Fluorometric Assay Kit for Measuring Cdc25A, B and C Phosphatase Activity

CycLex Protein Phosphatase Cdc25 Combo Fluorometric Assay Kit

150 Assays

Cat# CY-1355

Intended Use

The MBL Research product **Protein Phosphatase Cdc25 Combo Fluorometric Assay Kit** is a fluorometric and non-radioactive assay designed to measure the activity of Cdc25A, B and C protein phosphatase. This 96-well assay is useful for screening inhibitors and modulators of Cdc25A, B and C activities in HTS. The kit includes all necessary components, including recombinant, human Cdc25A, B and C (catalytic domain), 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 Recombinant Cdc25A, B and C!**

Introduction

Activation of cyclin-dependent kinases in higher eukaryotic cells can be achieved through dephosphorylation of two conserved residues, Thr14 and Tyr15 (1) by members of the Cdc25 phosphatase family, Cdc25A, Cdc25B and Cdc25C. The Cdc25 dual-specificity phosphatases control progression through the eukaryotic cell division cycle by activating cyclin-dependent kinases. Cdc25A plays an important role at the G1/S-phase transition. Cdc25A degradation during mitotic exit and in early G1 is mediated by the anaphase-promoting complex or cyclosome (APC/C)(Cdh1) ligase, and that a KEN-box motif in the N-terminus of the protein is required for its targeted degradation (2, 3). Cdc25B undergoes activation during S-phase and plays a role in activating the mitotic kinase Cdk1/cyclin B in the cytoplasm (4-6). Active Cdk1/cyclin B then phosphorylates and activates Cdc25C leading to a positive feedback mechanism and to entry into mitosis. In addition to their essential function in the normal cell cycle, cdc25 phosphatases are involved in the checkpoint-induced control of cell cycle progression (7-9). Finally, Cdc25A and B phosphatases have been shown to possess an important oncogenic potential and to be overexpressed in a variety of cancers and cancer cell lines (4, 10).

Principle of the Assay

The **Protein Phosphatase Cdc25 Combo 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 Cdc25A or B or C

↓ Incubate for 15 minutes at room temp.

Add 25 µL of Stop Solution

↓

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

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	**900 µL x 1**	**Below -20°C**
#2. 10X OMFP (3-o-methyl fluorescein phosphate)	**850 µL x 1**	**Below -20°C**
#3-A. Recombinant Cdc25A, Human	**250 µL x 1**	**-70°C**
#3-B. Recombinant Cdc25B, Human	**250 µL x 1**	**-70°C**
#3-C. Recombinant Cdc25C, Human	**250 µL x 1**	**-70°C**
#4. 100X Cdc25 Inhibitor (100 µM Na_3VO_4)	**100 µL x 1**	**Below -20°C**
#5. Stop Solution	**1,300 µL x 3**	**Below -20°C**
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 200-1,000 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.

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 "#3. Recombinant Cdc25" and keep all reagents on ice until use. Use them only after they are completely thawed and mixed.

1. Prepare **10X Cdc25 Inhibitor** by adding 5 µL of the **"#4. 100X Cdc25 Inhibitor"** to 45 µL of distilled (deionized) water. Mix well.

*Discard any unused **10X Cdc25 Inhibitor** after use.*

2. Prepare **Assay Mixture** by adding 5 µL of **"#1. 10X Assay Buffer"** and 5 µL of **"#2. 10X OMFP"** 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**
#1. 10X Assay Buffer	**5 µL**	**40 µL**	**80 µL**	**160 µL**	**240 µL**
#2. 10X 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**

Assay Procedure

In order to estimate the inhibitory effect on Cdc25A, B and C activities 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 Cdc25A, B and C activities, 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 Compound"** or **"10X Cdc25 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 Compound** **10X Cdc25 Inhibitor***	**5 µL** **-** **-**	**-** **5 µL** **-**	**-** **-** **5 µL**	**-** **5 µL** **-**
#3. Recombinant Cdc25 **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 Cdc25 Inhibitor (10 µM Na_3VO_4): See the section "Preparation of Reagents" above.

2. Initiate reactions by adding **5 µL** of **"#3. Recombinant Cdc25"** or distilled water to each well and mixing thoroughly at room temperature.

