In vitro assay for cyclin-dependent kinase activity in yeast
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Introduction

The activity of cyclin-dependent kinases (cdks) requires their association with positive regulatory subunits called cyclins (Ref. 1). In budding yeast, cyclin−cdk complexes have been shown to be involved in several cellular processes including cell cycle progression, phosphate metabolism, glycogen metabolism and transcription (Ref. 1, 2). Kinase assays have been developed for detecting the activity of cyclin−cdk complexes from yeast in vitro (Ref. 3). These assays can be used to demonstrate phosphorylation of endogenous or known exogenous substrates (Ref. 3, 4, 5, 6, 7). Here we describe a detailed protocol for in vitro kinase assays using immunoprecipitates from yeast lysates. The assay requires immunoprecipitation of the cyclin−cdk complex from yeast lysates using an appropriate antibody and incubation of the complexes with γ-[32P]ATP. The labelled substrates (endogenous or exogenously added) are then detected by SDS polyacrylamide electrophoresis and autoradiography. We describe a protocol that was developed by Mike Tyers for assaying Cln−Cdc28 kinase complexes in yeast extracts and has been used to assay other yeast cyclin−cdk complexes. However the immunoprecipitation−kinase assay should be readily adaptable for detecting the activity of cdks or other kinases from any whole cell extract (e.g., mammalian cell extracts).

Cell growth and collection
Yeast cells are grown in yeast extract−peptone−dextrose (YPD; Ref. 8). If maintenance of a plasmid is required, the cells can be grown in minimal medium containing the appropriate supplements (protein yields are typically lower than obtained for cells grown in YPD). For a single kinase reaction, a 100 ml yeast culture is grown to an OD600 of 0.6−0.8 (or cells may be counted and used at a density of approximately 2 × 10^7 cells/ml). The cells are collected by centrifugation (3000 rev/min for 3 min at 4°C). The cell pellet is then resuspended in 2 ml of ice-cold water and 1 ml transferred to each of two conical-bottom, screw-cap microfuge tubes (Sarstedt; 72-693-005). The cells are collected by brief centrifugation in a microfuge (3000 rev/min for 3 min at 4°C), the water is removed by aspiration and the cell pellet can then be used directly or frozen on dry ice or in liquid nitrogen and stored at −70°C for later use. It is important that the cells are kept cold at all times and that these steps are done quickly and efficiently.

Cell lysis
To minimize proteolytic degradation of proteins, all subsequent steps must be done in the cold room and as quickly as possible. To lyse the cells, one volume of lysis buffer containing protease inhibitors (equal to the volume of the cell pellet − typically 200 μl) and one volume of acid-washed glass beads (Biospec; 11079-105) are added to each tube. It works well if the frozen cell pellets are kept on dry ice, the beads are added, the tubes transferred to wet ice and then lysis buffer containing protease inhibitors is added quickly. The glass beads are prepared by soaking them in nitric acid for 1 h, rinsing them in water, followed by 1 M Tris−HCl pH 8.0 and then water again until the pH is neutral.

Lysis buffer
1 mM DTT
0.1% NP-40 (Boehringer Mannheim; 1332473)
250 mM NaCl
50 mM NaF
5 mM EDTA
50 mM Tris−HCl pH 7.5
**Lysis buffer with protease inhibitors**

As above, but also with:
- 1 mM PMSF (Sigma; P-7626)
- 1 μg/ml peptatin (Sigma; P-4265)
- 1 μg/ml leupeptin (Sigma; L-2884)
- 10 μg/ml soybean trypsin inhibitor (Sigma; T-6522)
- 10 μg/ml N-Tosyl-L-phenylalanine chloromethyl ketone (Sigma; T-4376)
- 0.6 mM dimethylaminopurine (Sigma; D-2629)

Cells are lysed by mechanical disruption using a vortex set at maximum speed for 1 min and subsequently placed on ice for 1 min. This is typically repeated 4–6 times, although different strains will require more or less cycles for optimal disruption. We use an adaptor for the vortex that will hold several tubes [vortexer at attachment (Scientecare Belart Products; H37080-0020)]. Cell lysis is monitored by examining the cells using a phase-contrast microscope. For efficient lysis, approximately 80–90% of the cells should appear black (indicating lysis) and there should be little cell debris in the field of view. The lystate is then spun in a microfuge at 13,000 rev/min for 10 min at 4°C. The supernatant is transferred to a cold Eppendorf tube and kept on ice for 1 h.

