



MAPKAP-kinase 2 Assay/Inhibitor Screening Kit

User's Manual

For Research Use Only, Not for use in diagnostic procedures

Non-Radioisotopic Kit for Measuring MAPKAP-kinase 2 activity

CycLex MAPKAP-kinase 2 Assay/Inhibitor Screening Kit

Cat# CY-1166

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Intended Use

The CycLex Research Product **CycLex MAPKAP-kinase 2 Assay/Inhibitor Screening Kit** is designed to measure the activities of purified MAPKAP-kinase 2 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-serine²⁰⁴ residue in LSP1, which is phosphorylated by MAPKAP-kinase 2.

Applications of this kit include:

- 1) Screening inhibitors or activators of MAPKAP-kinase 2.
- 2) Detecting the effects of pharmacological agents on MAPKAP-kinase 2 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

IAP kinase-activated protein kinase 2 (MAPKAP-kinase 2) was originally identified as a substrate for the p42/p44 MAPKs in vitro ⁽¹⁾. However, recent data indicate that in intact cells, the upstream kinase that regulates MAPKAP-kinase 2 is p38 MAPK ⁽²⁻⁵⁾. Treatment of cells with endotoxin, interleukin-1, tumor necrosis factor, or various stress stimuli activate p38 MAPK and MAPKAP-kinase 2 ⁽²⁻⁵⁾. Mice deficient in MAPKAP-kinase 2 showed a reduction in bacterial lipopolysaccharide-induced biosynthesis of tumor necrosis factor (TNF), interferon, interleukin (IL)-1, IL-6, and nitric oxide ⁽⁶⁾, suggesting a critical role of MAPKAP-kinase 2 in inflammatory cytokine production. In human neutrophils MAPKAP-kinase 2 is also activated by the chemotactic factor fMet-Leu-Phe and phorbol 12-myristate 13-acetate ⁽⁷⁾. The function of MAPKAP-kinase 2 is not known, but its upstream kinase p38 MAPK has been proposed to be involved in the biosynthesis of inflammatory cytokines ⁽⁸⁾, apoptosis ⁽⁹⁾ and platelet aggregation ⁽¹⁰⁾. MAPKAP-kinase 2 contains a C-terminal autoinhibitory domain ⁽¹⁰⁻¹³⁾ and is activated by phosphorylation at multiple sites ^(12,13).

Recently It was reported that the major substrate for MAPKAP-kinase 2 in human neutrophils is not HSP27 but is a protein termed p60 ⁽¹⁴⁾, which we was identified as LSP1, a 339-amino acid cytoskeletal protein, the expression of which is restricted to neutrophils, lymphocytes, and macrophages.

Measurement of MAPKAP-kinase 2 activity

The protocol generally regarded as most sensitive for the quantitative measurement of MAPKAP-kinase 2 activity involves incubation of the MAPKAP-kinase 2 sample with substrate, either a natural or synthetic polypeptide (such as Long S6 Kinase substrate peptide), in the presence of Mg²⁺ and ³²P-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 MAPKAP-kinase 2 Assay/Inhibitor Screening Kit** uses a peroxidase coupled anti-phospho-LSP1 serine204 monoclonal antibody as a reporter molecule in a 96-well ELISA format. This assay provides a non-isotopic, sensitive and specific method to detect MAPKAP-kinase 2 activity.



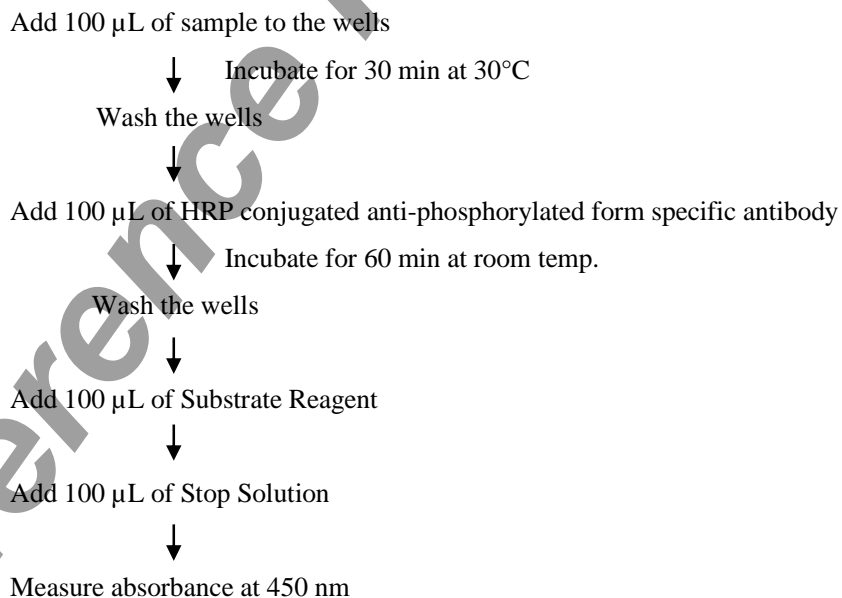
Principle of the Assay

The CycLex Research Product **CycLex MAPKAP-kinase 2 Assay/Inhibitor Screening Kit** is a single-site, semi-quantitative immunoassay for MAPKAP-kinase 2 activity. Plates are pre-coated with a substrate corresponding to recombinant LSP1 (Leukocyte Specific Protein), which contains a serine residue that are phosphorylated by MAPKAP-kinase 2 (MAPK-activated protein kinase 2).

