Non-Radioisotopic Kit for Measuring Plk1 Activity

# CycLex Polo-like kinase 1 Assay/Inhibitor Screening Kit

Cat# CY-1163



## Intended Use

The MBL Research Product **CycLex Polo-like kinase 1 Assay/Inhibitor Screening Kit** is designed to measure the activities of purified polo-like kinase 1 (Plk1) for the rapid and sensitive evaluation of inhibitors using recombinant Plk1. The phospho-threonine specific polyclonal antibody used in this assay kit has been demonstrated to recognize the phospho-threonine residue in protein-X, which is efficiently phosphorylated by Plk1.

Applications of this kit include:
1) Screening inhibitors or activators of Plk1.
2) Detecting the effects of pharmacological agents on Plk1 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

Polo-like kinases (Plk) have been shown to be important contributors to several cell-cycle events (1, 2). Genetic and biochemical experiments in various organisms indicate that polo-like kinases regulate diverse cellular events at multiple mitotic stages. Genetic studies in Drosophila and yeast indicate plks function in centrosome assembly and separation during the formation of the bipolar spindle. Drosophila polo mutants reveal phenotypes of hyper-condensed chromosomes, monopolar spindles, disorganized spindle poles, and abnormal chromosome segregation (3). Schizosaccharomyces pombe plo1 displays similar phenotypes, such as the formation of monopolar spindles or a failure in septum formation after nuclear division (4). The budding yeast polo-like kinase homolog, Cdc5, seems to play an important role in actin ring formation and cytokinesis (5). In mammalian cells, antibody microinjection suggests a role for Plk1 in centrosome maturation (6). Mammalian Plk1 was further shown to phosphorylate specifically at least three components of APC, and to activate APC to ubiquitinate cyclin B in an in vitro-reconstituted system (7). More recent studies demonstrated that polo kinase activity plays a pivotal role in the separation of sister chromatids during mitosis (8).

Elevated expression of Plk1 occurs in many different types of cancer, and Plk1 has been proposed as a diagnostic marker for several tumors. Liu and Erikson (9) used the vector-based small interfering RNA (siRNA) technique to specifically deplete Plk1 in cancer cells. They found that such depletion dramatically inhibited cell proliferation, decreased viability, and resulted in cell-cycle arrest with a 4 N DNA content. The formation of dumbbell-like chromatin structure suggested the inability of these cells to completely separate the sister chromatids at the onset of anaphase. Plk1 depletion induced apoptosis, as indicated by the appearance of sub-genomic DNA in FACS profiles, the activation of caspase-3, and the formation of fragmented nuclei. The p53 pathway was shown to be involved in Plk1 depletion-induced apoptosis. DNA damage occurred in Plk1-depleted cells and inhibition of ATM strongly potentiated the lethality of Plk1 depletion. The data supported the notion that disruption of Plk1 function could be an important application in cancer therapy.

## Measurement of polo-like kinase 1 activity

The protocol generally regarded as most sensitive for the quantitative measurement of Plk1 activity involves incubation of the Plk1 sample with substrate, either a natural or synthetic polypeptide (such as alpha casein), 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 Polo-like kinase 1 Assay/Inhibitor Screening Kit** uses anti-phospho-threonine polyclonal antibody (PPT-07) 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 Plk1.

## Principle of the Assay

The MBL Research Product **CycLex Polo-like kinase 1 Assay/Inhibitor Screening Kit** is a single-site, semi-quantitative immunoassay for Plk1 activity. Plates are pre-coated with a substrate corresponding to recombinant Protein-X, which contains threonine residues that can be efficiently phosphorylated by Plk1. The detector antibody specifically detects only the phosphorylated form of threonine residue on Protein-X. The **CycLex Polo-like kinase 1 Assay/Inhibitor Screening Kit** may be used to study the kinetics of a purified Plk1 as well as to screening Plk1 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 PPT-07, a anti-phospho-threonine 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 Plk1 activity in the sample. For kinetic analysis, the Plk1 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 minutes at 30°C
Wash the wells
↓
Add 100 µL of anti-phospho-threonine polyclonal antibody (PPT-07).
↓ Incubate for 30 minutes at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti-rabbit IgG
↓ Incubate for 30 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-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 Protein-X as Plk1 substrate.

