Non-Radioisotopic Kit for Measuring Human Mps1/TTK Activity

CycLex Human Mps1/TTK kinase Assay/Inhibitor Screening Kit

Cat# CY-1179

Intended Use

The CycLex Research Product **CycLex Human Mps1/TTK kinase Assay/Inhibitor Screening Kit** is designed to measure the activities of purified human Mps1/TTK 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 recombinant human Mps1-substrate, which is phosphorylated by human Mps1/TTK.

Applications of this kit include:

1) Screening inhibitors or activators of human Mps1/TTK.
2) Detecting the effects of pharmacological agents on human Mps1/TTK 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

Recently, the vertebrate orthologues of the yeast MPS1 kinase were identified and found to localize to kinetochores (1.2). MPS1 encodes a tyrosine and serine/threonine dual-specificity kinase (3) that was originally identified in a genetic screen for mutants defective in spindle pole duplication (4). Subsequently, it was discovered to be an essential component of the mitotic checkpoint (5). Consistent with yeast MPS1, mouse Mps1 is localized at centrosomes throughout the cell cycle and is essential for accurate centrosome duplication (6). However, a recent study indicated that human Mps1 was not localized at centrosomes in human U2OS cells (1). Despite the discrepancy in the centrosome localization of Mps1 in mouse and human cells, it is clear that Mps1 is present at kinetochores during mitosis, where it may participate in the checkpoint.

The human Mps1/TTK kinase was originally identified in a screen for novel tyrosine kinases by using a phosphotyrosine antibody to screen a T-cell cDNA expression library (7). Using a similar strategy, the mouse homologue, esk, was also cloned from an embryonal carcinoma cell line (8). It was determined subsequently that esk was the mouse orthologue of yeast MPS1 (6). Human Mps1 is detectable in all proliferating human cells and tissues. Expression of the human Mps1 gene is markedly reduced or absent in resting cells and in tissues with a low proliferative index. Levels of human Mps1 mRNA and protein are very low in starved cells. When cells are induced to enter the cell cycle, levels of human Mps1 mRNA, protein and kinase activity increase at the G1/S phase of the cell cycle and peak in G2/M. Human Mps1 mRNA levels, as well as kinase activity, drop sharply in early G1, whereas protein levels are largely maintained. Human Mps1 may play a role in cell cycle control.

Measurement of Human Mps1/TTK activity

The protocol generally regarded as most sensitive for the quantitative measurement of human Mps1/TTK activity involves incubation of the human Mps1/TTK sample with substrate, either a natural or synthetic polypeptide (such as MBP), 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 is 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 Research Product **CycLex Human Mps1/TTK kinase Assay/Inhibitor Screening Kit** uses a peroxidase coupled anti-phospho-serine specific monoclonal antibody as a reporter molecule in a 96-well ELISA format. This assay provides a non-isotopic, sensitive and specific method to detect human Mps1/TTK activity.

Principle of the Assay

The CycLex Research Product **CycLex Human Mps1/TTK kinase Assay/Inhibitor Screening Kit** is a single-site, semi-quantitative immunoassay for human Mps1/TTK activity. Plates are pre-coated with a substrate corresponding to recombinant human Mps1-substrate (newly designed by CycLex), which contains a serine residue that is phosphorylated by human Mps1/TTK.

The detector antibody specifically detects only the phosphorylated form of serine residue on human Mps1/TTK-substrate. The CycLex Research Product **CycLex Human Mps1/TTK kinase Assay/Inhibitor Screening Kit** can be used to study the kinetics of a purified or partially purified human Mps1 as well as to screening these kinases inhibitor. To perform the test, the sample is diluted in Kinase Buffer, pipetted into the wells and allowed to phosphorylate the bound substrate in the presence of Mg^{2+} and ATP. The amount of phosphorylated substrate is measured by binding it with a horseradish peroxidase conjugate of TK-21B, an anti-phospho-serine 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 quantified by spectrophotometry and reflects the relative amount of human Mps1 activity in the sample. For kinetic analysis, the sample containing human Mps1 is added to the wells in a similar fashion and at varying times the reaction is stopped by the addition of a chelator, sodium ethylenediaminetetraacetate (EDTA) and the amount of phosphorylated substrate determined as before.

