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1-866-9 KINASE (54-6273)

CDK6/CyclinD3, Active

Full-length recombinant proteins expressed in Sf9 cells

Catalog # C35-10H-10
Lot # V060-1

Product Description

Recombinant full-length human CDK6 and CyclinD3 were co-expressed by baculovirus in Sf9 insect cells using an N-terminal His tag on both proteins. The gene accession numbers for CDK6 and CyclinD3 are NM_001259 and NM_001760, respectively.

Gene Aliases

CDK6: PLSTIRE, MGC59692
Cyclin D3: CCND3

Concentration

0.1 µg/µl

Formulation

Recombinant protein stored in 50mM sodium phosphate, pH 7.0, 300mM NaCl, 150mM imidazole, 0.1mM PMSF, 0.25mM DTT, 25% glycerol.

Storage, Shipping and Stability

Store product at –70°C. For optimal storage, aliquot target into smaller quantities after centrifugation and store at recommended temperature. For most favorable performance, avoid repeated handling and multiple freeze/thaw cycles.
Stability is 1yr at –70°C from date of shipment. Product shipped on dry ice.

Scientific Background

CDK6 is a member of the cyclin-dependent family of protein kinases that are important regulators of cell cycle progression. CDK6 activity is regulated by the D-type cyclins and members of the INK4 family of CDK inhibitors (1). The CDK6 kinase activity is detected in mid-G1 phase of the cell cycle and is responsible for the phosphorylation and regulation of the activity of tumor suppressor protein Rb. Although CDK6 and CDK4 can both phosphorylate multiple residues in the Rb protein, they do so with different residue selectivities in vitro; CDK6 phosphorylates Thr821 while CDK4 phosphorylates Thr826 on Rb protein (2).

References

1. Meyerson, M. et al: Identification of G1 kinase activity for cdk6, a novel cyclin D partner. Molec. Cell. Biol. 14: 2077-2086, 1994.
2. Takaki, T. et al: Preferences for phosphorylation sites in the retinoblastoma protein of D-type cyclin-dependent kinases, Cdk4 and Cdk6, in vitro. J Biochem. 2005 Mar;137(3):381-6.

Purity

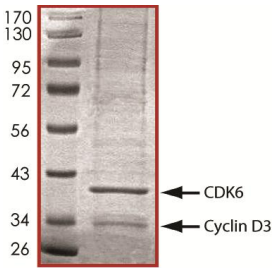
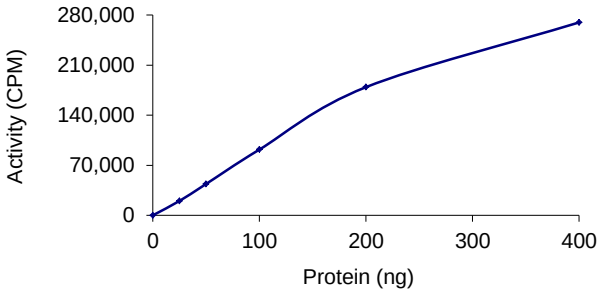


Figure 1. SDS-PAGE gel image

The purity of CDK6/CyclinD3 was determined to be **>75%** by densitometry, CDK6 approx. MW **40kDa** and CyclinD3 Approx. MW **35kDa**.

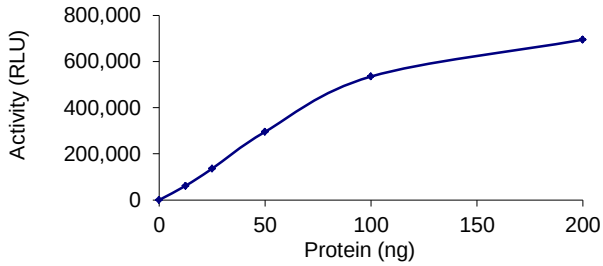
Specific Activity

Figure 2. Radiometric Assay Data



The specific activity of CDK6/CyclinD3 was determined to be **33 nmol /min/mg** as per activity assay protocol.
(For Radiometric Assay Protocol on this product please see pg. 2)

Figure 3. ADP-Glo™ Assay Data



The specific activity of CDK6/CyclinD3 was determined to be **90 nmol /min/mg** as per activity assay protocol.
(For ADP-Glo™ Assay Protocol on this product please see pg. 3)

Activity Assay Protocol

Reaction Components

Active Kinase (Catalog #: C35-10H)

Active CDK6/CyclinD3 (0.1μg/μl) diluted with Kinase Dilution Buffer III (Catalog #: K23-09) and assayed as outlined in sample activity plot. (Note: these are suggested working dilutions and it is recommended that the researcher perform a serial dilution of Active CDK6/CyclinD3 for optimal results).

