Promega

Technical Bulletin

SignaTECT® cdc2 Protein Kinase Assay System

INSTRUCTIONS FOR USE OF PRODUCT V6430.

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PRINTED IN USA.
Revised 2/06

Part# TB227

Promega

SignaTECT® cdc2 Protein Kinase Assay System

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I. Description

Cyclin-dependent protein kinases (cdks) are a family of serine/threonine kinases related to $p34^{cdc2}$ that control the major cell cycle transitions (1,2). Members of this family of enzymes include cdc2, cdk2 to cdk7 and the RbKs. The cdks require a kinase catalytic subunit and a cyclin regulatory subunit for activity but also interact with other positive and negative protein regulators. In mammalian cells, the prototype, $p34^{cdc2}$, regulates the entry into mitosis (i.e., the transition from G2 $\rightarrow$ M phases) by phosphorylating a group of key proteins (3). The $p34^{cdc2}$ protein itself is regulated by its association with cyclin B and by its phosphorylation state on amino proximal tyrosine and threonine residues as well as an internal threonine residue (Thr_{14}, Tyr_{15} and Thr_{161} based on the human sequence; 4,5). Following mitosis, cyclin B is degraded, and $p34^{cdc2}$ is phosphorylated at the amino proximal sites and dephosphorylated internally, resulting in the inhibition of its kinase activity. This dependence on cyclin B, the phosphorylation state, and the relatively constant level of $p34^{cdc2}$ throughout the cell cycle make it difficult to assess cdc2 kinase activity in cell lysates. One approach used to assay cdk activity employs antibodies that specifically recognize either the cdk proteins or the cyclins (5,6). Antibodies capable of

I. Description (continued)

immunoprecipitating the cdk enzymes can be used to select the enzyme of interest from the cell lysate, which frequently contains a variety of other kinases and phosphatases. This approach is useful but time-consuming and frequently results in low activities because often only a fraction of the enzyme is immunoprecipitated and cannot be quantitated easily. In addition, antibody binding to the enzyme may sterically hinder the catalytic activity of the enzyme.

The SignaTECT® cdc2 Protein Kinase Assay System[a] uses a biotinylated peptide substrate derived from histone H1 to measure cdc2 kinase activity directly in cell lysates. This substrate (PKTPKKAKKL) is highly selective for cdc2 and possibly cdk2 and cdk3, providing the specificity necessary to perform cdc2 kinase assays on a variety of biological samples including crude cell lysates (7). The radiolabeled, phosphorylated substrate is recovered from the reaction mix with the SAM2® Biotin Capture Membrane (SAM2® Membrane), which is a novel streptavidin matrix (8). The SAM2® Membrane is prenumbered and partially cut so that individual squares can be easily identified, separated and placed in scintillation vials. Alternatively, the intact SAM2® Membrane can be analyzed using a phosphoimaging system or by conventional autoradiography. Olomoucine, a specific inhibitor of a subset of cdk enzymes including cdc2, cdk2 and cdk5 (9), is also provided in the system and can be used to confirm the specificity of the reaction. With this assay, the analysis of cdc2 kinase is simple, rapid and amenable to high throughput. The assay detects olomoucine-sensitive cdc2 kinase activities at levels approaching pmol/min/µg of mitotic cell lysate protein.

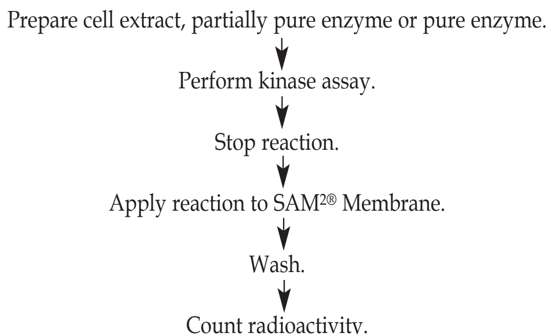


Figure 1. Overview of the procedure for measurement of cdc2 kinase activity from partially purified enzyme preparations and cell extracts with the SignaTECT® cdc2 Protein Kinase Assay System.

