Non-Radioisotopic Kit for Measuring Met Kinase Activity

CycLex Met Kinase Assay/Inhibitor Screening Kit

Cat# CY-1080

Intended Use

The MBL Research Product **CycLex Met Kinase Assay/Inhibitor Screening Kit** is designed to measure the activities of recombinant catalytic domain of Met for the rapid and sensitive evaluation of inhibitors or activators. The phosphotyrosine specific monoclonal antibody used in this assay kit has been demonstrated to recognize the phosphotyrosine residue in recombinant "Tyrosine kinase-substrate-1", which is efficiently phosphorylated by Met *in vitro*.

Applications of this kit include:
1) Screening inhibitors or activators of recombinant catalytic domain of Met.
2) Detecting the effects of pharmacological agents on recombinant catalytic domain of Met.

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 *MET* protooncogene was discovered because of the ability of oncogenic Met to mediate chemically induced transformation of a human osteogenic sarcoma cell line (1). This receptor tyrosine kinase is synthesized as a single-chain precursor, which undergoes intracellular proteolytic cleavage at a basic amino acid site, yielding a disulfide-linked heterodimer. Its C-terminal, intracellular region contains a multifunctional docking site that binds to various signaling molecules.

The ligand of the Met receptor is HGF/scatter factor, known to stimulate invasive growth of epithelial cells (2). It is a multifunctional factor affecting a number of cell targets including epithelium, endothelium, myoblasts, spinal motor neurons, and hematopoietic cells. Signaling pathways activated by the HGF-Met interaction mediate cell adhesion and motility. These cellular phenotypes, coupled to tightly regulated changes in cell growth, morphology, and survival, define a general pattern of invasive growth that occurs widely in normal development.

In addition, Met is involved in malignant cell transformation. Increased Met expression has been found in a significant percentage of human cancers and is amplified during the transition between primary tumors and metastasis. Point mutations in *MET* have been identified in hereditary and sporadic papillary renal carcinomas (3–5), hepatocellular and gastric carcinomas (6, 7), and head and neck squamous carcinomas (8). Numerous experimental and clinical data indicate a particular role of HGF and Met in tumor invasive growth, a stage of tumor progression leading to metastases. Dysregulation of Met activity in cells is thought to be a key event underlying tumor metastasis, and indeed, Met overexpression and hyperactivation are reported to correlate with metastatic ability of the tumor cells (9).

Measurement of Met Kinase Activity

The protocol generally regarded as most sensitive for the quantitative measurement of Met kinase activity involves incubation of the Met kinase sample with substrate, either a natural or synthetic polypeptide (such as poly[Glu,Tyr]4:1), in the presence of Mg^{2+}, Mn^{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 MBL Research Product **CycLex Met Kinase Assay/Inhibitor Screening Kit** uses a horseradish peroxidase coupled anti-phosphotyrosine monoclonal antibody as a reporter molecule in a 96-wells ELISA format. This assay provides a non-isotopic, sensitive and specific method to detect kinase activity of recombinant Met catalytic domain.

Principle of the Assay

The MBL Research Product **CycLex Met Kinase Assay/Inhibitor Screening Kit** is a single-site, semi-quantitative immunoassay for kinase activity of recombinant catalytic domain of Met. Plates are pre-coated with a newly designed "Tyrosine kinase-substrate-1", which can be easily phosphorylated by recombinant catalytic domain of Met. The detector antibody is PY-39, an antibody that specifically detects the phosphotyrosine residue on "Tyrosine kinase-substrate-1".

The MBL Research Product **CycLex Met Kinase Assay/Inhibitor Screening Kit** might be used to follow the kinetics of recombinant catalytic domain of Met as well as screening Met inhibitor or activator. To perform the test, the recombinant catalytic domain of Met is diluted in Kinase Buffer, pipetted into the wells and allowed to phosphorylate "Tyrosine kinase-substrate-1" on the wells in the presence of Mg^{2+}, Mn^{2+} and ATP. The amount of phosphorylated "Tyrosine kinase-substrate-1" is measured by binding it with a horseradish peroxidase conjugate of PY-39, a anti-phosphotyrosine monoclonal antibody, which then catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stopping reagent). The color is quantified by spectrophotometry and reflects the relative amount of Met kinase activity in the sample. For kinetic analysis, the recombinant catalytic domain of Met 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 MBL Research Product **CycLex Met Kinase Assay/Inhibitor Screening Kit** is designed to determine non-isotopic kinetic analysis of the Met catalytic domain kinase activity. Careful attention to operation and the assay protocol will provide the investigator with a reliable tool for the evaluation of inhibitor or activator of Met kinase.