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

4. Add **25 µL** of **"#5. Stop 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 **"#4. Stop Solution"** for a few hours at 4°C. The microtiter plate must be sealed to prevent evaporation and kept from excessive light.

Alternate procedure

1'. Following Table.1 above, first, add **"Assay mixture"** to microtiter plate wells. Second, add **"Test Compound"** or **"Vehicle of Test Compound"** or **"10X Cdc25 Inhibitor"** to each well of the microtiter plate and mix well.

2'. Initiate reactions by adding **5 µL** of **"#3. Recombinant Cdc25"** 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 controls 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.

Evaluation of Results

Analysis of Inhibitor Effect

% 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:

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

Note: This % Intensity is a rough value of enzyme activity or inhibition. For greater accuracy, plot a standard curve of Cdc25 for each new set of reactions and estimate the % Activity (see below).

Fig.1-1 Cdc25A Inhibition Curve by SOV (Na_3VO_4; Sodium orthovanadate)

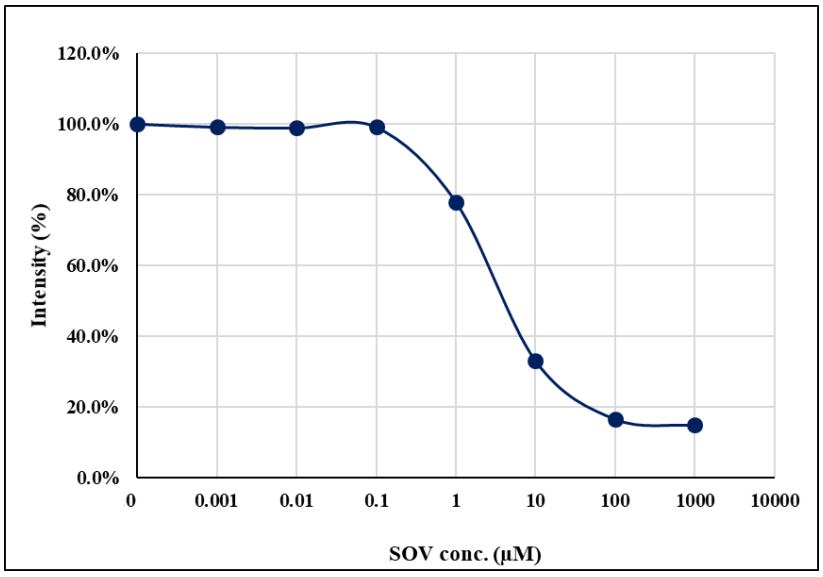


Fig.1-2 Cdc25B Inhibition Curve by SOV (Na_3VO_4; Sodium orthovanadate)

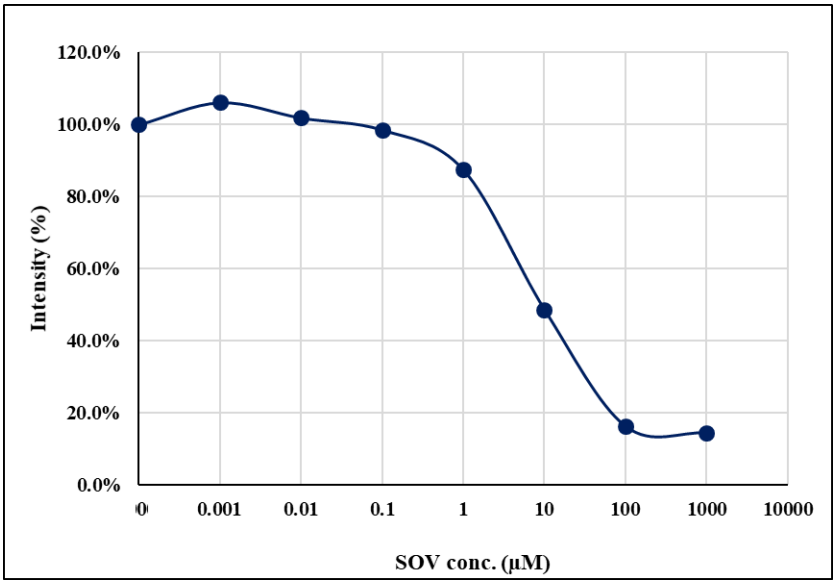
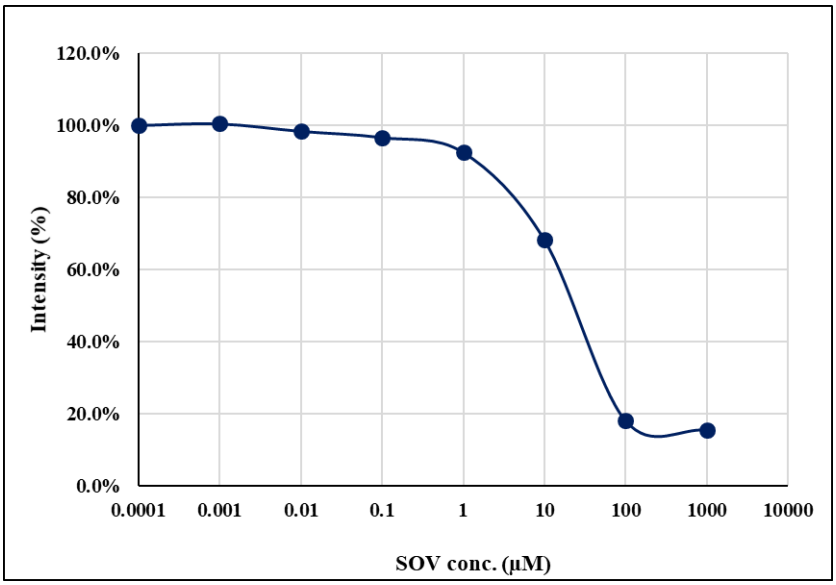