The CycLex Research Product **CycLex Human Mps1/TTK kinase Assay/Inhibitor Screening Kit** is designed to accurately determine the presence and relative amount of human Mps1 kinase activity in purification column fractions, and to determine non-isotopic kinetic analysis of human Mps1 kinase activity. Careful attention to methods of chromatography and the assay protocol will provide the investigator with a reliable tool for the evaluation of human Mps1 activity.

Summary of Procedure

Add 100 µL of sample to the wells
↓ Incubate for 60 min at 30°C
Wash the wells
↓
Add 100 µL of HRP conjugated anti-phosphorylated form specific antibody
↓ Incubate for 60 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 human Mps1-substrate as a 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 12 mL of HRP (horseradish peroxidase) conjugated anti-phospho-serine monoclonal antibody (TK-21B). 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

- **Human Mps1/TTK Positive Control:** Available from CycLex (Cat # CY-E1179); One vial contains 4 units/200 µL human Mps1 enzyme. Positive control should be added to the first well at 10 m units/well. For instance, diluted positive control 1:10, use 10 µL for 1 assay. (Unused human Mps1 enzyme should be stored in aliquots at **-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 pipe**t**ors 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 ATP at -20°C in aliquots. 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 pipet 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 Research Product **CycLex Human Mps1/TTK 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 experimental conditions may vary, an aliquot of the human Mps1/TTK positive control (Cat # CY-E1179), available separately from CycLex, should be included in each assay as a positive control. Disposable pipette tips and reagent troughs should be used for all liquid 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** 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). All samples should be assayed in duplicate.

3. To assay partially purified recombinant human Mps1, add **10 µL** of **each fraction** to the wells of the assay plate on ice. Duplicate wells containing **20 m units/10 µL human Mps1/TTK positive control** (Cat # CY-E1179) 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** 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 step 5.

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.

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 human Mps1/TTK positive control.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the Mps1/TTK positive control, perform a second reading at 405 nm. A new O.D. values, measured at 405 nm, is used to determine human Mps1 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 human Mps1, add **10 µL** of **each fraction** to the wells of the assay plate on ice. Duplicate wells containing **20 m units/10 µL human Mps1/TTK positive control** (Cat# CY-E1179) 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 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 five times 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 and inhibitors

In order to estimate the inhibitory effect on human Mps1/TTK 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 human Mps1/TTK 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<0.2).

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 Staurosporin (10 µM)*	**-**	**-**	**10 µL**
Human Mps1/TTK Positive Control (2 m unit/µL) or your enzyme fraction**	**10 µL**	**10 µL**	**10 µL**

* 10X Staurosporin: See page 4, section "Materials Required but not Provided"
** Cat # CY-E1179: 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 "Diluted human Mps1/TTK positive control" to each well and mixing thoroughly at room temperature. Cover with plate sealer. Incubate **at 30°C for 60 minutes**.

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

Special considerations when measuring precise Human Mps1 activity

In order to measure the activity of human Mps1/TTK 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 human Mps1/TTK 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 Staurosporin (10 µM)	**-**	**10 µL**	**-**	**-**	**-**
Your enzyme fraction	**10 µL**	**10 µL**	**10 µL**	**-**	**-**
Human Mps1 Positive Control (2 m unit/µL)**	**-**	**-**	**-**	**10 µL**	**-**
Buffer for Your enzyme fraction	**-**	**-**	**-**	**-**	**10 µL**

* 10X Staurosporin: See page 4, section "Materials Required but not Provided"
** Cat # CY-E1179: 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. Incubate **at 30°C for 60 minutes**.

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

Evaluation of Results

1. Average the absorbance values for the human Mps1/TTK positive control duplicates and all experimental sample duplicate values (when applicable). When the human Mps1 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 human Mps1.

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 **CycLex Human Mps1/TTK kinase Assay/Inhibitor Screening Kit** has been shown to detect the activity of recombinant human Mps1 in column fractions. The assay shows good linearity of sample response. The assay may be used to follow the purification of recombinant human Mps1.

