Non-Radioisotopic Kit for Measuring Pim-1 Kinase Activity

CycLex Pim-1 Kinase Assay/Inhibitor Screening Kit

Cat# CY-1167

Intended Use

The CycLex Research Product **CycLex Pim-1 Kinase Assay/Inhibitor Screening Kit** is designed to measure the activities of purified Pim-1 Kinase for the rapid and sensitive evaluation of inhibitors using recombinant Pim-1. The phospho-threonine specific polyclonal antibody used in this assay kit has been demonstrated to recognize the phospho-threonine145 residue in p21waf1, which is efficiently phosphorylated by Pim-1.

Applications of this kit include:
1) Screening inhibitors or activators of Pim-1.
2) Detecting the effects of pharmacological agents on Pim-1 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 pim-1 oncogene encodes a serine/threonine kinase (Pim-1) involved in the transduction of cytokine triggered mitogenic signals. The pim-1 oncogene was originally identified as a genetic locus frequently activated by the proviral insertion of Moloney murine leukemia virus into mouse T cell lymphomas (1–3). The pim-1 oncogene has also been implicated in human hematopoietic malignancies, with its overexpression frequently detected in human hematopoietic cell lines as well as in fresh tumor cells from patients with leukemia (4, 5). During embryonal development, the pim-1 gene is expressed mainly in developing fetal hematopoietic tissues (5). The pim-1 gene product (Pim-1), identified as a serine/threonine kinase (6–8), has been thought to play a critical role in the transduction of mitogenic signals from cytokines since Pim-1 expression is rapidly induced after cytokine stimulation, and the proliferative response to cytokines is impaired in cells from pim-1-deficient mice (9–11).

When overexpressed, pim genes can efficiently cooperate with myc or bcl-2 oncogenes in lymphomagenesis (12-14). Pim-1 has been reported to protect thymocytes against glucocorticoid-induced apoptosis (15) and to promote cell proliferation or survival in several IL-3- or IL-6-dependent hematopoietic cell lines (16–17).

Measurement of Pim-1 Kinase activity

The protocol generally regarded as most sensitive for the quantitative measurement of Pim-1 activity involves incubation of the Pim-1 sample with substrate, either a natural or synthetic polypeptide (such as histone H1 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 Pim-1 Kinase Assay/Inhibitor Screening Kit** uses anti-phospho-p21waf1 threonine145 polyclonal antibody (PWT-01) and peroxidase coupled anti-rabbit IgG 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 Pim-1.

Principle of the Assay

The CycLex Research Product **CycLex Pim-1 Kinase Assay/Inhibitor Screening Kit** is a single-site, semi-quantitative immunoassay for Pim-1 activity. Plates are pre-coated with a substrate corresponding to recombinant p21waf1, which contains threonine residues that can be efficiently phosphorylated by Pim-1. The detector antibody specifically detects only the phosphorylated form of threonine145 residue on p21waf1. The **CycLex Pim-1 Kinase Assay/Inhibitor Screening Kit** may be used to study the kinetics of a purified Pim-1 as well as to screening Pim-1 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 PWT-01, a anti-phospho-p21waf1 threonine145 polyclonal antibody, followed by binding with horseradish peroxidase conjugated anti-rabbit IgG, 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 Pim-1 activity in the sample. For kinetic analysis, the Pim-1 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

Add 100 µL of sample to the wells
↓ Incubate for 30 min at 30°C
Wash the wells
↓
Add 100 µL of anti-phospho-threonine polyclonal antibody (PWT-01).
↓ Incubate for 30 min at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti-rabbit IgG
↓ Incubate for 30 min at room temp.
Wash the wells
↓
Add 100 µL of Substrate Reagent
↓
Add 100 µL of Stop Solution
↓
Measure absorbance at 450 nm

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 p21waf1 as Pim-1 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: Lyophilized ATP Na_2 salt. Reconstitute contents of vial with **0.8 mL** of ddH_2O. (See section "Preparation of Working Solution" page 7.)

