

cdc2 Kinase Assay Kit

Catalog # 17-137

Lot # 14977

Kit Components

Assay Dilution Buffer (ADB), Catalog #20-108, Lot # 14327

2 vials, each containing 1.0ml of assay dilution buffer (20mM MOPS, pH 7.2, 25mM β -glycerol phosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM dithiothreitol).

Substrate Cocktail, Catalog #20-125, Lot # 14991

2 vials, each containing 1.0ml of substrate cocktail (2mg/ml Histone H1 [Catalog #14-155]) in assay dilution buffer.

Inhibitor Cocktail, Catalog #20-126, Lot # 14992

2 vials, each containing 1.0ml of inhibitor cocktail in assay dilution buffer (20 μ M PKC inhibitor peptide [Cat. #12-121], 2 μ M protein kinase A inhibitor peptide (PKI) [Catalog #12-151], and 20 μ M Compound R24571).

Magnesium/ATP Cocktail, Catalog #20-113, Lot # 14994

2 vials, each containing 1.0ml of Mg²⁺/ATP cocktail (75mM magnesium chloride and 500 μ M ATP in assay dilution buffer). 90 μ l of the Mg²⁺/ATP cocktail should be added to 100 μ Ci (10 μ l) of the [³²P]ATP (\approx 3000 Ci/mmol).

P81 Phosphocellulose Squares, Catalog #20-134, Lot #14021

1 pouch containing 200 prelabeled squares.

Other components required but not included as part of kit are:

Enzyme Preparation containing cdc2 Kinase

10-200 μ g protein/immunoprecipitate diluted into assay dilution buffer or 25-100ng of purified cdc2 Kinase.

vortex mixer

plexiglass shielding

30°C incubating water bath

timer

variable volume (5-200 μ l) pipet + tips

0.75% phosphoric acid

acetone

scintillation vials

scintillation fluid

scintillation counter

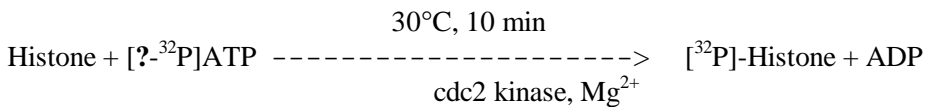
[³²P]ATP - \approx 3000 Ci/mmol, obtained from Dupont-New England Nuclear.

50ml disposable conical centrifuge tubes

Quantity: 200 kinase assays per kit.

Storage and Shelf Life: 1 year at -20°C.

Use: The assay kit is designed to measure the phosphotransferase activity of cdc2 kinase in immunoprecipitates and column fractions. Crude cell lysates may also be used but detergents/biochemicals contained in the cell lysis buffer may inhibit cdc2 kinase activity. Furthermore, although 3 inhibitors are included with the kit, editors may suggest other unknown kinases found in crude lysates are responsible for histone phosphorylation. The assay kit is based on phosphorylation of a specific substrate (histone) using the transfer of the γ -phosphate of adenosine-5'-[^{32}P] triphosphate ([γ - ^{32}P] ATP) by cdc2 kinase. The phosphorylated substrate is then separated from the residual [γ - ^{32}P] ATP using P81 phosphocellulose paper and quantitated by using a scintillation counter. The assay is linear for incubation times of up to 30 min and incorporation of up to 20% of total ATP. Further incubation or incorporation may not be linear and may therefore not be a true indication of cdc2 kinase activity in the sample extract. The enzyme assay is rapid, convenient and fairly specific for cdc2 kinase. Each kit contains sufficient reagents for 200 individual cdc2 kinase assays.



Safety Warnings and Precautions: The cdc2 kinase assay kit is designed for research use only and not recommended for internal use in humans or animals. Since the kit involves the use of radioactive [γ - ^{32}P]ATP, please follow the manufacturers instructions relating to the handling, use, storage and disposal of such materials. All chemicals should be considered to be potentially hazardous and handled with the principles of good laboratory practice.

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cdc2 KINASE ASSAY KIT PROCEDURES

All assay components must be rapidly thawed and mixed completely before proceeding with assay. Do not use extended thawing time. Once the assay components are thawed, mix thoroughly by vortexing. The assay components can be refrozen at -20°C for extended periods.

