Chapter 3

Expression and Purification of Recombinant CDKs: CDK7, CDK8, and CDK9

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Abstract

Cyclin-dependent kinases have established roles in the regulation of cell cycle, in gene expression and in cell differentiation. Many of these kinases have been considered as drug targets and numerous efforts have been made to develop specific and potent inhibitors against them. The first step in all of these attempts and in many other biochemical analyses is the production of highly purified and reliable kinase, most frequently in a recombinant form. In this chapter we describe our experience in the cloning, expression, and purification of CDKs using CDK7/CycH, CDK8/CycC, and CDK9/CycT1 as an example.

Key words Cyclin-dependent kinases, CDK expression, CDK purification

1 Introduction

Cyclin-dependent kinases (CDKs) are Ser/Thr protein kinases, which are critically dependent on their association with a cyclin partner [1–3]. CDKs have a firmly established role as key regulators of the division of cells. Their cyclin partners are expressed and degraded in tightly regulated temporal fashion thus furnishing the orchestrated oscillating kinase activities required for the proper progression though the cell cycle. Other members of the CDK family play roles in gene transcription and in cell differentiation [3]. Some of the cyclins that couple with these kinases do not display oscillating abundance, but still remain critical for the activity of the kinase. Most cyclins associate with one or two kinases [3]; however, some of the budding yeast CDKs can associate with as many as nine cyclins [2].

In addition to association with cyclins, CDKs are regulated by a variety of mechanisms including phosphorylation, association with inhibitory proteins and assembly factors, and protein degradation [1]. In particular, the activity of many CDKs is dependent on their phosphorylation by CAK (CDK Activating Kinase). CAK itself is a CDK, CDK7/CycH [4]. The phosphorylation by CAK and other posttranslational modifications provide yet another layer

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of complexity in the regulation of CDKs and pose challenges to the expression of active recombinant kinases that can be used in vitro.

Many studies on CDKs have been carried out with natural or epitope-tagged kinases that have been immunoprecipitated from cell extracts. This approach benefits from the natural environment for posttranslational modifications of the enzymes. Normally, the presence of contaminating kinase in such preps is addressed by testing for kinase activity in a blank control. Additional reassurance is provided through the use of specific kinase inhibitors or the use of multiple substrates. However, many CDKs exist in vivo in more than one complex (*see* below) and some of these complexes display substantial variations in their substrate preferences [5–9]. The uncertainty on the homogeneity of these kinase preparations often obfuscates the correct interpretation of the data. Clearly, these small-scale procedures have provided valuable insights, but are incompatible with large-scale analyses including the search and characterization of inhibitors and drug candidates.

Here we describe strategies for large-scale expression and purification of recombinant CDKs with a focus on CDK7/CycH, CDK8/CycC, and CDK9/CycT1 (*see* Fig. 1 for outline). These kinases do not directly regulate the progression through the cell cycle. CDK7 exits in several forms including a core tripartite CDK7/CycH/MAT1 complex known as CAK (CDK activating kinase) and as a component in the general pol II transcription factor TFIIH [10, 11]. CDK8/CycC exists as a bipartite complex, but has also been found in a variety of complexes that contain RNApol II and general RNApol II transcription factors [12]. CDK9 has been initially identified as P-TEFb (Positive Transcription Elongation Factor-b) [13, 14]. Independently, CDK9/CycT1 has been isolated as the HIV-tat associated kinase TAK [15, 16]. All these kinases have multiple targets, but share the carboxy-terminal domain (CTD) of RNA pol II as a common substrate [10].

As mentioned earlier, most CDKs are regulated by posttranslational modifications. The enzymes that exert these modifications are not available in prokaryotic cells. Consequently, the expression and/or assembly of CDKs in *E. coli* frequently generates inactive kinases. Practice has shown that insect cells, albeit more expensive and cumbersome, provide the proper environment for the expression of active CDKs. We recommend insect Sf9 cells and baculovirus vectors for the expression of these enzymes.

2 Materials

2.1 Growth and Maintenance of Sf9 Cells

1. 1× Grace's Medium; 100× yeastolate solution; 100× lactalbumin hydrolysate solution; 100× antibiotic/antimycotic solution; fetal bovine serum (*see* **Note 1**).