Anti-phospho-p21waf1 threonine145 polyclonal antibody (PWT-01): One vial containing 12 mL of anti-phospho-p21waf1 threonine145 polyclonal antibody (PWT-01). Ready to use.

HRP conjugated anti-rabbit IgG: One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-rabbit IgG. 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

- **Pim-1 positive control:** Available from CycLex (Pim-1 positive control: Cat# CY-E1167, One vial containing 2 units/100 µL Pim-1 enzyme. Positive control should be added to the first well at 10 m units/well. For instance, For instance, diluted positive control 1:20 with enzyme dilution buffer, use 10 µL for 1 assay (Unused Pim-1 enzyme should be stored in aliquots at -70°C). *The Pim-1 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 Pim-1 positive control.

- **10X Staurosporine (20 µM):** Staurosporine is available from Sigma, Cat#. S-4400. 2 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**

Precautions and Recommendations

- Store the Pim-1 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 Pim-1 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 Pim-1 positive control (Cat# CY-E1167), 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 **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 (ATP plus)** by mixing following reagents.

	96 assays	**10 assays**	**1 assay**
Kinase Buffer (provided) **20X ATP** (provided)	**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 (ATP plus) per assay well. Mix well. Discard any unused Kinase Reaction Buffer (ATP plus) after use.

4. Prepare **Enzyme dilution buffer** by mixing 9-parts of Kinase buffer and 1-part of 10X BSA (100 µg/mL), which is supplied with Pim-1 positive control.

The Pim-1 Positive control should be diluted with an enzyme dilution buffer to avoid inactivating the enzyme activity in low protein concentration condition..

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. To assay partially purified recombinant Pim-1, add **10 µL** of **each fraction** to the wells of the assay plate on ice. Duplicate wells containing 10 m units/10 µL of Pim-1 positive control (Cat # CY-E1167) 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 30 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 **anti-phospho-p21waf1 threonine145 polyclonal antibody PWT-01** into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**.

7. Wash wells five times as same as in step 5.

8. Pipette **100 µL** of **HRP-conjugated anti-rabbit IgG** into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any unused conjugate after use.

9. Wash wells five times as same as in step 5.

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

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

12. 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 Pim-1 positive control.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the Wee1 positive control, perform a second reading at 405 nm. A new O.D. values, measured at 405 nm, is used to determine Pim-1 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. To assay partially purified recombinant Pim-1, add **10 µL** of **each fraction** to the wells of the assay plate on ice. Duplicate wells containing 10 m units/10 µL of Pim-1 positive control (Cat # CY-E1167) should be included in each assay as a positive control for phosphorylation.

4. Begin kinase reaction by addition of **90 µL Kinase Reaction Buffer** in duplicate per well in timed intervals (suggested interval is 4 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 **anti-phospho-p21waf1 threonine145 polyclonal antibody PWT-0** into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**.

8. Wash wells five times as same as in step 6.

9. Pipette **100 µL** of **HRP-conjugated anti-rabbit IgG** into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any unused conjugate after use.

10. Wash wells five times as same as in step 6.

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

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

13. 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 Pim-1 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 Pim-1 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 (20 µM)*	**-**	**-**	**10 µL**
Pim-1 positive control (1 m unit/µL) or Purified enzyme sample**	**10 µL**	**10 µL**	**10 µL**

* Cat# S-4400: See Page 4, section "Materials Required but not Provided"
** Pim-1 positive control: Cat# CY-E1167: See Page 5, 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 **"Pim-1 positive control"** to each well and mixing thoroughly at room temperature. Cover with plate sealer or lid, and incubate **at 30°C for 30 minutes**.