Troubleshooting

1. The human Mps1/TTK positive control should be run 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 that are 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 Human Mps1/TTK 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 human Mps1 enzyme reaction

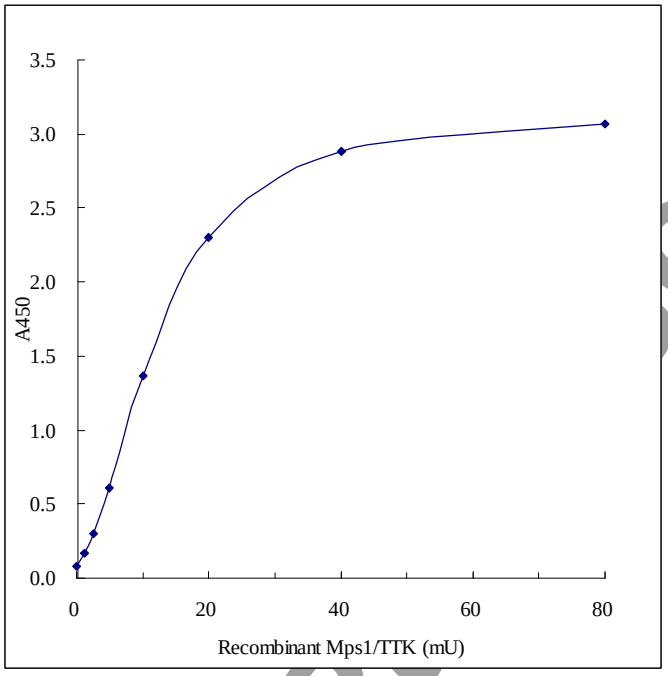


Fig.2 Time course of recombinant human Mps1 enzyme reaction

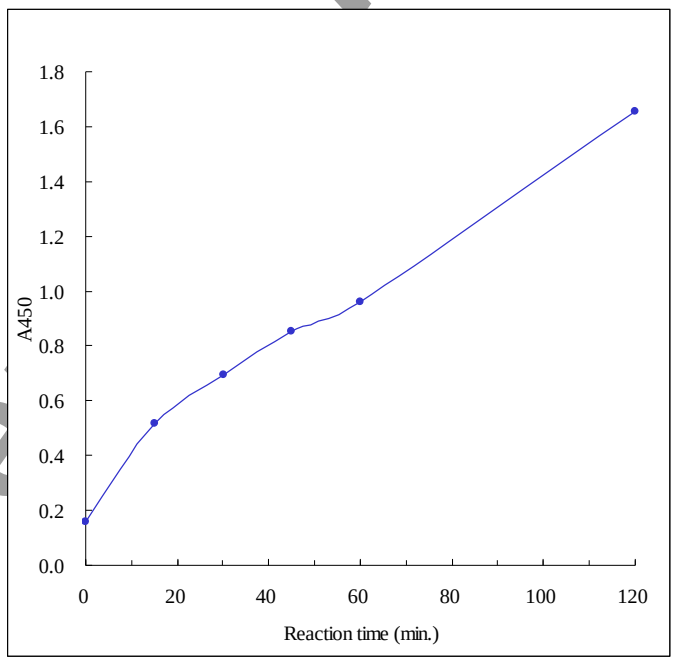
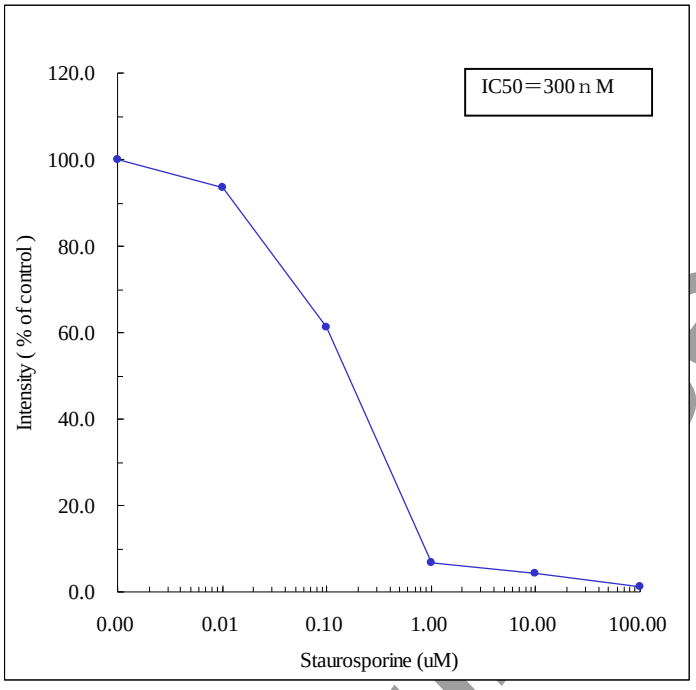


Fig.3 Effect of broad protein kinase inhibitor Staurosporine on activity of recombinant human Mps1



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Related Products

***Human Mps1 Positive control: Cat# CY-E1179**

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