Summary of Procedure

Add 100 µL of reaction mixture to the wells
↓ Incubate for 60 minutes at 30°C
Wash the wells
↓
Add 100 µL of HRP conjugated anti-phosphotyrosine antibody
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of Substrate Reagent
↓ Incubate for 5-15 minutes at room temp.
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-wells 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 "Tyrosine kinase-substrate-1".

10X Wash Buffer: One bottle containing 100 mL of 10X buffer containing 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 bottle containing 12 mL of HRP (horseradish peroxidase) conjugated anti-phosphotyrosine monoclonal antibody (PY-39).

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

- **Met Positive Control:** Recombinant Met catalytic domain, available from MBL, Cat# CY-E1080.
- **(Optional) 10X Staurosporine (100 µM):** A broad spectrum protein kinase inhibitor, available from Sigma, Cat# S-4400. 10 mM stock solution (DMSO) diluted 1:100 in Kinase Buffer.
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 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**
- **Microplate washer:** optional (Manual washing is possible but not preferable)
- **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.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1,000 mL graduated cylinder**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**
- 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 the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of human origin, and these reagents. 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 MBL Research Product **Met Kinase Assay/Inhibitor Screening** 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. Due to technical reason, this kit is adjusted to measure kinase activity of the recombinant catalytic domain of Met (Met Positive Control: not provided, Cat# CY-E1080), which should be used in all assays. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solutions

1. Prepare a working solution of **Wash Buffer** by adding 100 mL of the **10X Wash Buffer** (provided) to 900 mL of deionized (distilled) water (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.

4. Prepare **Met Positive Control (0.1 unit/µL)** or serial dilution of Met Positive Control by diluting Met Positive Control (Cat# CY-E1080*) with the **Kinase Buffer** (provided).
**See the section "Materials Required but not Provided" above.*

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. Add **10 µL** of **Met Positive Control (0.1 unit/µL)** or serial dilution of Met Positive Control to the wells of the assay plate on ice. All assays should be done in duplicate.

3. Begin the kinase reaction by addition of **90 µL** of **Kinase Reaction buffer** per well, cover with plate sealer, and incubate **at 30°C for 60 minutes**.

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

5. 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.

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

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

9. 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 Met Positive Control.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the Met Positive Control, perform a second reading at 405 nm. A new O.D. values, measured at 405 nm, is used to determine Met 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. Add **10 µL** of **Met Positive Control (0.1 unit/µL)** or serial dilution of Met Positive Control to the wells of the assay plate on ice. All assays should be done in duplicate.

3. Begin kinase reaction by addition of **90 µL** of **Kinase Reaction Buffer** 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**.

4. 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).

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 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.

Recommendations

Special considerations when screening activators and inhibitors

In order to estimate the inhibitory effect on Met 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 Met 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<0.4).

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 (100 µM)*	**-**	**-**	**10 µL**
Met Positive Control (0.1 unit/µL)	**10 µL**	**10 µL**	**10 µL**

* 10X Staurosporine (100 µM): See the section "Materials Required but not Provided" above.

1. Following the above table, add the Reagents to each well of the microplate. Finally, initiate reaction by adding 10 µL of "**Met Positive Control (0.1 unit/µL)**" to each well and mixing thoroughly at room temperature. Cover with plate sealer. Incubate **at 30°C for 60 minutes**.

2. Follow the step 4 to 9 of "**Standard Assay**" above.

Evaluation of Results

1. Average the absorbance values for the Met sample duplicates (Positive Control) and all experimental sample duplicate values (when applicable). When the Met Positive Control (1 unit/assay) is included as an internal control for the phosphorylation reaction, the absorbance value should be greater than 1.0 with a background less than 0.2.

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 MBL Research Product **CycLex Met Kinase Assay/Inhibitor Screening Kit** has been shown to detect the kinase activity of recombinant catalytic domain of Met. The assay shows good linearity of sample response.

Troubleshooting

1. All samples and controls should be assayed 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 MBL Research Product **CycLex Met 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. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