Kinase Dilution Buffer III (Catalog #: K23-09)

Kinase Assay Buffer I (Catalog #: K01-09) diluted at a 1:4 ratio (5X dilution) with final 50ng/μl BSA solution.

Kinase Assay Buffer I (Catalog #: K01-09)

Buffer components: 25mM MOPS, pH 7. 2, 12.5mM β-glycerol-phosphate, 25mM $MgC1_2$, 5mM EGTA, 2mM EDTA. Add 0.25mM DTT to Kinase Assay Buffer prior to use.

[^{33}P]-ATP Assay Cocktail

Prepare 250μM [^{33}P]-ATP Assay Cocktail in a designated radioactive working area by adding the following components: 150μl of 10mM ATP Stock Solution (Catalog #: A50-09), 100μl [^{33}P]-ATP (1mCi/100μl), 5.75ml of Kinase Assay Buffer I (Catalog #: K01-09). Store 1ml aliquots at –20°C.

10mM ATP Stock Solution (Catalog #: A50-09)

Prepare ATP stock solution by dissolving 55mg of ATP in 10ml of Kinase Assay Buffer I (Catalog #: K01-09). Store 200μl aliquots at –20°C.

Substrate (Catalog #: R05-55G)

Rb (733-928) protein substrate, 0.2 mg/ml concentration.

Assay Protocol

Step 1. Thaw [^{33}P]-ATP Assay Cocktail in shielded container in a designated radioactive working area.

Step 2. Thaw the Active CDK6/CyclinD3, Kinase Assay Buffer, Substrate and Kinase Dilution Buffer on ice.

Step 3. In a pre-cooled microfuge tube, add the following reaction components bringing the initial reaction volume up to 20μl:

- **Component 1.** 10μl of diluted Active CDK6/CyclinD3 (Catalog #C35-10H)
- **Component 2.** 10μl of 0.2mg/ml stock solution of substrate (Catalog # R05-55G)

Step 4. Set up the blank control as outlined in step 3, excluding the addition of the substrate. Replace the substrate with an equal volume of distilled H_2O.

Step 5. Initiate the reaction by the addition of 5μl [^{33}P]-ATP Assay Cocktail bringing the final volume up to 25μl and incubate the mixture in a water bath at 30°C for 15 minutes.

Step 6. After the 15 minute incubation period, terminate the reaction by spotting 20μl of the reaction mixture onto individual pre-cut strips of phosphocellulose P81 paper.

Step 7. Air dry the pre-cut P81 strip and sequentially wash in a 1% phosphoric acid solution (dilute 10ml of phosphoric acid and make a 1L solution with distilled H_2O) with constant gentle stirring. It is recommended that the strips be washed a total of 3 intervals for approximately 10 minutes each.

Step 8. Count the radioactivity on the P81 paper in the presence of scintillation fluid in a scintillation counter.

Step 9. Determine the corrected cpm by removing the blank control value (see Step 4) for each sample and calculate the kinase specific activity as outlined below.

Calculation of [P^{33}]-ATP Specific Activity (SA) (cpm/pmol)

Specific activity (SA) = cpm for 5 μl [^{33}P]-ATP / pmoles of ATP (in 5 μl of a 250 μM ATP stock solution, i.e., 1250 pmoles)

Kinase Specific Activity (SA) (pmol/min/μg or nmol/min/mg)

Corrected cpm from reaction / [(SA of ^{33}P-ATP in cpm/pmol)*(Reaction time in min)*(Enzyme amount in μg or mg)]*[(Reaction Volume) / (Spot Volume)]

ADP-Glo™ Activity Assay Protocol

Reaction Components

CDK6/CyclinD3 Kinase Enzyme System (Promega, Catalog #:V4510)

CDK6/CyclinD3, Active, 10µg (0.1µg/µl)
Histone H1 Protein, 1ml (1mg/ml)
Reaction Buffer A (5X), 1.5ml
DTT (0.1M), 25µl

ADP-Glo™ Kinase Assay Kit (Promega, Catalog #: V9101)

Ultra Pure ATP, 10 mM (0.5ml)
ADP, 10 mM (0.5ml)
ADP-Glo™ Reagent (5ml)
Kinase Detection Buffer (10ml)
Kinase Detection Substrate (Lyophilized)

Reaction Buffer A (5X)

200mM Tris-HCl, pH 7. 5, 100mM $MgCl_2$ and 0.5 mg/ml BSA.

