Fluorometric Assay Kit for Measuring Cdi1/KAP Phosphatase Activity

CycLex Protein Phosphatase Cdi1/KAP Fluorometric Assay Kit

100 Assays

Cat# CY-1356

Intended Use... 1
Storage.. 1
Introduction 2
Principle of the Assay............................. 2
Materials Provided 3
Materials Required but not Provided...... 3
Precautions and Recommendations......... 3-4
Detailed Protocol.................................... 4-6
Evaluation of Results 7-9
Troubleshooting 10
Reagent Stability 10
References... 11
Related Products..................................... 11-12

Intended Use

The CycLex Research product **Protein Phosphatase Cdi1/KAP Fluorometric Assay Kit** is a fluorometric and non-radioactive assay designed to measure the activity of Cdi1/KAP protein phosphatase. This 96-well assay is useful for screening inhibitors and modulators of Cdi1/KAP activity in HTS. The kit includes all necessary components, including recombinant, human Cdi1/KAP (full length), for use in preinvestigational drug discovery assays.

This assay kit is for research use only and not for use in human, diagnostic, or therapeutic procedures.

Storage

- Upon receipt, store the kit at -70°C.
- Don't expose reagents to excessive light.
- **AVOID REPEATED FREEZE THAW CYCLES OF "③Recombinant Cdi1/KAP"!**

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Introduction

A Cdk-interacting protein called Cdi1 (cyclin-dependent kinase interactor 1)/KAP (kinase associated phosphatase) was first identified as a novel G1- and S-phase dual-specificity phosphatase that associates with Cdk2 and/or Cdc2 by the interaction trap, a yeast genetic selection for interacting proteins (1, 2). Using yeast two hybrid system, Cdi1/KAP interacts with cyclin-dependent kinases, including human Cdc2, Cdk2 and Cdk3, but not with Cdk4. In HeLa cells, Cdi1/KAP is expressed at the G1 to S transition, and the protein forms stable complexes with Cdk2. Further studies demonstrated that Cdi1/KAP binds to Cdk2 and dephosphorylates Thr160 when the associated cyclin subunit is degraded or dissociated (3). It means that Cdi1/KAP may inactivate a Cdk2 or Cdc2, by removing phosphates from the cyclin complexes, and this may contribute to cell cycle control (2). However, the physiological substrate(s) for tyrosine dephosphorylation of Cdi1/KAP has not yet been identified.

Phosphatases have also been shown to play an important role in regulating a variety of signal transduction pathways that have a bearing on cancer (4-6). It was reported that the Cdi1/KAP gene is overexpressed in human breast and prostate cancer using differential screening and that breast and prostate malignancies are associated with high levels of KAP expression (7).

Principle of the Assay

The **Protein Phosphatase Cdi1/KAP Fluorometric Assay Kit** is based on an exclusive fluorescence substrate, OMFP (3-o-methylfluorescein phosphate). This homogenous assay kit is sensitive and convenient. This method of measurement should raise the efficiency of inhibitor screening and biochemical analysis of this enzyme.

Summary of Procedure

Mix 40 µL of Assay mixture and 5 µL of test compound in the wells

Add 5 µL of Recombinant Cdi1/KAP

Incubate for 15 min at room temp.

Add 25 µL of Stop Solution

Measure fluorescence at 510-540 nm emission / 482-502 nm excitation

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Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for one hundred assays.

Components of Kit

Components	Quantity	Storage
① **10X Assay Buffer**	**600 µL x 1**	**Below -20°C**
② **10X 3-o-methyl fluorescein phosphate (OMFP)**	**550 µL x 1**	**Below -20°C**
③ **Recombinant Cdi1/KAP, Human (0.4 µg/µL)**	**550 µL x 1**	**-70°C**
④ **100X Cdi1 Inhibitor : 100 µM Na_3VO_4 in H_2O**	**100 µL x 1**	**Below -20°C**
⑤ **Stop Solution**	**300 µL x 1**	**Below -20°C**
⑥ **Instruction Manual**	**1**	**Room temp.**

Materials Required but not Provided

- **Microtiter plate suitable for use with a fluorometric plate reader**
- **Fluorometric plate reader or microtiter plate fluorometer:** Use a fluorescence microplate reader equipped with appropriate filters. OMFP has excitation/emission maxima of approximately 485/525 nm. We have found that standard filters for blue-fluorescent dyes (e.g., excitation = 485 ±12.5 nm, emission = 525 ± 20 nm) can be used to detect OMFP.
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1000 µL precision pipettors with disposable tips
- **Multi-channel pipette**
- **Microtiter plate shaker**
- **Distilled water (DW)** or equivalent high quality water
- **Microcentrifuge and tubes** for sample preparation
- **Reagent reservoirs**
- **Ice bucket** to keep reagents cold until use

Precautions and Recommendations

- Upon receipt, store the kit at -70°C.
- Do not expose reagents to excessive light.
- Do not use kit components beyond the indicated kit expiration date.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- 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.