The detector antibody specifically detects only the phosphorylated form of serine-204 on LSP1. The CycLex Research Product **CycLex MAPKAP-kinase 2 Assay/Inhibitor Screening Kit** can be used to study the kinetics of a purified or partially purified MAPKAP-kinase 2 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 AT-1E6, an anti-phospho-LSP1 serine-204 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 MAPKAP-kinase 2 activity in the sample. For kinetic analysis, the sample containing MAPKAP-kinase 2 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 MAPKAP-kinase 2 Assay/Inhibitor Screening Kit** is designed to accurately determine the presence and relative amount of MAPKAP-kinase 2 kinase activity in purification column fractions, and to determine non-isotopic kinetic analysis of MAPKAP-kinase 2 activity. Careful attention to methods of chromatography and the assay protocol will provide the investigator with a reliable tool for the evaluation of MAPKAP-kinase 2 activity.

Summary of Procedure





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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 full length LSP1 as substrate of MAPKAP-kinase 2.

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 Na₂ salt.

HRP conjugated Detection Antibody: One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-phospho-LSP1 S204 (AT-1E6) 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 H₂SO₄. Ready to use.

Materials Required but not Provided

- **MAPKAP-kinase 2 positive control:** Available from CycLex (Cat # CY-E1166); One vial contains 4 units/100 μ L MAPKAP-kinase 2 enzyme. Positive control should be added to the first well at 20 m units/well. For instance, diluted positive control 1:20, use 10 μ L for 1 assay. (Unused MAPKAP-kinase 2 enzyme should be stored in aliquots at **-70°C**.)
- **10X Staurosporine (500 μ M):** Staurosporine is available from Sigma, Cat#. S-4400. 50 mM stock solution (DMSO) diluted 1:100 in Kinase Buffer.
- **Pipettors:** 2-20 μ L, 20-200 μ L and 200-1000 μ L 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

- 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.**



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Detailed Protocol

The CycLex Research Product **CycLex MAPKAP-kinase 2 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 MAPKAP-kinase 2 (Cat # CY-E1166), 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 ddH₂O. 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 ddH₂O to the vial of **20X ATP** (provided, lyophilized). Mix gently until dissolved. the Final concentration of the **20X ATP Solution** should be **2.5 mM**. Store the solution in small aliquots (e.g. 100 µL) at -20°C.
3. Prepare **Kinase Reaction Buffer** by mixing following reagents.

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

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

Standard Assay

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.
2. Prepare all samples (diluted with Kinase Buffer. as needed). All samples should be assayed in duplicate.
3. Duplicate wells containing 20 m units/10 µL MAPKAP-kinase 2 (Cat # CY-E1166) 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 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.



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

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 individual column fractions, add **10 μ L** of **each fraction** to the wells of the assay plate on ice. Duplicate wells containing 20 m units/10 μ L MAPKAP-kinase 2 (Cat # CY-E1166) should be included in each assay as a positive control for phosphorylation.
4. Begin kinase reaction by addition of **90 μ 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 μ 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 μ L** 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 after use.
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 μ L** of **Substrate Reagent** to each well and incubate **at room temperature (ca.25°C) for 10-15 minutes.**
10. Add **100 μ 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



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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 MAPKAP-kinase 2 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 MAPKAP-kinase 2 activity, the level of A450 is weakened as compared with "Solvent control". The high level of A450 is not observed in "Inhibitor control" (usually $A_{450} < 0.2$).

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 Staurosporine (500 μM)*	-	-	10 μL
MAPKAP-kinase 2 Positive Control (2 m unit/μL)** or your enzyme fraction	10 μL	10 μL	10 μL

* 10X Staurosporine (500 μ M): See page 4, section "Materials Required but not Provided"

** Cat # CY-E1166: 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 μ L of "Diluted MAPKAP-kinase 2 positive control" to each well and mixing thoroughly at room temperature. Cover with plate sealer. Incubate **at 30°C for 30-60 minutes**.
2. Follow the **Standard Assay**, steps 5-10, page 6-7.



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Special considerations when measuring precise MAPKAP-kinase 2 activity

In order to measure the activity of MAPKAP-kinase 2 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 MAPKAP-kinase 2 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 (500 µM)*	-	10 µL	-	-	-
Your enzyme fraction	10 µL	10 µL	10 µL	-	-
MAPKAP-kinase 2 Positive Control (2 m unit/µL)**	-	-	-	10 µL	-
Buffer	-	-	-	-	10 µL

* 10X Staurosporine (500 µM): See page 4, section "Materials Required but not Provided"

** Cat # CY-E1166: 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 30-60 minutes**.
2. Follow the **Standard Assay**, steps 5-10, page 6-7.