**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.

**Anti-Phospho-Threonine Polyclonal Antibody:** One vial containing 12 mL of anti-phospho-threonine polyclonal antibody (PPT-07). 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

- **Plk1 Positive Control:** Available from MBL, Cat# CY-E1163
- **(Optional) 10X Staurosporine (200 µM):** A broad spectrum protein kinase Inhibitor, available from Sigma, Cat# S-4400. 20 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.
- **Wash bottle or multichannel dispenser** for plate washing.
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **(Optional) Microplate washer:** 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 **CycLex Polo-like kinase 1 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 Plk1 Positive Control (See the section "Materials Required but not Provided" above) 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 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 (dd$H_2O$). 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 dd$H_2O$ 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) | **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. Add **10 µL** of diluted enzyme samples to the wells of the assay plate on ice. Duplicate wells containing 10 µL of Plk1 Positive Control (10 µ units/µL) should be included in each assay as a positive control for phosphorylation.

4. Begin the kinase reaction by addition of **90 µL** of **Kinase Reaction Buffer** per well, cover with plate sealer or lid, 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-Threonine Polyclonal Antibody** into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any unused antibody after use.

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 7.

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.

## 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). All samples should be assayed in duplicate.

3. Add **10 µL** of diluted enzyme samples to the wells of the assay plate on ice. Duplicate wells containing 10 µL of Plk1 Positive Control (10 µ units/ µL) should be included in each assay as a positive control for phosphorylation.

4. Begin the kinase reaction by addition of **90 µL** of **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, 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 **Anti-Phospho-Threonine Polyclonal Antibody** into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any unused antibody after use.

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 8.

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 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 Stop Solution.

## Recommendations

**Special considerations when screening activators or inhibitors**

In order to estimate the inhibitory effect on individual Plk1 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 Plk1 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 (200 µM)*** | **-** | **-** | **10 µL** |
| **Plk1 positive control (10 µ unit/µL)*** **or** **Your enzyme samples** | **10 µL** | **10 µL** | **10 µL** |

* 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 "Plk1 Positive Control" or "Your enzyme samples"to each well and mixing thoroughly at room temperature. Cover with plate sealer or lid, and incubate **at 30°C for 30-60 minutes**.

2. Follow the step 5 to 12 of "**Standard Assay**" above.

**Special considerations when measuring precise Polo-like kinase 1 activity**

In order to measure the activity of Plk1 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 Plk1 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 (200 µM)*** | **-** | **10 µL** | **-** | **-** | **-** |
| **Your enzyme samples** | **10 µL** | **10 µL** | **10 µL** | **-** | **-** |
| **Plk1 Positive Control (10 µ unit/µL)*** | **-** | **-** | **-** | **10 µL** | **-** |
| **Buffer** | **-** | **-** | **-** | **-** | **10 µL** |

* 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 the reaction by adding 10 µL of "Your enzyme samples" or "Plk1 Positive Control" or "Buffer" to each well and mixing thoroughly at room temperature. Cover with plate sealer or lid, and incubate **at 30°C for 30-60 minutes**.

2. Follow the step 5 to 12 of "**Standard Assay**" above.

## Evaluation of Results

1. Average the absorbance values for the Plk1 sample duplicates (positive control) and all experimental sample duplicate values (when applicable). When Plk1 Positive Control (100 µ 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 MBL Research Product **CycLex Polo-like kinase 1 Assay/Inhibitor Screening Kit** has been shown to detect the activity of purified recombinant Plk1. The assay shows good linearity of sample response.

## Troubleshooting

1. All samples and standards 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 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 MBL Research Product **CycLex Polo-like kinase 1 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.