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NOTE: THE FOLLOWING PROCEDURES ARE INTENDED ONLY AS A GUIDELINE. THE OPTIMAL EXPERIMENTAL CONDITIONS WILL VARY DEPENDING ON THE PARAMETERS BEING INVESTIGATED, AND MUST BE DETERMINED BY THE INDIVIDUAL USER.

Detailed Protocol

Preparation of Reagents

Thaw the reagents at room temperature except "Recombinant Cdi1/KAP" and keep all reagents on ice until use. Use them only after they are completely thawed and mixed.

1. Prepare **10X Cdi1 Inhibitor** by adding 5 µL of the ④ **100X Cdi1 Inhibitor** (provided) to 45 µL of distilled (deionized) water. Mix well.

*Discard any unused **10X Cdi1 Inhibitor** after use.*

2. Prepare **Assay Mixture** by adding 5 µL of the ①10X Assay Buffer (provided) and 5 µL of the 10X Fluoro-Phospho-Substrate (provided) to 30 µL of distilled (deionized) water per one assay. Mix well.

Assay Mixture

Assay reagents	1 assay	8 assays	16 assays	32 assays	48 assays
Distilled water	**30 µL**	**240 µL**	**480 µL**	**960 µL**	**1,440 µL**
①10X Assay Buffer	**5 µL**	**40 µL**	**80 µL**	**160 µL**	**240 µL**
②10X OMFP	**5 µL**	**40 µL**	**80 µL**	**160 µL**	**240 µL**
Total volume of Assay Mixture	**40 µL**	**320 µL**	**640 µL**	**1,240 µL**	**1,920 µL**

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Assay Procedure

In order to estimate the inhibitory effect on Cdi1/KAP activity by the test compounds correctly, it is necessary to conduct the control experiment of "**Vehicle 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 Table.1 (below). When test chemicals cause an inhibitory effect on Cdi1/KAP activity, the level of increase of fluorescence intensity is weakened as compared with "**Vehicle control**". The increase in fluorescence intensity is not observed in "**Inhibitor control**".

1. Following Table.1 below, first, add **"Assay mixture"** to microtiter plate wells. Second, add **"Test Compound"** or **"Vehicle of Test Compounds"** or **"10X Cdi1 Inhibitor"** to each well of the microtiter plate and mix well.

Table.1: Reaction mixture

Assay reagents	Test Sample	Vehicle Control	Inhibitor Control	No Enzyme Control
Assay Mixture	**40 µL**	**40 µL**	**40 µL**	**40 µL**
Test Compound **Vehicle of Test Compounds** **10X Cdi1 Inhibitor***	**5 µL** **-** **-**	**-** **5 µL** **-**	**-** **-** **5 µL**	**-** **5 µL** **-**
③Recombinant Cdi1/KAP (0.1µg/µL) **Distilled water**	**5 µL** **-**	**5 µL** **-**	**5 µL** **-**	**-** **5 µL**
Total Volume of the Reaction mixture	**50 µL**	**50 µL**	**50 µL**	**50 µL**

**10X Cdi1 Inhibitor (10 µM Na_3VO_4): See Page 4, section Preparation of Reagents*

2. Initiate reactions by adding **5 µL of "③Recombinant Cdi1/KAP"** or distilled water to each well and mixing thoroughly at room temperature.

3. Incubate for 15 min or desired length of time at room temperature.

4. Add **25 µL of "⑤Stop Solution"** to each well of the microtiter plate, and mix thoroughly.

5. Measure fluorescence intensity using a microtiter plate fluorometer with excitation at 482-502 nm and emission at 510-540 nm.

6. The efficacy of the Test compound is the difference in fluorescence intensity between "Vehicle control" and "Test sample".

Note: If necessary, it is possible to store the microtiter plate after adding **"④Stop Solution"** for a few hours at 4°C. The microtiter plate must be sealed to prevent evaporation and kept from excessive light.

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Alternate procedure

1'. Following Table.1 above, first, add **"Assay mixture"** to microtiter plate wells. Second, add **"Test Compound"** or **"Vehicle of Test Compounds"** or **"10X Cdi1/KAP Inhibitor"** to each well of the microtiter plate and mix well.