**Protein A-Sepharose CL-4B** (Sigma; P-3391) should be pre-swelled in water for 2 h (100 mg Protein A-Sepharose per ml). Let the Protein A-Sepharose settle, remove the water and add the same volume of fresh lysis buffer (1:1 slurry). Mix the Protein A-Sepharose slurry well and add 15 μl to each immunoprecipitation. Gently mix on a Nutator at 4°C for 45 min.

The immunoprecipitated complexes are then washed by adding 1 ml of cold lysis buffer without protease inhibitors directly to each reaction. The beads are collected by gently spinning the tubes in a microfuge (2000 rev/min for 2 min at 4°C). Most of the supernatant is then removed using a cold Pasteur pipet; the pellet of beads and a small amount of residual fluid should be left in the tube. This is repeated three more times. The beads are then washed twice with 1 ml of 1× kinase buffer.

**2× Kinase buffer**
- 100 mM Tris–HCl pH 7.5
- 20 mM MgCl₂
- 2 mM DTT
- 2 μM ATP

Remove the last wash using a Pasteur pipet and leave behind a small amount of buffer. We aspirate off the residual buffer using a 27-1/2 gauge needle attached to a 1 ml syringe. The end of the needle should be bent to an angle of 45°. The top end of the syringe is cut off and the syringe attached to rubber tubing which is connected to an aspirator. The aspirator is turned on, the needle tip placed directly in the beads and all residual buffer removed. The kinase reaction mix described below should be added immediately to prevent the beads from drying out.

**Kinase reaction**

**Kinase reaction mix**
- 2.5 μl 2× kinase buffer
- 1.0 μl γ-[³²P]ATP (10 μCi) (fresh)
- 1.0–1.5 μl of exogenous substrate (if one is used)
- water to 5 μl

Commonly used exogenous substrates include 1 μg histone H1 (generally Cdc28-specific), 100 ng Pho4 (generally Pho85-specific), 1 μg myelin basic protein (MBP) and 1 μg β-casein (Sigma; C-8157).

Add 5 μl of the kinase reaction mix to each tube, gently mix the beads and place the reaction at 30°C for 20 min.
FIGURE 1. Pcl2 immunoprecipitation—kinase assay. Anti-Pcl2 polyclonal antibodies were used to immunoprecipitate Pcl2—Pho85 and test for phosphorylation of several exogenous substrates (lanes 5, 6, 7 and 8). Of these, only Pho4 (lane 8) is phosphorylated strongly. Phosphorylation of endogenous substrates (lanes 2, 5, 6, 7 and 8) was also observed. This experiment includes a wild-type strain (lanes 1, 2, 5, 6, 7 and 8) and strains deleted for PCL2 (lane 3) and PHO85 (lane 4) as controls, along with a pre-immune control (lane 1). (Reproduced with permission from Ref. 4).

To stop the reaction, add 10 µl of 2x SDS sample buffer (100 mM Tris–HCl pH 6.8, 20% glycerol, 4% SDS, 0.1% bromophenol blue, 2% β-mercaptoethanol). The sample can be frozen at this point or analysed immediately. The samples should be boiled for 2 min, spun briefly in a microfuge to pellet the beads, and 7 µl of supernatant separated by SDS polyacrylamide gel electrophoresis. The gel is then fixed for 15 min in 5% methanol, 7% acetic acid and dried at 60°C for 30 min (these times should be increased for gels thicker than 1 mm). An autoradiograph of the dried gel is then obtained.

An example of a Pcl2—Pho85 kinase assay is shown (Fig. 1).

References
Products Used

Acid-washed glass beads: Acid-washed glass beads from Sigma

glass beads: glass beads from Sigma

NP-40: NP-40 from Boehringer Mannheim

PMSF: PMSF from Sigma

pepstatin: pepstatin from Sigma

dimethylaminopurine: dimethylaminopurine from Sigma

TAC: TAC from Research Genetics

protein assay: protein assay from Bio-Rad

Protein A-Sepharose CL-4B: Protein A-Sepharose CL-4B from Sigma