II. Product Components and Storage Conditions

Product	Size	Cat.#
SignaTECT® cdc2 Protein Kinase Assay System	96 reactions	V6430

Each system contains sufficient reagents to perform 96 kinase reactions. Includes:

- 1 SAM2® Biotin Capture Membrane
- 600µl ATP, 0.5mM
- 200µl BSA, 10mg/ml
- 1.4ml Termination Buffer (2 × 700µl)
- 300µl cdc2 Protein Kinase Biotinylated Peptide Substrate, 0.25mM
- 750µl cdc2 Protein Kinase Assay 5X Buffer
- 0.5mg Olomoucine cdc2 Protein Kinase Inhibitor
- 1 Protocol

Storage Conditions: Store all components of the SignaTECT® cdc2 Protein Kinase Assay System at –20°C, where they are stable for at least six months from the date of purchase. Avoid multiple freeze-thaw cycles. For storage for less than 1 month, the SAM2® Biotin Capture Membrane may be stored at 4°C (return the unused portion of the SAM2® Membrane to the resealable plastic bag). The Termination Buffer may be stored at room temperature (20–25°C).

III. Quantitation of cdc2 Protein Kinase Activity

III.A. Preparation of Cell Lysates

Materials to Be Supplied by the User
(Solution compositions are provided in Section IV.)

- cell culture medium
- nocodazole (microtubule assembly inhibitor; Sigma Cat.# M1404)
- phosphate buffered saline (PBS)
- extraction buffer
- homogenizer or sonicator (for lysis of cultured cells)
- protein analysis assay

Cyclin-dependent kinases are activated at different stages of the cell cycle. To obtain maximal activity and avoid degradative enzymes, cells in the appropriate stage must be used. This can be accomplished by a number of means, including synchronization, elutriation and stage-specific blockers of the cell cycle.

The following procedure provides one approach used successfully at Promega for preparing active cdc2 kinase from HeLa cells (6). Section IV provides the formulation of buffers that have been used successfully at Promega. The extract preparations should be stored at –70°C for maximal stability.

III.A. Preparation of Cell Lysates (continued)

Mitotic Cell Extract Preparation

1. Plate 1.5–2.0 × 10^6 cells per T-150 cm^2 flask in 20ml of culture medium containing 10% fetal bovine serum and allow the cells to adhere overnight.
2. Add nocodazole to a final concentration of 1µg/ml 24 hours after plating the cells.
3. Harvest the mitotic (synchronized) cells 7–18 hours after adding the nocodazole by carefully removing the medium and any cell debris. Rinse with 20ml of culture medium per flask to remove loosely adherent mitotic cells (do not scrape off tightly adherent nonmitotic cells).
4. Centrifuge the cells at 200 × *g* for 10 minutes at 4°C.
5. Rinse the cell pellet with 5ml of PBS and centrifuge at 200 × *g* for 10 minutes at 4°C.
6. Resuspend the cells in 3–5 pellet volumes of extraction buffer.
7. Lyse the cells with a homogenizer or by brief sonication (2 × 10-second pulses).
8. Clarify the lysate by centrifugation at 100,000 × *g* for 1 hour at 4°C.
9. Determine total protein concentration of the lysate.

III.B. cdc2 Protein Kinase Assay System Protocol

Materials to Be Supplied by the User
(Solution compositions are provided in Section IV.)

- dimethyl sulfoxide (DMSO)
- [γ-^{32}P]ATP (3,000Ci/mmol, 10µCi/µl)
- 2M NaCl
- 2M NaCl in 1% H_3PO_4
- washing container (e.g., Nalgene® plastic utility box, 7.5 × 6 × 4 inches)
- 30°C heating block or water bath
- scintillation counter or phosphoimaging system
- deionized water
- **optional:** orbital platform shaker
- **optional:** heat lamp

Olomoucine Preparation: Add 168µl of dimethyl sulfoxide (DMSO) to the tube containing 0.5mg of Olomoucine to prepare a 10mM solution. This solution can be stored at –20°C as a master stock solution. Further dilutions can be made in water.

1. Thaw the Termination Buffer at 20–30°C, then vortex well. If Olomoucine will be used, thaw at room temperature (DMSO will freeze at 0°C). Thaw the rest of the frozen components on ice.