2'. Initiate reactions by adding 5 μL of **"③Recombinant Cdi1/KAP"** or distilled water to each well and mixing thoroughly at room temperature.

3'. Read fluorescence intensity for 20 to 30 minutes at 1 to 2 minute intervals using microtiter plate fluorometer with excitation at 482-502 nm and emission at 510-540 nm.

4'. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Caution and Significance

• All samples and "Recombinant Cdi1/KAP" should be assayed in duplicate.

• Use of a microtiter plate shaker is recommended for complete mixing.

• If the test compounds or samples themselves emit fluorescence at excitation wavelength: 482-502 nm and fluorescence wavelength: 510-540 nm, the test assay cannot be evaluated correctly.

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Evaluation of Results

Analysis of Inhibitor Effect #1

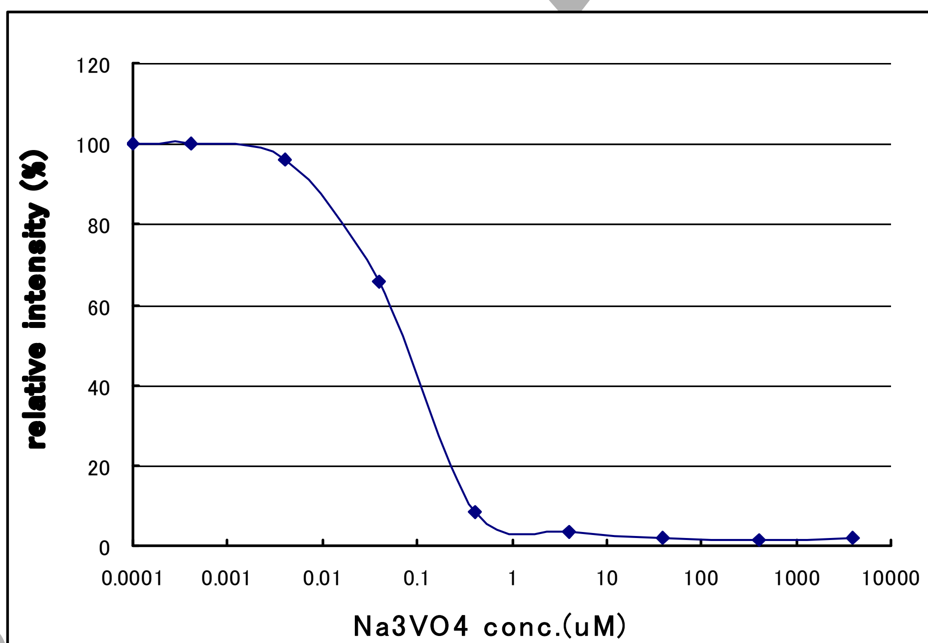
% Intensity

1. Run reactions with test compounds and Vehicle as described in the **Detailed Protocol**.
2. Subtract fluorescence intensity of "No Enzyme Control" from all experimental samples (Test Samples and Vehicle Control).
3. Calculate the % Intensity:

$$\%\ \text{Intensity} = \frac{\text{Fluorescence Intensity of Test Sample}}{\text{Fluorescence Intensity of Vehicle Control}} \times 100$$

Note: This % Intensity is a rough value of enzyme activity or inhibition. For greater accuracy, plot a standard curve of Cdi1/KAP for each new set of reactions and estimate the % Activity (see below).

Fig.1 Cdi1/KAP Inhibition Curve by Na_3VO_4 (Na_3VO_4,Sodium Orthovanadate)