Assay Protocol

The CDK6/CyclinD3 assay is performed using the CDK6/CyclinD3 Kinase Enzyme System (Promega; Catalog #: V4510) and ADP-Glo™ Kinase Assay kit (Promega; Catalog #: V9101). The CDK6/CyclinD3 reaction utilizes ATP and generates ADP. Then the ADP- Glo™ Reagent is added to simultaneously terminate the kinase reaction and deplete the remaining ATP. Finally, the Kinase Detection Reagent is added to convert ADP to ATP and the newly synthesized ATP is converted to light using the luciferase/luciferin reaction. For more detailed protocol regarding *the ADP-Glo™ Kinase Assay,* see the technical Manual #TM313, available at www.promega.com/tbs/tm313/tm313.html.

Step 1. Thaw the ADP-Glo™ Reagents at ambient temperature. Then prepare Kinase Detection Reagent by mixing Kinase Detection Buffer with the Lyophilized Kinase Detection Substrate. Set aside.

Step 2. Thaw the components of CDK6/CyclinD3 Enzyme System, ADP and ATP on ice.

Step 3. Prepare 1ml of 2X Buffer by combining 400µl Reaction Buffer A, 1µl DTT and 599µl of dH_20.

Step 4. Prepare 1ml of 250µM ATP Assay Solution by adding 25µl ATP solution (10mM) to 500µl of 2X Buffer and 475µl of dH_20.

Step 5. Prepare diluted CDK6/CyclinD3 in 1X Buffer (diluted from 2X buffer) as outlined in sample activity plot. (Note: these are suggested working dilutions and it is recommended that the researcher perform a serial dilution of Active CDK6/CyclinD3 for optimal results).

Step 6. In a white 96-well plate (Corning Cat # 3912), add the following reaction components bringing the initial reaction volume up to 20µl:

Component 1.	10µl of diluted Active CDK6/CyclinD3
Component 2.	5µl of 1mg/ml stock solution of substrate
Component 3.	5µl of 2X Buffer

Step 7. Set up the blank control as outlined in step 6, excluding the addition of the substrate. Replace the substrate with an equal volume of distilled H_2O.

Step 8. At the same time as the CDK6/CyclinD3 kinase reaction, set up an ATP to ADP conversion curve at 50µM ATP/ADP range as described in the *ADP-Glo™ Kinase Assay* technical Manual #TM313.

Step 9. Initiate the CDK6/CyclinD3 reactions by the addition of 5µl of 250 µM ATP Assay Solution thereby bringing the final volume up to 25µl. Shake the plate and incubate the reaction mixture at 30°C for 15 minutes.

Step 10. Terminate the reaction and deplete the remaining ATP by adding 25µl of ADP-Glo™ Reagent. Shake the 96-well plate and then incubate the reaction mixture for another 40 minute at ambient temperature.

Step 11. Add 50µl of the Kinase Detection Reagent, shake the plate and then incubate the reaction mixture for another 30 minute at ambient temperature.

Step 12. Read the 96-well reaction plate using the Kinase-Glo™ Luminescence Protocol on a GloMax® Microplate Luminometer (Promega; Cat # E6501).

Step 13. Using the conversion curve, determine the amount of ADP produced (nmol) in the presence (step 6) and absence of substrate (Step 7) and calculate the kinase specific activity as outlined below. For a detailed protocol of how to determine nmols from RLUs, see Kinase Enzyme Systems Protocol at: http://www.promega.com/KESProtocol

Kinase Specific Activity (SA) (nmol/min/mg)

(ADP (step 6) – ADP (Step 7)) in nmol) / (Reaction time in min)*(Enzyme amount in mg)