Perform all preincubation reactions at 1°C over an ice bath. The kinase assay can also be performed at room temperature but will not give linear results. Aliquot 10µl of substrate cocktail, 10µl of inhibitor cocktail, and 10µl of enzyme preparation into the bottom of a microcentrifuge tube. Start the reaction by adding 10µl of the Mg²⁺/ATP cocktail containing [³²P]ATP, vortex gently and incubate the microcentrifuge tube at 30°C for 10 min. Stop the reaction by removing 25µl of the reaction mixture and pipet slowly onto the center of a P81 phosphocellulose paper. Allow the radiolabelled substrate to bind to the filter paper for 30 seconds before immersing the paper into a beaker containing 0.75% phosphoric acid. Wash the papers thoroughly in the beaker with up to 10 rinses of 0.75% phosphoric acid. Do not wash more than 30 papers per beaker. Dispose of each rinse according to local radioisotope regulations. After washing, add acetone and wash for 2 minutes, remove the paper with forceps and stuff the paper into a 5ml scintillation vial. Add scintillation cocktail and quantitate the bound radioactivity on the paper in a scintillation counter for 1 minute. Alternatively, assay squares can be washed by adding to a 50ml conical tube containing 40ml 0.75% phosphoric acid. Gently shake the assay squares for 5 minutes on a rotator. Discard the wash in a liquid radioisotope waste container and repeat the wash step twice. Wash the squares in 20ml of acetone for 5 minutes. Drain and add scintillation cocktail.

Suitable blanks should always be performed to correct for non-specific binding of [³²P]ATP and its breakdown products to the phosphocellulose paper. Controls for endogenous phosphorylation of proteins in the sample extract can be performed by substituting assay dilution buffer for substrate cocktail.

Assay Protocol Summary:

1. Add 10µl of the substrate cocktail to a microcentrifuge tube.
2. Add 10µl of the inhibitor cocktail or ADB to a microcentrifuge tube.
3. Add 10µl of cdc2 Kinase (25-100ng purified enzyme/assay or 10-200µg protein/immunoprecipitate).
4. Add 10µl of the cold adenosine 5-triphosphate and [³²P]ATP mixture.
5. Add assay dilution buffer for a total volume of 50µl.
6. Incubate for 10 minutes at 30°.
7. Blot a 25µl aliquot on the numbered P81 paper.
8. Wash the assay squares with 0.75% phosphoric acid.
9. Wash assay squares once with acetone.
10. Transfer assay squares to a scintillation vial and add scintillation cocktail.
11. Read in scintillation counter. Compare CPM of enzyme samples to CPM of control samples that contain no enzyme (background control).

cdc2 Kinase Assay Data: cdc2 kinase activity was measured using the cdc2 kinase assay kit (Catalog # 17-137). This kit uses Histone H1 as a kinase substrate and contains a separate inhibitor cocktail which blocks the activity of other serine/threonine kinases such as protein kinase A, protein kinase C, and calmodulin dependent kinases. Some of the actual test results are shown below:

cdc2 Enzyme	Inhibitors Present	Substrate	CPM Mean	Comments
none (B)	PKA, PKC and CdK	none	4,842	Background
25ng (C)	none	none	5,762	cdc2 background phosphorylation
25ng (A)	PKA, PKC and CdK	Histone H1	55,384	cdc2 activity
25ng	none	Histone H1	62,315	total kinase activity

Determination of cdc2 Kinase Activity:

1. Determine the specific radioactivity of the Mg^{+2} /cold ATP-hot ATP mixture. Assume that the amount of hot ATP is negligible. In the above experiment, 5 μ l of the ATP solution gave 5.9×10^6 CPM, therefore 10 μ l would give 11.8×10^6 CPM = (500 μ M x 10^{-5} L x 10^3 nmoles/ μ mole = 5nmoles ATP.

$$11.8 \times 10^6 \text{ CPM} / 5 \text{ nmoles ATP}$$

$$2.4 \times 10^6 \text{ CPM/nmole ATP} = 2360 \text{ CPM/pmole ATP}$$

= Specific Radioactivity (S.R.)

The [32 P] incorporated into the substrate is quantitatively measured by its binding to the phosphocellulose paper. In the presence of sample extract, the [32 P] counted on the paper is the sum of non-specific [32 P]ATP binding, specific binding of phosphorylated substrate and binding of phosphorylated endogenous proteins in the sample extracts (A).

In the absence of enzyme, the [32 P] counted on the paper is due to non-specific binding of [32 P]ATP and its breakdown products (B).

In the absence of substrate the [32 P] counted on the papers is due to non-specific binding of [32 P]ATP and its breakdown products and binding of phosphorylated endogenous proteins in the sample extracts(C).

Therefore, the [32 P] incorporated into the substrate is obtained from (A-B) - (C-B), that simplifies to [A-C].

Since only 25 μ l of the incubation mixture was spotted onto the P81 paper out of a total volume of 50 μ l, the total [32 P] incorporated into the substrate is given by [A - C] X 2.0.

$$\frac{[A - C] \times 2.0}{\text{S.R.} \times 10 \text{ min}} = \text{pmoles phosphate incorporated into histone/min/25ng of enzyme}$$

In the above example:

$$\frac{[55,384 - 5,762] \times 2.0}{2360 \times 10 \text{ minutes}}$$

$$= 4.20 \text{ pmoles incorporated into histone/min/25ng}$$

$$= 0.17 \mu\text{moles incorporated into histone/min/mg}$$