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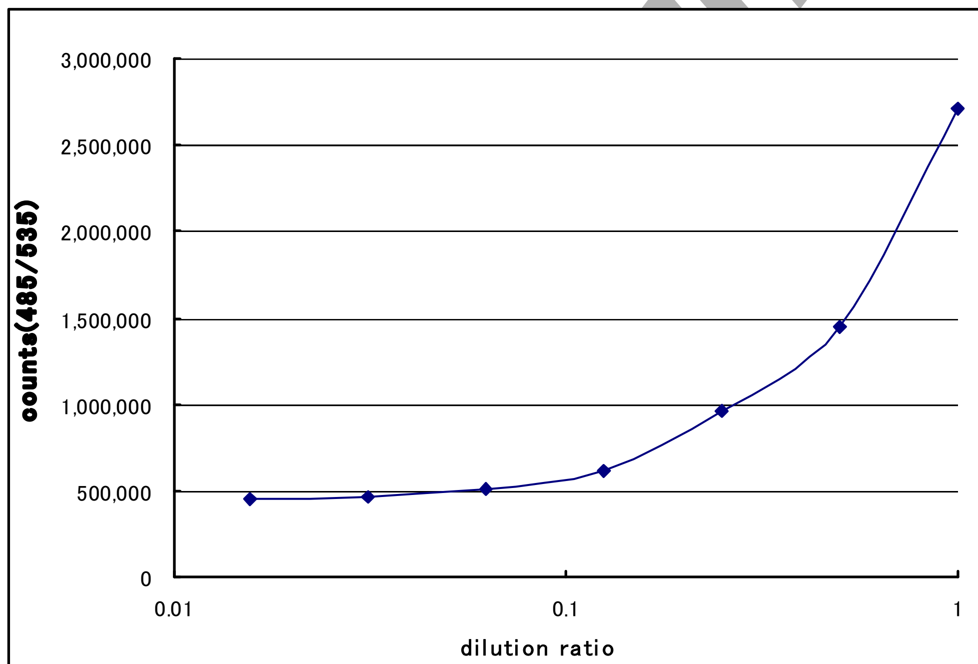
Analysis of Enzyme Activity

Cdi1/KAP Standard Curve and % Activity

1. Dilute the ①10X Assay buffer 1:10 with distilled water to make 1X Assay Buffer.
2. Make serial dilutions of Recombinant Cdi1/KAP with 1X Assay Buffer (ex. 100%, 50%, 25%, 12.5% 6.25% , 3.13% and 0%).
3. Run reactions with Vehicle and serial dilutions of Recombinant Cdi1/KAP as described in the Detailed Protocol.
4. Plot standard curve data (dose dependent curve data) as fluorescence intensity at 510-540 nm versus dose of Cdi1/KAP (ng/assay)
5. Obtain a line-fit to the data using appropriate calculations.
6. Use the slope and Y-intercept to calculate the amount of Cdi1/KAP activity for the experimental data.

Fig.2 Dose Dependency of Recombinant Cdi1/KAP



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Analysis of Kinetics

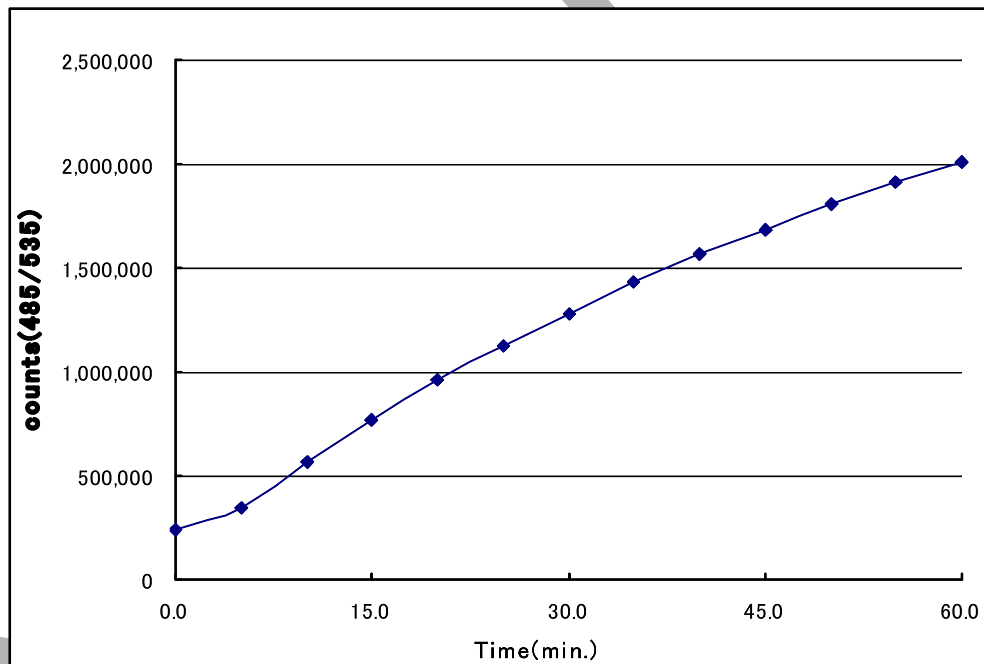
Time Course Curve

1. Run reactions as described in the *Detailed Protocol*.
2. Subtract fluorescence intensity at the 0 time from all reaction time points.
3. Plot fluorescence intensity at 510-540 nm versus reaction time.
4. Determine the reaction time range in which the increase in fluorescence intensity at 510-540 nm is linear.
5. Calculate activity:

$$\text{Activity (reaction velocity)} = \frac{\text{Fluorescence Intensity of Test Sample}}{\text{Reaction time (min.)}}$$

Note: Usually, the linear range is from 0 to 30 min. This value is variable depending on reaction conditions and storage/handling of the Recombinant Cdi1/KAP. Decreasing the amount of Recombinant Cdi1/KAP in the assay may help to lengthen the time range.