Fig.1-3 Cdc25C Inhibition Curve by SOV (Na_3VO_4; Sodium orthovanadate)



Analysis of Enzyme Activity

Cdc25 Standard Curve and % Activity

1. Dilute "#1.10X Assay Buffer" 1:10 with distilled water to make 1X Assay Buffer.
2. Make serial dilutions of "#3. Recombinant Cdc25" with 1X Assay Buffer (e.g. 100%, 50%, 25%, 12.5% 6.25%, 3.13% and 0%).
3. Run reactions with vehicle and serial dilutions of Recombinant Cdc25 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 Cdc25.
5. Obtain a line-fit to the data using appropriate calculations.
6. Use the slope and Y-intercept to calculate the amount of Cdc25 activity for the experimental data.

Fig.2-1 Dose Dependency of Recombinant Cdc25A catalytic domain

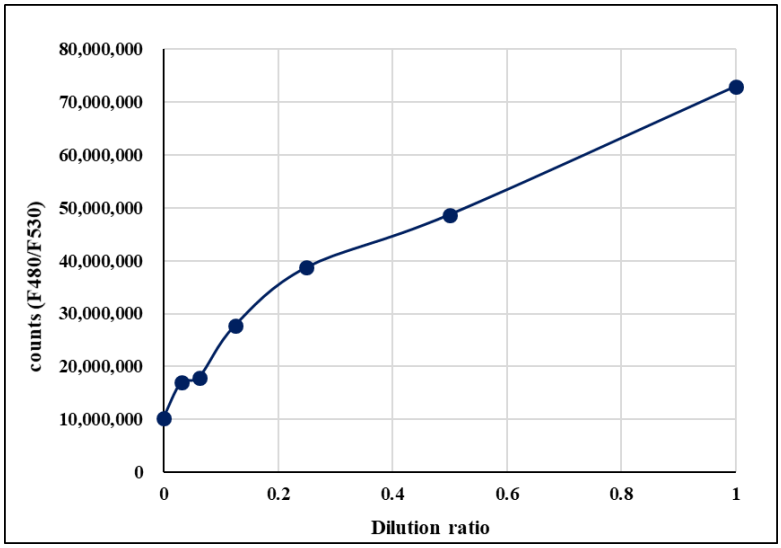


Fig.2-2 Dose Dependency of Recombinant Cdc25B catalytic domain

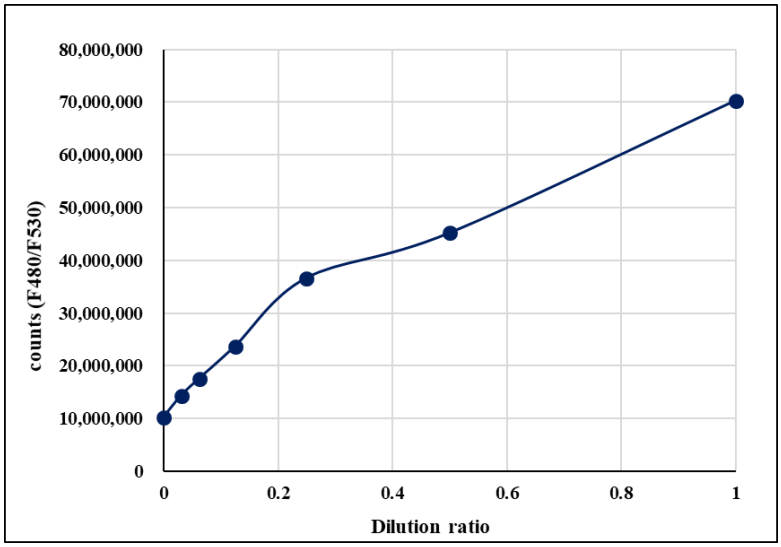
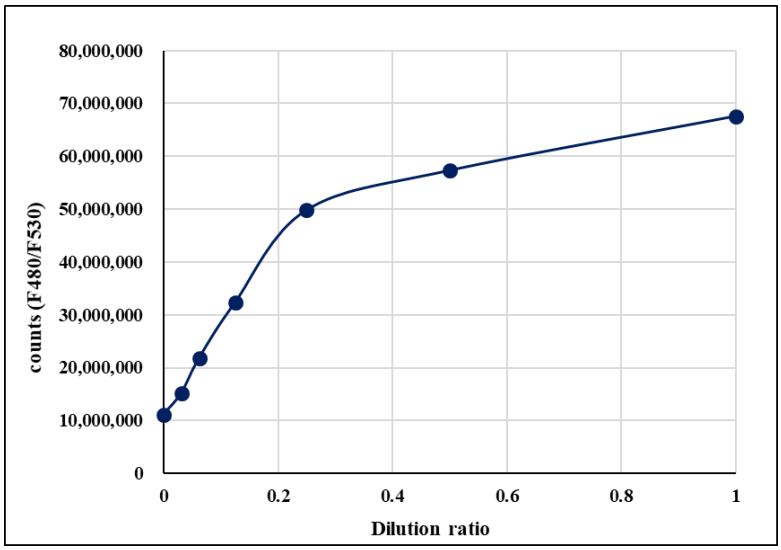


Fig.2-3 Dose Dependency of Recombinant Cdc25C catalytic domain



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:

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

Note: Usually, the linear range is from 0 to 30 minutes. This value is variable depending on reaction conditions and storage/handling of the Recombinant Cdc25. Decreasing the amount of Recombinant Cdc25 in the assay may help to lengthen the time range.