III.B. cdc2 Protein Kinase Assay System Protocol (continued)

2. Wearing gloves, cut (using scissors or a razor blade) the required number of squares from the SAM2® Biotin Capture Membrane. The squares may remain connected as a partial sheet to minimize handling. Return the unused SAM2® Membrane to the resealable plastic bag at 4°C or –20°C. The template provided in Section VII can be used to identify samples using the SAM2® Membrane square numbers.

3. Prepare the ATP mix as follows:

Component	Final per Reaction	10 Reactions
0.5mM ATP	2.5µl	25µl
[γ-^{32}P]ATP (3,000Ci/mmol, 10µCi/µl)	0.1µl	1.0µl

4. Prepare the following reaction in 0.5ml or 1.5ml microcentrifuge tubes:

Component	Final per Reaction	10 Reactions
cdc2 Protein Kinase Assay 5X Buffer	5.0µl	50µl
ATP mix (Step 3)	2.6µl	26µl
cdc2 Protein Kinase Biotinylated Peptide Substrate*	2.5µl	25µl
water/inhibitors	9.9µl	99µl

*Final peptide substrate concentration is 25µM, which is tenfold above the K_m (see data in Section III.E). Other concentrations can be used, and the peptide also can be added separately to the reaction. One set of control reactions should exclude substrate to determine background due to endogenous phosphorylation of proteins within the extract sample that may bind to the SAM2® Membrane.

Note: Final ATP concentration is 50µM. Higher ATP concentrations can be used but will lead to lowered assay sensitivity.

5. Mix gently and preincubate the reaction mix (Step 4) at 30°C for 1–5 minutes.

6. Initiate the reaction by adding 5µl of the enzyme sample/cell lysate (Section III.A, Step 9) to the reactants in Section III.B, Step 4. Total reaction volume will be 25µl.

   Note: Other volumes of sample can be used by adjusting the volume of water. If more than 10µg of cell extract protein per reaction is used, the specific activity of the cdc2 kinase may be reduced because of inhibitors present in the extract. To obtain maximal activity, a dilution series of extract should be performed (see Figure 2, Panel A). An appropriate dilution buffer (recipe provided in Section IV) can be made using the cdc2 Assay 5X Buffer and the supplied BSA Solution.

7. Incubate the reaction at 30°C for 10 minutes (other time points and temperatures may be tested if desired).

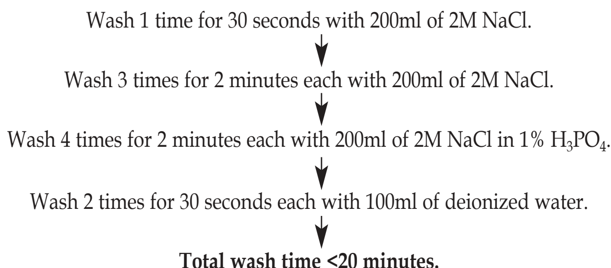
III.B. cdc2 Protein Kinase Assay System Protocol (continued)

8. Terminate the reaction by adding 12.5µl of Termination Buffer to each reaction; mix well.

9. Spot 15µl of each terminated reaction onto a prenumbered SAM2® Membrane square. After all samples have been spotted follow the wash and rinse steps as described below. Save the reaction tubes for Step 12.

   Note: Larger volumes can be spotted; however, if more than 15µl will be spotted, separate the squares first to prevent cross-contamination. **Do not** exceed 30µl per square. (Minor seepage of liquid onto adjacent squares should not cause contamination, because the biotinylated peptide is rapidly immobilized to the SAM2® Membrane before liquid migration is complete.)

10. Place the SAM2® Membrane squares containing samples from Step 9 into a washing container. Wash using an orbital platform shaker set on low or by manual shaking, as follows:



Total wash time <20 minutes.

Notes:

- Dispose of the radioactive wash solution in accordance with the regulations of your institution.
- More or less washing may be appropriate to achieve acceptably low background counts; this should be determined empirically.
- For rapid drying, a final, 15-second 95% ethanol wash (100ml) can be used. Longer washes with ethanol may cause the ink to run slightly.