Evaluation of Results

1. Average the absorbance values for the MAPKAP-kinase 2 sample duplicates (positive control) and all experimental sample duplicate values (when applicable). When the MAPKAP-kinase 2 positive control (20 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 MAPKAP-kinase 2.
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 MAPKAP-kinase 2 Assay/Inhibitor Screening Kit** has been shown to detect the activity of MAPKAP-kinase 2 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 MAPKAP-kinase 2.



Troubleshooting

1. The MAPKAP-kinase 2 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 MAPKAP-kinase 2 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. 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 Dose dependency of recombinant MAPKAP-kinase 2 enzyme reaction

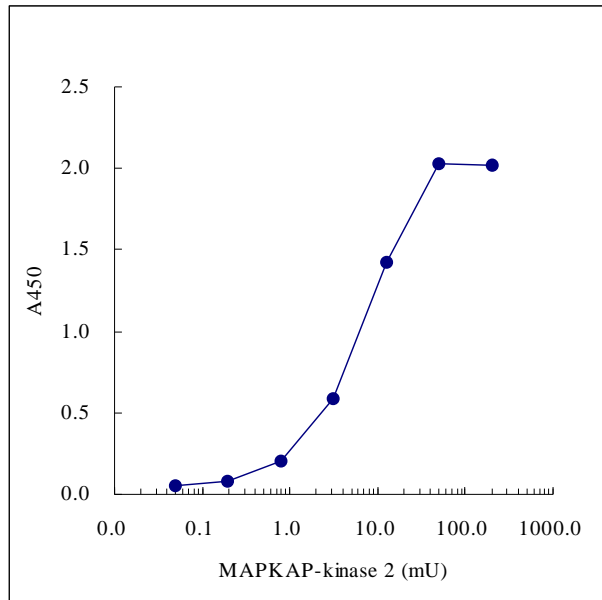
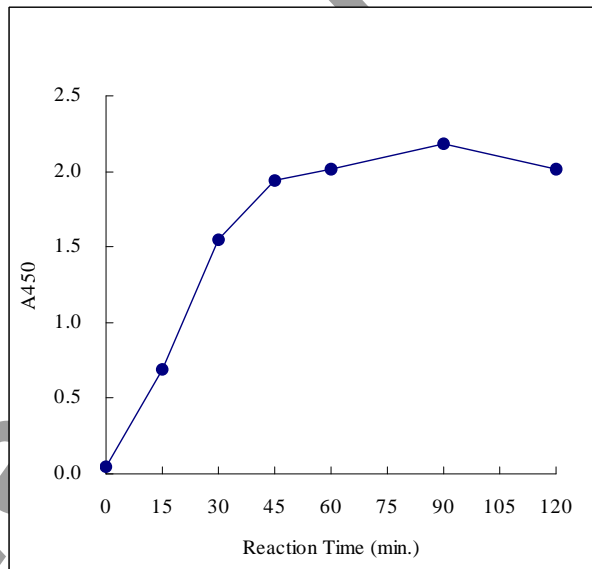


Fig.2 Time course of recombinant MAPKAP-kinase 2 enzyme reaction



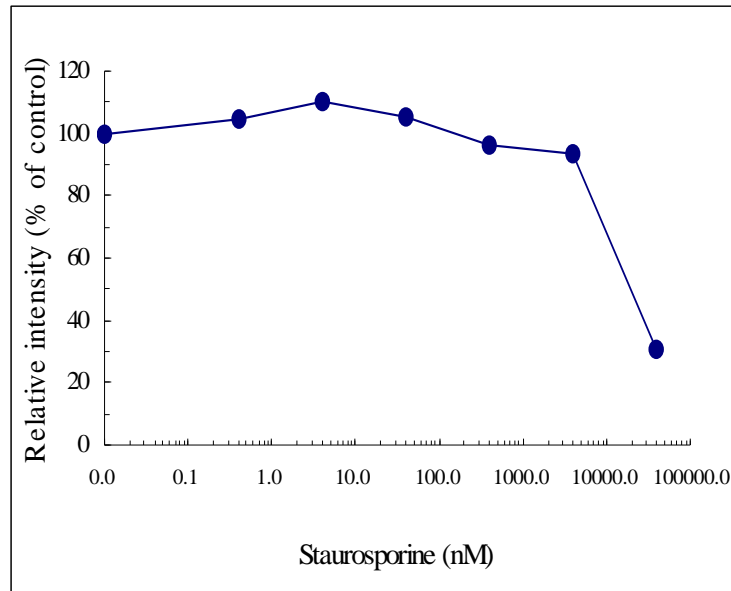


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Fig.3 Effect of broad protein kinase inhibitor Staurosporine on activity of recombinant MAPKAP-kinase
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Related Products

* MAPKAP-kinase 2 Positive control: Cat# CY-E1167

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