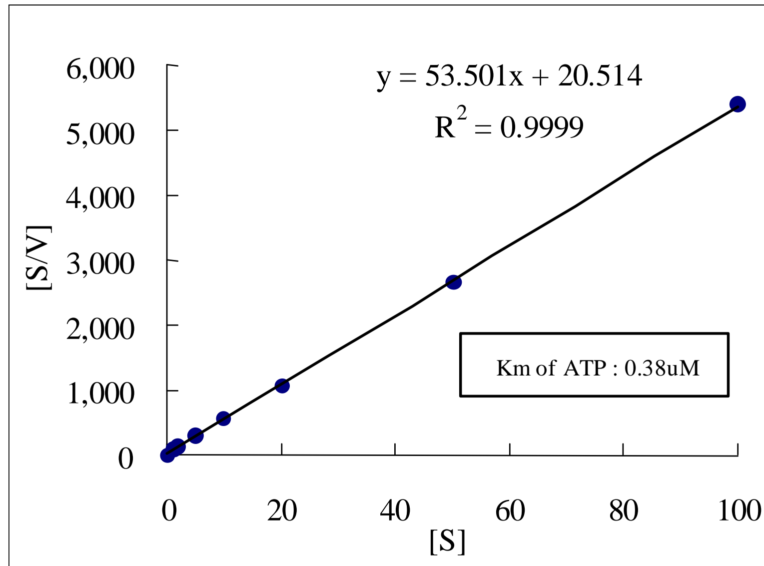

Fig.4-1 Effect of broad-spectrum kinase inhibitor staurosporine on Chk1 activity

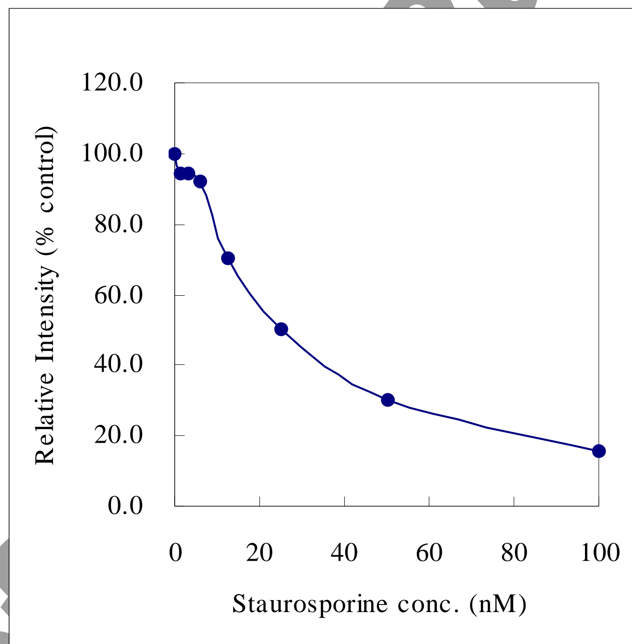

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Fig.4-2 Effect of broad-spectrum kinase inhibitor staurosporine on Chk2 activity

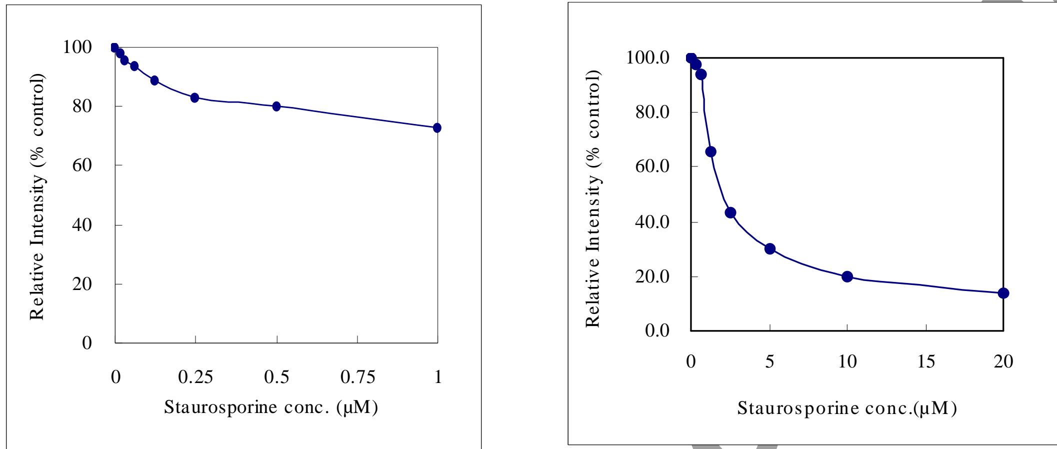
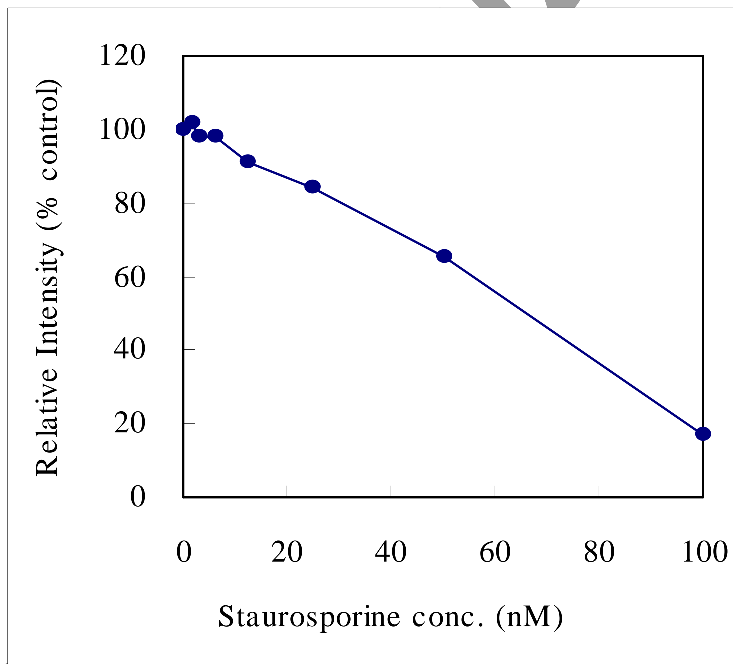


Fig.4-3 Effect of broad-spectrum kinase inhibitor staurosporine on C-TAK1 activity



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Related Products

*** Chk1 Positive control: Cat# CY-E1162-1**
*** Chk2 Positive control: Cat# CY-E1162-2**
*** C-TAK1 Positive control: Cat# CY-E1162-3**
*** Anti-phospho-Cdc25C-Ser216 monoclonal antibody (1F1): Cat# CY-M1018**

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