11. Dry the SAM2® Membrane squares on a piece of aluminum foil under a heat lamp for 5–10 minutes or air-dry at room temperature 30–60 minutes. (If the SAM2® Membrane has been washed with ethanol, shorten the drying time to 2–5 minutes under a heat lamp or 10–15 minutes at room temperature.)

III.B. cdc2 Protein Kinase Assay System Protocol (continued)

12. Determine total counts for calculation of the specific activity of [γ-^{32}P]ATP as follows: remove 5µl aliquots from any 2 reaction tubes from Step 8 and spot onto individual SAM2® Membrane squares. For this step, dry SAM2® Membrane squares **without** washing. After analysis use these results to calculate the specific activity of [γ-^{32}P]ATP in Section III.C.

    Note: If 5µl is not available from a single tube you may combine the contents of several tubes for this step.

13. **Analysis by Scintillation Counting:** If still connected, separate the SAM2® Membrane squares (from Steps 11 and 12) using forceps, scissors or a razor blade and place the squares or 3mm filter discs into individual scintillation vials. Add scintillation fluid to the vials and count.

    Analysis by Phosphoimaging: Alternatively, the SAM2® Membrane may remain intact and the intact SAM2® Membrane may be analyzed using a phosphoimaging system.

III.C. Calculation of the Specific Activity of [γ-^{32}P]ATP

$$\text{The specific activity of } [\gamma\text{-}^{32}\text{P}]\text{ATP in cpm / pmol of ATP} = \frac{(37.5/5)(X)}{1{,}250}$$

where:

- 37.5 is the sum of the reaction volume (25µl) + the Termination Buffer volume (12.5µl).
- 5 is the volume in microliters of sample used from Section III.B, Step 12.
- X is the average counts/minute of the 5µl samples from Section III.B, Step 12.
- 1,250 is the number of picomoles of ATP in the reaction.

III.D. Determination of cdc2 Protein Kinase Activity

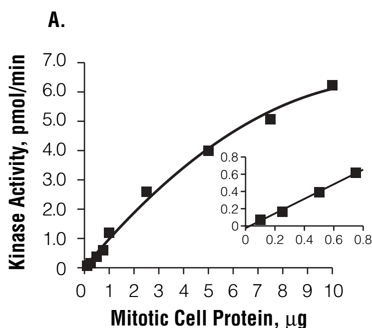
Enzyme activity in pmol ATP/min/µg of protein =

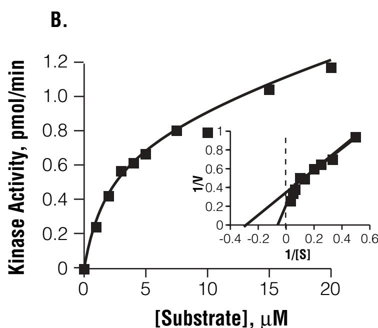
$$\frac{(\text{cpm}_{\text{reaction with substrate}} - \text{cpm}_{\text{reaction without substrate}}) \times (37.5)}{(15) \times (\text{time}_{\text{min}}) \times (\mu\text{g of protein in reaction}) \times (\text{specific activity of } [\gamma\text{-}^{32}\text{P}]\text{ATP})}$$

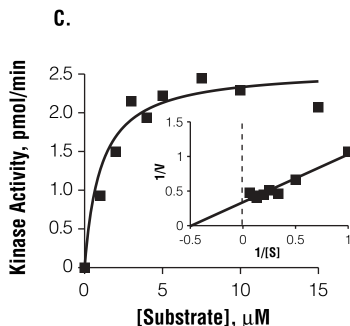
where:

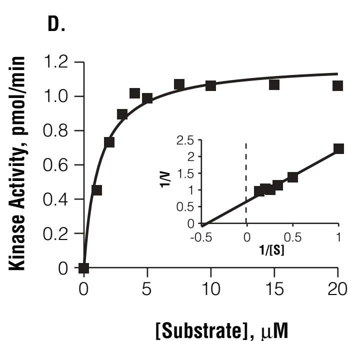
- 37.5 is the sum of the reaction volume (25µl) + the Termination Buffer volume (12.5µl).
- 15 is the volume in microliters of the sample from Section III.B, Step 9.