## Example of Test Results

Fig.1 Dose dependency of recombinant Plk1 enzyme reaction

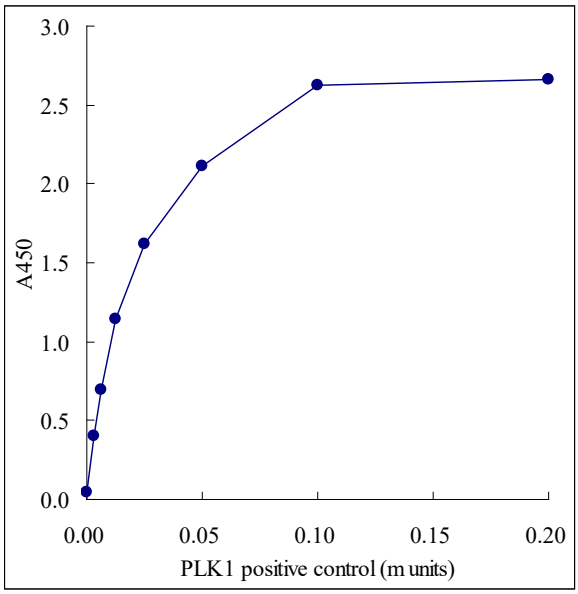



Fig.2 Time course of recombinant Plk1 enzyme reaction

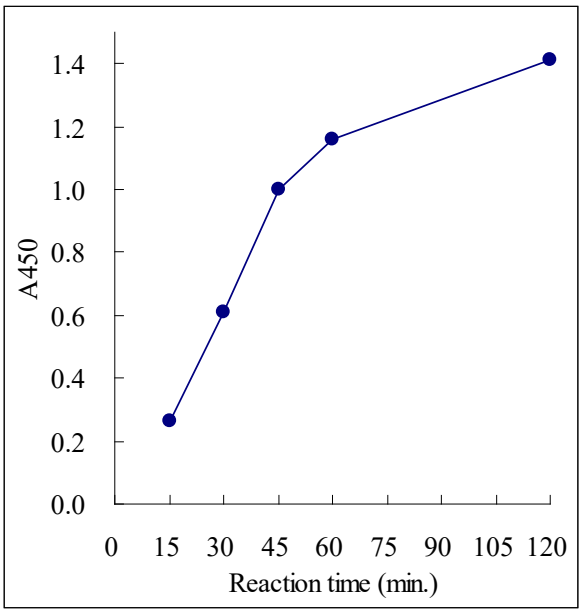


Fig.3 Km for ATP (recombinant Plk1)

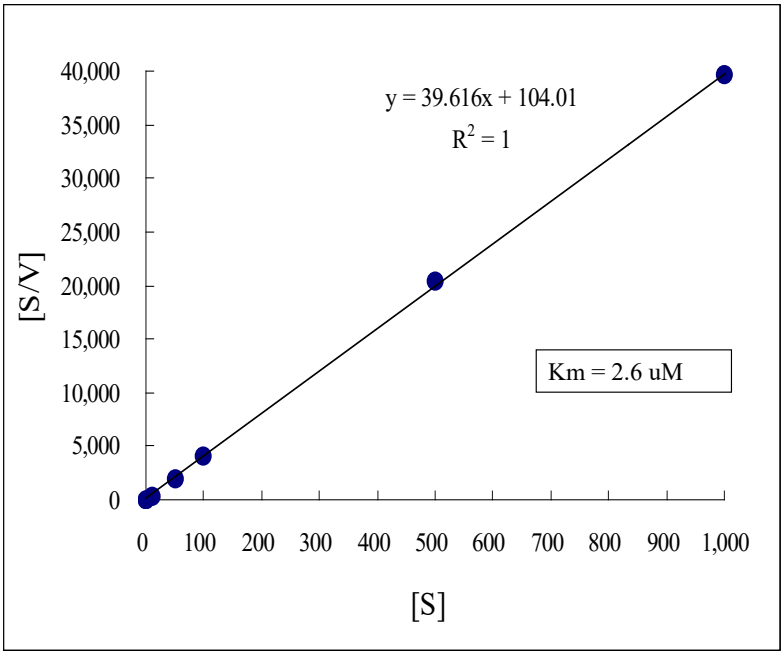
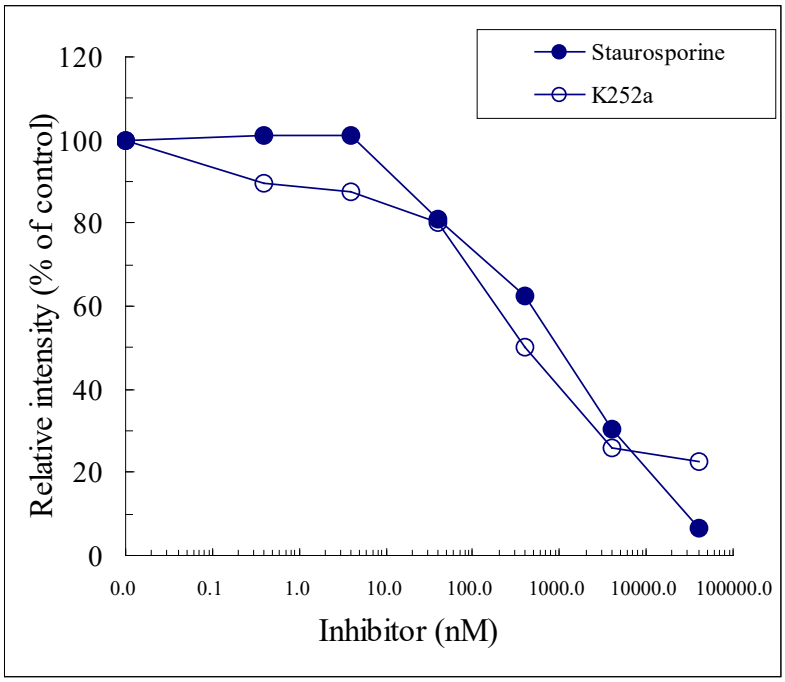



Fig.4 Effect of broad-spectrum kinase inhibitor staurosporine and K252a on Plk1 activity



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