Example of Test Results

Fig.1 Dose dependency of recombinant Met catalytic domain enzyme reaction

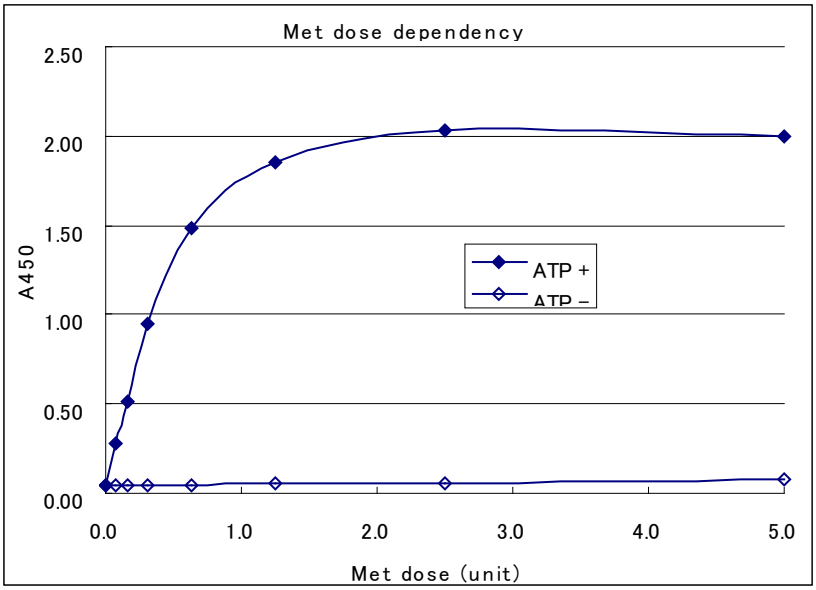


Fig.2 Time course of recombinant Met catalytic domain enzyme reaction

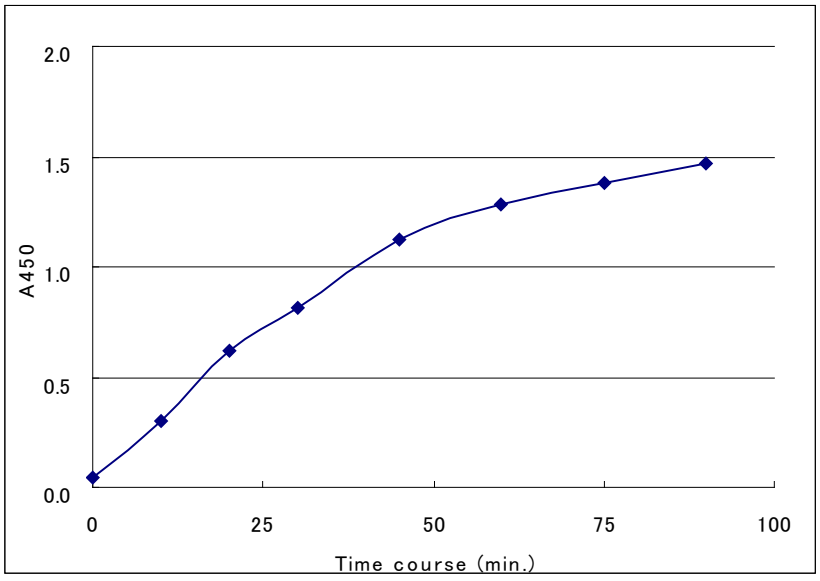


Fig.3-1 Dose dependency of ATP (recombinant Met catalytic domain)

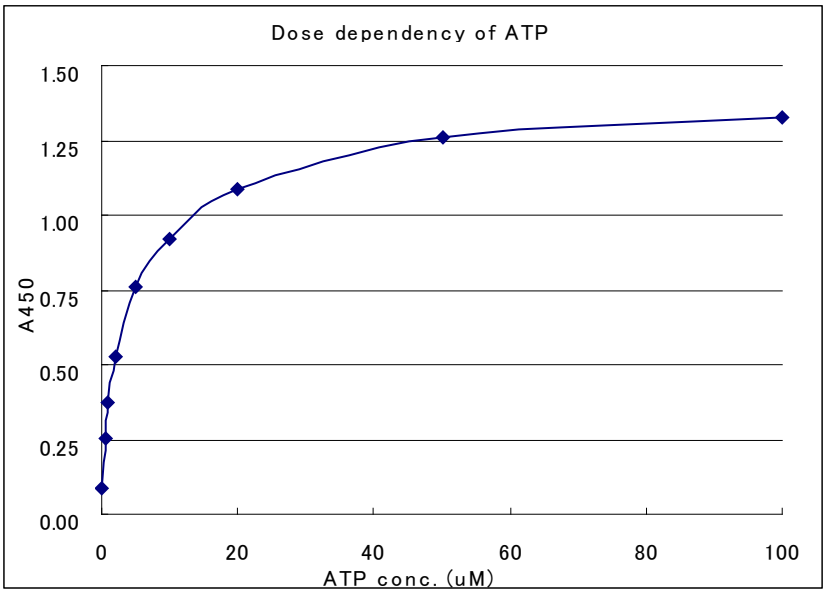


Fig.3-2 Km for ATP (recombinant Met catalytic domain)