Fig.3 Time Course Curve of Recombinant Cdi1/KAP



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Troubleshooting

1. The Recombinant Cdi1/KAP 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 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 inaccurate dispensing of assay reagents. If all instructions in the *Detailed Protocol* were followed accurately, such results indicate a need for multi-channel pipette maintenance.

Reagent Stability

All of the reagents included in the **Protein Phosphatase Cdi1/KAP Fluorometric Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, all kit reagents, except Recombinant Cdi1/KAP, should be stored at -20°C. Recombinant Cdi1/KAP should be stored at -70°C. After use, return kit reagents to -20°C as soon as possible.

For research use only, not for use in human, diagnostic or therapeutic procedures

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References

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Related Products

*** CycLex Protein Tyrosine Phosphatase 1B (PTP1B) Fluorometric Assay Kit: Cat# CY-1350**
*** CycLex T Cell Protein Tyrosine Phosphatase (TC-PTP) Fluorometric Assay Kit: Cat# CY-1351**
*** CycLex Protein Phosphatase Cdc25A Fluorometric Assay Kit: Cat# CY-1352**
*** CycLex Protein Phosphatase Cdc25B Fluorometric Assay Kit: Cat# CY-1353**
*** CycLex Protein Phosphatase Cdc25C Fluorometric Assay Kit: Cat# CY-1354**
*** CycLex Protein Phosphatase Cdc25 Combo Fluorometric Assay Kit: Cat# CY-1355**
*** CycLex Protein Phosphatase Cdi1/KAP Fluorometric Assay Kit: Cat# CY-1356**
*** CycLex Protein Phosphatase LMW-PTP/ACP1 Fluorometric Assay Kit: Cat# CY-1358**
*** CycLex Protein Phosphatase DUSP1/MKP-1 Fluorometric Assay Kit: Cat# CY-1373**

*** Protein Tyrosine Phosphatase PTPRA 1st Catalytic Domain: Cat# CY-E1301**
*** Protein Tyrosine Phosphatase PTPRA 2nd Catalytic Domain: Cat# CY-E1302**
*** Protein Tyrosine Phosphatase PTPRD 2nd Catalytic Domain: Cat# CY-E1307**
*** Protein Tyrosine Phosphatase PTPRE 1st Catalytic Domain: Cat# CY-E1308**
*** Protein Tyrosine Phosphatase PTPRF 1st Catalytic Domain: Cat# CY-E1310**
*** Protein Tyrosine Phosphatase PTPRK 1st Catalytic Domain: Cat# CY-E1316**
*** Protein Tyrosine Phosphatase PTPRQ: Cat# CY-E1323**
*** Protein Tyrosine Phosphatase PTP4A2: Cat# CY-E1341**
*** Recombinant Cdc25A (Catalytic domain): Cat# CY-E1352**
*** Recombinant Cdc25B (Catalytic domain): Cat# CY-E1353**
*** Recombinant Cdc25C (Catalytic domain): Cat# CY-E1354**
*** Recombinant Cdi1/KAP: Cat# CY-E1356**
*** Protein Phosphatase PP5: Cat# CY-E1359**
*** Protein Tyrosine Phosphatase PTPN3/PTPH1: Cat# CY-E1360**
*** Protein Tyrosine Phosphatase PTPN6/SHP-1: Cat# CY-E1363**
*** Protein Tyrosine Phosphatase PTPN7/HePTP: Cat# CY-E1364**

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*** Protein Tyrosine Phosphatase PTPN8/PTPN22: Cat# CY-E1365**
*** Protein Tyrosine Phosphatase PTPN9/MEG2: Cat# CY-E1366**
*** Protein Tyrosine Phosphatase PTPN11/SHP-2: Cat# CY-E1367**
*** Protein Tyrosine Phosphatase PTPN12/PTP-PEST: Cat# CY-E1368**
*** Protein Tyrosine Phosphatase PTPN13/FAP-1: Cat# CY-E1369**
*** Protein Tyrosine Phosphatase PTPN14/PEZ: Cat# CY-E1370**
*** Protein Tyrosine Phosphatase PTPN21/PTPD1: Cat# CY-E1372**
*** Protein Phosphatase DUSP1/MKP-1: Cat# CY-E1373**

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