2. Follow the **Standard Assay** steps 5-12, page 8.

Special considerations when measuring precise Pim-1 kinase activity

In order to measure the activity of Pim-1 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 Pim-1 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 (ATP minus)	**-**	**-**	**90 µL**	**-**	**-**
10X Staurosporine (20 µM)*	**-**	**10 µL**	**-**	**-**	**-**
Purified enzyme sample	**10 µL**	**10 µL**	**10 µL**	**-**	**-**
Pim-1 positive control (1 m unit/µL)**	**-**	**-**	**-**	**10 µL**	**-**
Buffer	**-**	**-**	**-**	**-**	**10 µL**

* Cat# S-4400: See Page 4, section "Materials Required but not Provided"
** Pim-1 positive control: Cat# CY-E1167: See Page 5, 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 "Purified enzyme sample" or "Buffer" to each well and mixing thoroughly at room temperature. Cover with plate sealer or lid, and incubate **at 30°C for 30 minutes**.

2. Follow the **Standard Assay** steps 5-12, page 8.

Evaluation of Results

1. Average the absorbance values for the Pim-1 sample duplicates (positive control) and all experimental sample duplicate values (when applicable). When Pim-1 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 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 **CycLex Pim-1 Kinase Assay/Inhibitor Screening Kit** has been shown to detect the activity of purified recombinant Pim-1. The assay shows good linearity of sample response.

Troubleshooting

1. The **CycLex Pim-1 positive control** (Cat# CY-E1167) 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.

Reagent Stability

All of the reagents included in the CycLex Research Product **CycLex Pim-1 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 Pim-1 enzyme reaction

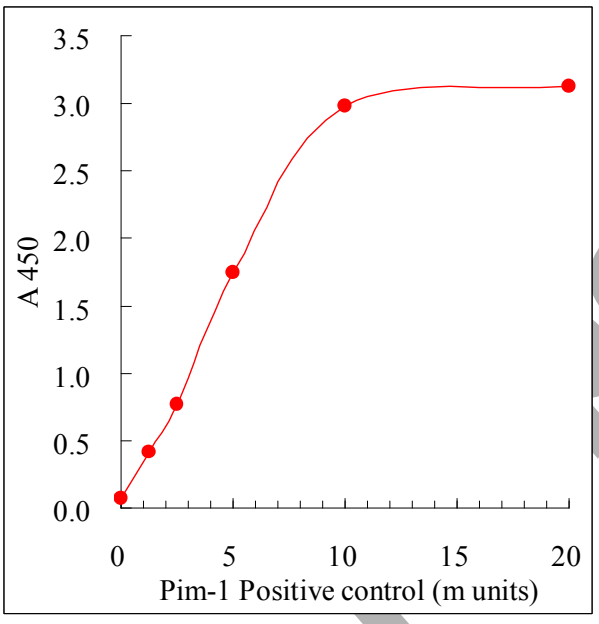


Fig.2 Time course of recombinant Pim-1 enzyme reaction

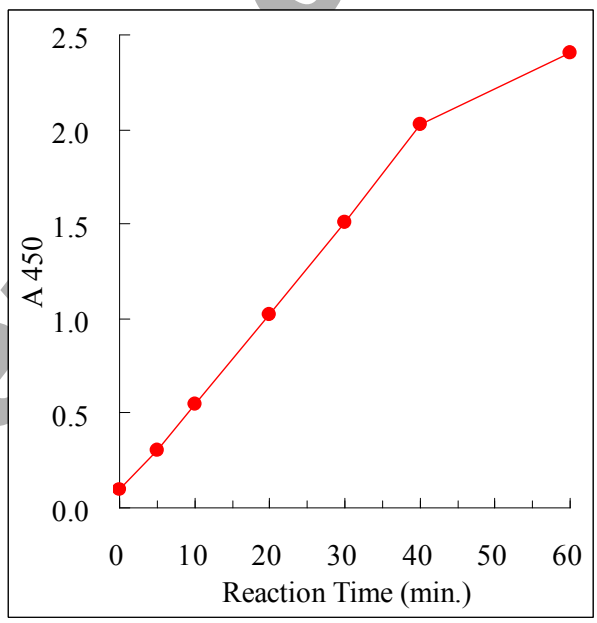


Fig.3 Km for ATP (recombinant Pim-1)