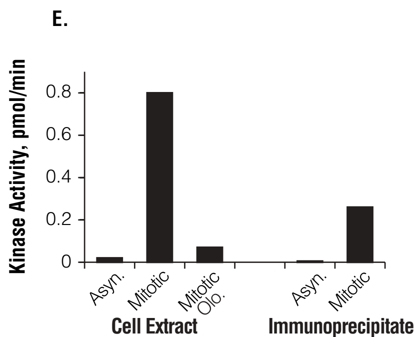
III.E Expected Results











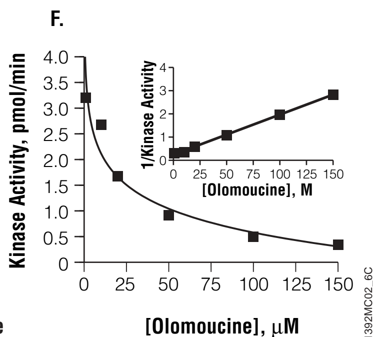


Figure 2. Analysis of cdk activities using the SignaTECT® cdc2 Protein Kinase Assay System. Panel A. Sensitivity determination. The indicated amounts of cell lysate prepared from HeLa cells, blocked in mitosis (as described in Section III.A), were assayed for 15 minutes using 50μM ATP and 1μCi of [γ-^{32}P]ATP/reaction. Results were obtained with less than 1μg of extract protein. **Kinetic analysis of extracts.** Activated starfish oocyte extract (**Panel B**) and mitotic HeLa cell extract (**Panel C**) were incubated for 10 minutes with increasing concentrations of the peptide substrate. Double reciprocal plots are shown. An apparent K_m value of 2.0μM was observed in HeLa extracts, while a biphasic curve with apparent K_m values of 3.3μM and 24μM was observed in starfish oocyte extracts. **Panel D. Kinetic analysis of purified cdc2 Kinase.** cdc2 Kinase purified from starfish was incubated with increasing concentrations of the peptide substrate. A double reciprocal plot with a K_m value of 2.3μM is shown. **Panel E. Specificity.** Extracts prepared from actively growing HeLa cells (Asyn.) and HeLa cells blocked in mitosis (Mitotic) were analyzed for cdc2 Kinase activity. Activity inhibited by 100μM Olomoucine (Mitotic Olo.), a selective inhibitor of cdc2 Kinase, was only detected in mitotic cell extracts. To confirm the specificity, 10μg of extract protein was immunoprecipitated with an anti-p34^{cdc2} kinase C-terminus polyclonal antibody and protein A agarose. Approximately one-third of the cdc2 Kinase activity was recovered in the immunoprecipitates. **Panel F. Inhibition.** Activated starfish oocyte extract was incubated with 50μM ATP and the indicated concentrations of Olomoucine. The single reciprocal plot indicates simple inhibition kinetics in the range reported for cdc2 Kinase.

Note: Results presented here were obtained using a different assay buffer than that supplied with this system. The composition of the buffer used to obtain these data is 25mM MOPS, 10mM $MgCl_2$, 2mM EDTA, 1mM DTT, 40mM β-glycerophosphate, 20mM *p*-nitrophenylphosphate, 0.1mM sodium vanadate. The buffer supplied with this system provides improved reaction conditions for assaying cdc2 Kinase activity, as measured in both extracts and purified enzyme, and thus optimized measurement of cdc2 Kinase activity.

IV. Composition of Buffers and Solutions

extraction buffer

50mM	Tris-HCl (pH 7.4)
250mM	NaCl
1mM	EDTA
50mM	NaF
1mM	DTT
0.1%	Triton® X-100
10µM	leupeptin
100µg/ml	aprotinin

Store at –20°C. Just before use, add 0.5ml of PMSF stock solution (100mM PMSF in absolute ethanol) per 100ml of extraction buffer.

phosphate buffered saline (PBS)