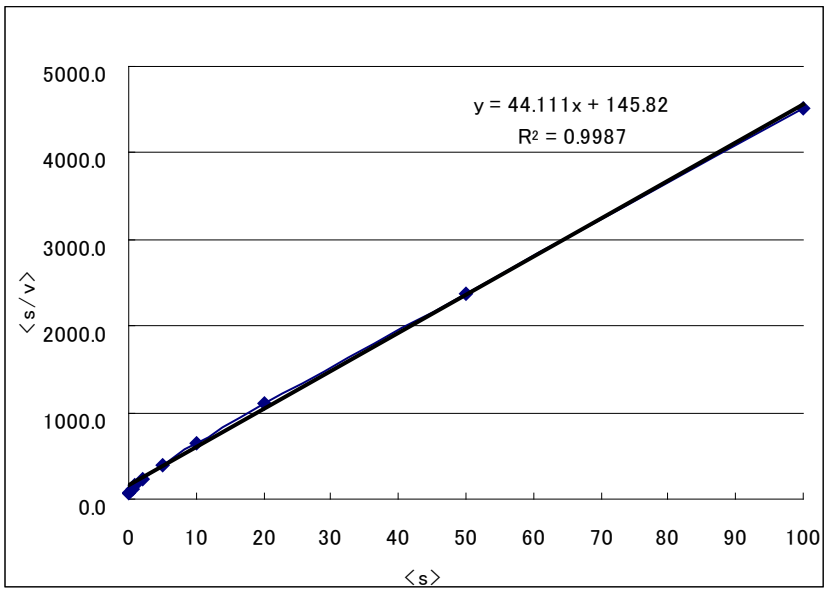


Fig.4-1 Effect of broad-spectrum kinase inhibitor Staurosporine on activity of recombinant Met catalytic domain enzyme reaction.

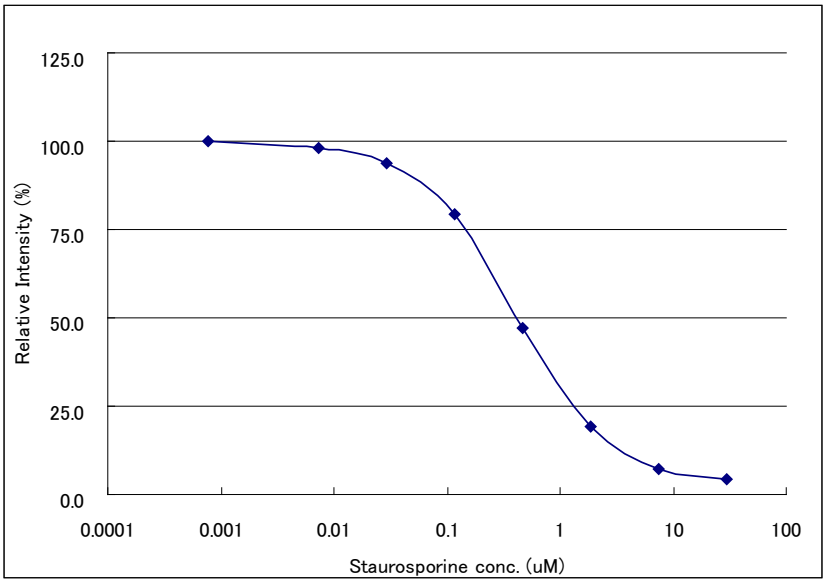
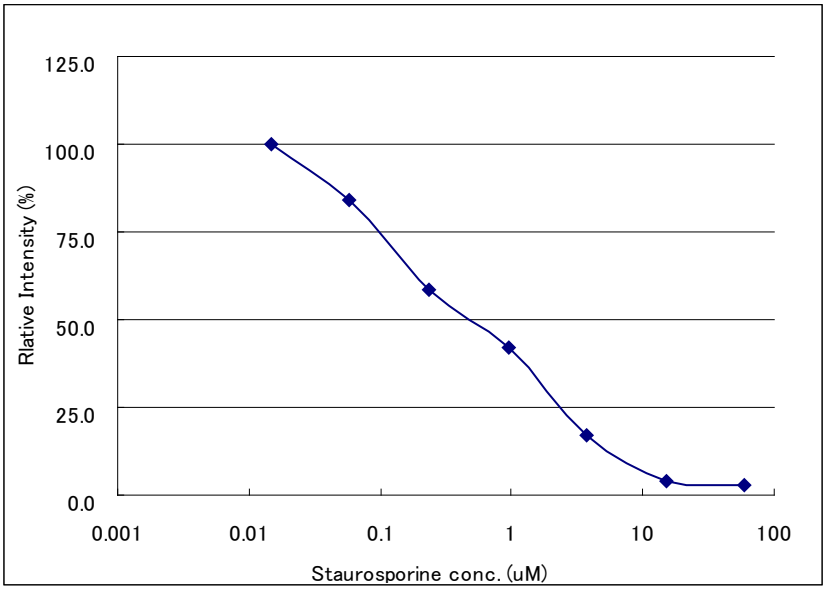


Fig.4-2 Effect of broad-spectrum kinase inhibitor Staurosporine on activity of recombinant Met catalytic domain enzyme reaction using radioisotope gamma-^{32}P-ATP.



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