Fig.3-1 Time Course Curve of Recombinant Cdc25A

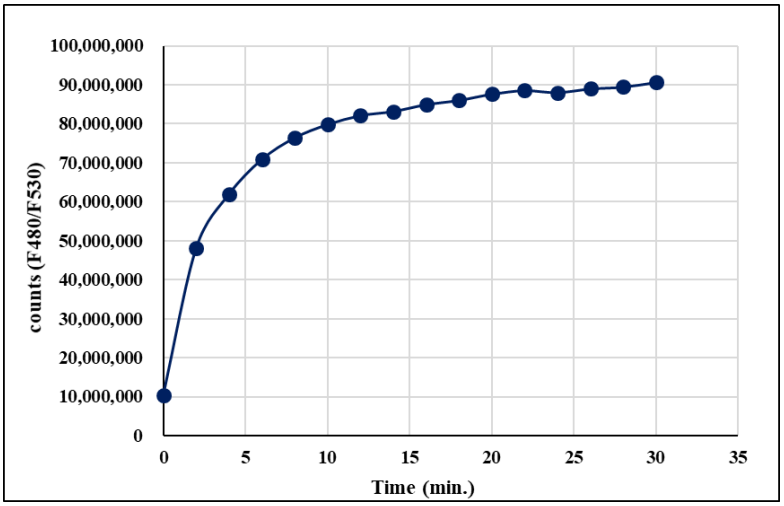


Fig.3-2 Time Course Curve of Recombinant Cdc25B

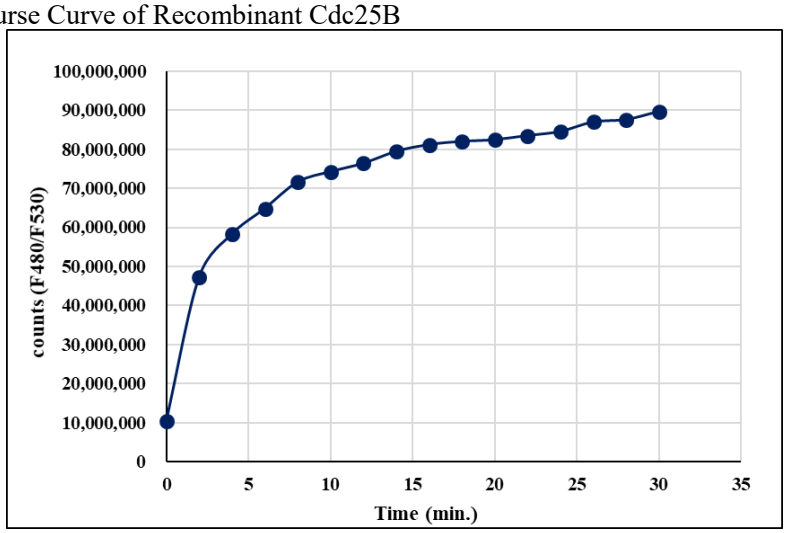
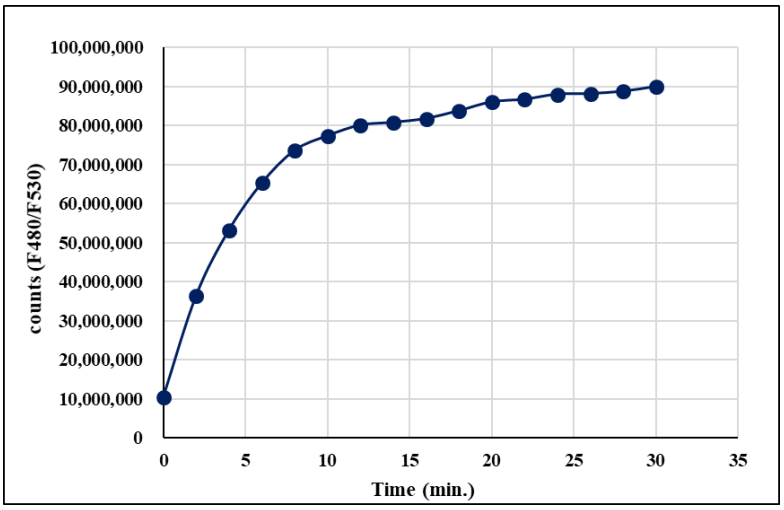


Fig.3-3 Time Course Curve of Recombinant Cdc25C



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 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 Cdc25 Combo Fluorometric Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, all the kit should be stored at -70°C. After use, return the kit to -70°C as soon as possible.

References

1. Morgan, D. O. (1997) Annu. *Rev. Cell Dev. Biol.* **13**, 261-291

2. Jinno, S., Suto, K., Nagata, A., Igarashi, M., Kanaoka, Y., Nojima, H., and Okayama, H. (1994) *EMBO J.* **13**, 1549-1556

3. Hoffmann, I., Draetta, G., and Karsenti, E. (1994) *EMBO J.* **13**, 4302-4310

4. .Nagata, A., Igarashi, M., Jinno, S., Suto, K., and Okayama, H. (1991) *New Biol.* **3**, 959-968

5. Karlsson, C., Katich, S., Hagting, A., Hoffmann, I., and Pines, J. (1999*) J. Cell Biol.* **146**, 573-583

6. Hoffmann, I., Clarke, P. R., Marcote, M. J., Karsenti, E., and Draetta, G. (1993) *EMBO J.* **12**, 53-63

7. Furnari, B., Rhind, N., and Russell, P. (1997) *Science* **277**, 1495-1497

8. Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S. J. (1997) *Science* **277**, 1497-1501
9. Peng, C.-Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S., and Piwnica-Worms, H. (1997) *Science* **277**, 1501-1505

10. Galaktionov, K., Lee, A. K., Eckstein, J., Draetta, G., Meckler, J., Loda, M., and Beach, D. (1995) *Science* **269**, 1575-1577

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