0.2g/L	KCl
8.0g/L	NaCl
0.2g/L	KH_2PO_4
1.15g/L	Na_2HPO_4

Termination Buffer

7.5M	guanidine hydrochloride

dilution buffer

50mM	Tris-HCl (pH 7.5)
10mM	$MgCl_2$
1mM	EGTA
2mM	DTT
0.1mg/ml	BSA
40mM	β-glycerophosphate
20mM	*p*-nitrophenylphosphate
0.1mM	sodium vanadate

cdc2 Protein Kinase Assay 5X Buffer

250mM	Tris-HCl (pH 7.5)
50mM	$MgCl_2$
5mM	EGTA
10mM	DTT
200mM	β-glycerophosphate
100mM	*p*-nitrophenylphosphate
0.5mM	sodium vanadate
0.05%	Brij 35

V. Related Products

Product	Size	Cat.#
cdc2 Kinase, Human, Recombinant	200u	V4781
PepTag® Non-Radioactive PKC Assay	120 reactions	V5330
PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay	120 reactions	V5340
Tyrosine Phosphatase Assay System	96 reactions	V2471
Serine/Threonine Phosphatase Assay System	96 reactions	V2460
SignaTECT® Protein Tyrosine Kinase Assay System	96 reactions	V6480
SignaTECT® Protein Kinase C (PKC) Assay System	96 reactions	V7470
SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System	96 reactions	V7480
SignaTECT® DNA-Dependent Protein Kinase Assay System	96 reactions	V7870
SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System	96 reactions	V8161

V. Related Products (continued)

Product	Size	Cat.#
ProFluor® Src-Family Kinase Assay	4 plate	V1270
	8 plate	V1271
ProFluor® PKA Assay	4 plate	V1240
	8 plate	V1241
ProFluor® Ser/Thr PPase Assay	4 plate	V1260
	8 plate	V1261
ProFluor® Tyrosine Phosphatase Assay	4 plate	V1280
	8 plate	V1281
Kinase-Glo® Luminescent Kinase Assay	10ml	V6711
	10 × 10ml	V6712
	100ml	V6713
	10 × 100ml	V6714
Kinase-Glo® Plus Luminescent Kinase Assay	10ml	V3771
	10 × 10 ml	V3772
	100ml	V3773
	10× 100ml	V3774

VI. References

1. Lewin, B. (1990) Driving the cell cycle: M phase kinase, its partners, and substrates. *Cell* **61**, 743–52.
2. King, R.W., Jackson, P.K. and Kirschner, M.W. (1994) Mitosis in transition. *Cell* **79**, 563–71.
3. Nurse, P. (1990) Universal control mechanism regulating onset of M-phase. *Nature* **344**, 503–8.
4. Solomon, M.J. *et al.* (1990) Cyclin activation of p34cdc2. *Cell* **63**, 1013–24.
5. Draetta, G. *et al.* (1988) Human cdc2 protein kinase is a major cell-cycle regulated tyrosine kinase substrate. *Nature* **336**, 738–44.
6. Pagano, M. *et al.* (1993) Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts. *J. Cell. Biol.* **121**, 101–11.
7. Beaudette, K.N., Lew, J. and Wang, J.H. (1993) Substrate specificity characterization of a cdc2-like protein kinase purified from bovine brain. *J. Biol. Chem.* **268**, 20825–30.
8. Goueli, B.S. *et al.* (1995) A novel and simple method to assay the activity of individual protein kinases in a crude tissue extract. *Anal. Biochem.* **225**, 10–7.
9. Vesely, J. *et al.* (1994) Inhibition of cyclin-dependent kinases by purine analogues. *Eur. J. Biochem.* **224**, 771–86.

VII. SAM2® Biotin Capture Membrane Template

Promega					SAM2® Biotin Capture Membrane						
1	9	17	25	33	41	49	57	65	73	81	89
2	10	18	26	34	42	50	58	66	74	82	90
3	11	19	27	35	43	51	59	67	75	83	91
4	12	20	28	36	44	52	60	68	76	84	92
5	13	21	29	37	45	53	61	69	77	85	93
6	14	22	30	38	46	54	62	70	78	86	94
7	15	23	31	39	47	55	63	71	79	87	95
8	16	24	32	40	48	56	64	72	80	88	96

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[a]U.S. Pat. Nos. 6,066,462, 6,348,310 and 6,753,157 and European Pat. No. 0 760 678 B9 have been issued to Promega Corporation for quantitation of protein kinase activity. Other patents are pending.

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