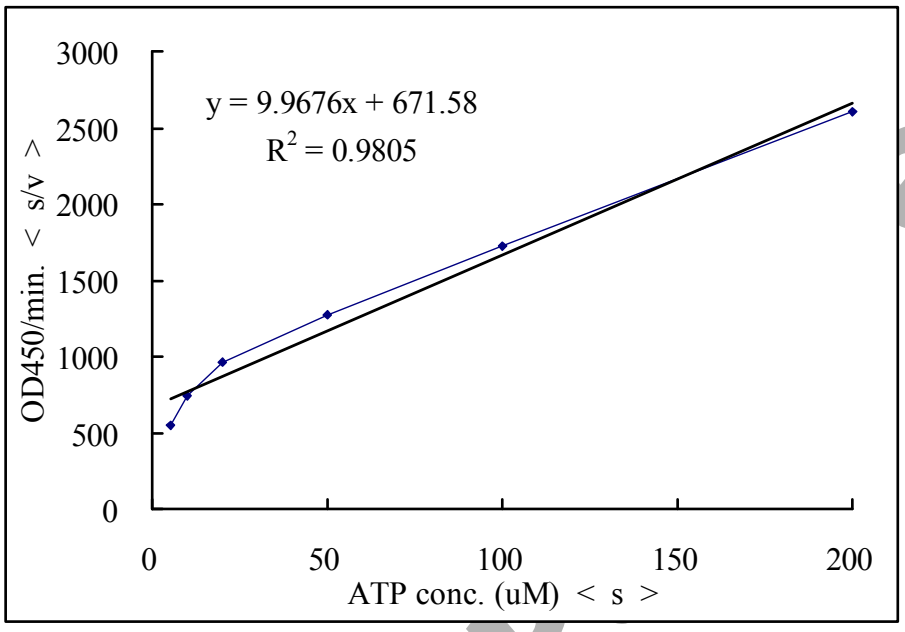
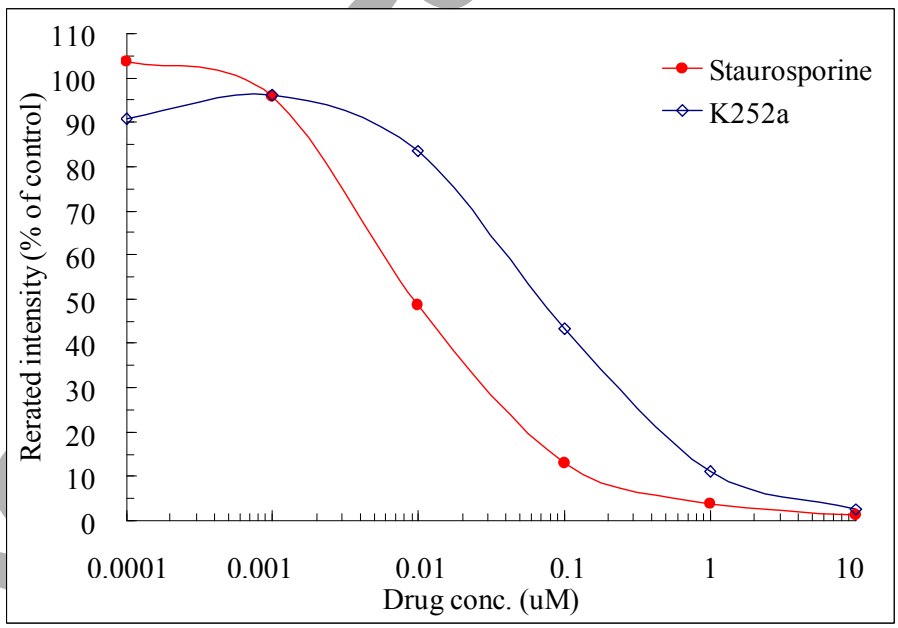


Fig.4 Effect of broad-spectrum kinase inhibitor staurosporine and K252a on Pim-1 kinase activity



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Related Products

*** Pim-1 Positive control: Cat# CY-E1167**

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