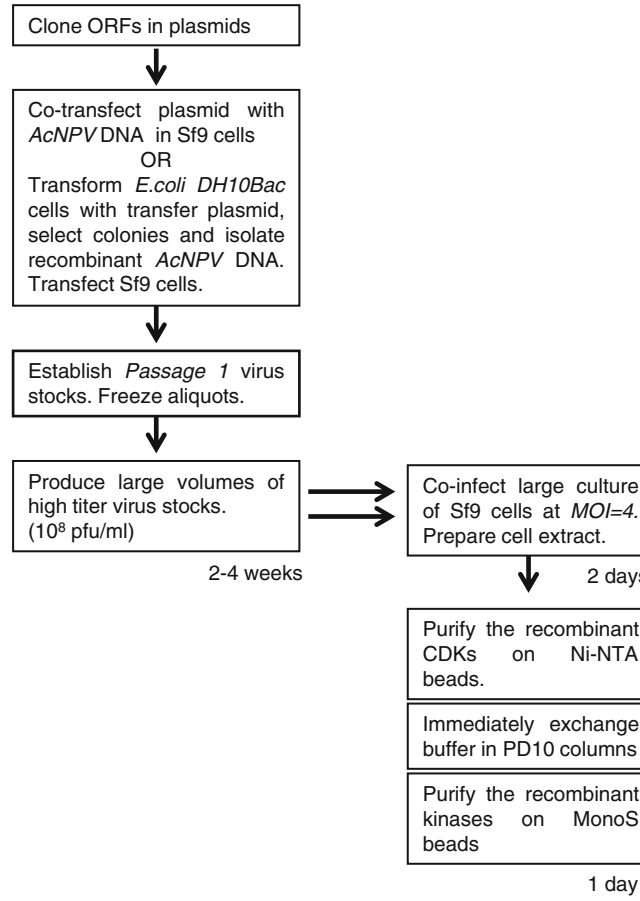

Fig. 1 An outline of the expression and purification of CDK complexes. Cloning of the proteins of interest in transfer plasmids and the production of recombinant AcNPV usually takes 2–4 weeks. The subsequent expression of the kinases takes 2 days. The purification of the kinases over Ni-NTA and MonoS resins should be performed in a single day

2. Tissue culture hood.
3. 28 °C incubator.
4. Tissue culture flasks (25, 175 cm^2).

2.2 Expression and Purification of Recombinant Kinases

1. Cell lysis buffer: 10 mM Tris–HCl pH 7.5, 10 mM NaCl, 2 mM β-mercaptoethanol, 0.5 mM EDTA, 10 mM 2-glycerophosphate, 0.5 mM Na-vanadate Na3VO4, 2 mM NaF. Add 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin (*see* **Note 2**), 0.2 % (v/v) NP-40. Add 50 μg/ml PMSF immediately before use.
2. 5 M NaCl.
3. 80 % glycerol.

4. 0.5 M imidazole–HCl pH 7.6.
5. Dounce homogenizer, 40 ml.
6. SW50.1 rotor and a compatible ultracentrifuge.

2.3 Purification of CDK Complexes by Ni^{2+}-NTA

1. Ni^{2+}-NTA Agarose.
2. Equilibration buffer: 10 mM Tris–HCl pH 7.6, 0.5 M NaCl, 5 mM imidazole, 10 % (v/v) glycerol. Add 50 μg/ml PMSF immediately before use.
3. Ni-NTA Elution buffer: 10 mM Tris–HCl pH 7.6, 100 mM NaCl, 10 % (v/v) glycerol. Supplement with 5, 15, 25, 100, and 400 mM imidazole–HCl as indicated. Add 50 μg/ml PMSF immediately before use.
4. Disposable 10 ml columns.

2.4 Purification of CDK Complexes by Mono S Chromatography

1. PD10 buffer exchange columns.
2. 5 ml Econo-Pac Mono S cartridge.
3. S-buffer: 25 mM Hepes.Na pH 7.6, 0.1 mM EDTA, 1 mM DTT, 5 % (v/v) glycerol, supplemented with 80 or 500 mM NaCl. Add 50 μg/ml PMSF immediately before use.

2.5 Expression and Purification of GST-CTD Substrate

1. Competent *E. coli BL21*(*DE3*)*lysS* cells.
2. LB (Luria Broth)/amp plates and LB/amp liquid medium.
3. Bacterial shaker/incubator set at 37 °C.
4. IPTG powder.
5. TEN buffer: 20 mM Tris–HCl pH 7.5, 5 mM EDTA, 200 mM NaCl. Add 50 μg/ml PMSF before use.
6. Protease inhibitors (pepstatin, leupeptin, aprotinin) at 1 mg/ml (*see* **Note 2**).
7. Glutathione-agarose beads.
8. 1 M glutathione reduced.
9. 10 % Triton X-100 (TX100).
10. Disposable 10 ml columns.
11. One 10 % SDS-PAGE gel.
12. A mid-speed centrifuge (*Sorvall Evolution RC* or similar), rotors (*Sorvall* SA300, SLA1500 or similar), 250 ml centrifuge bottles, 35 ml centrifuge tubes.
13. A sonicator, Misonix XL or similar.

2.6 Kinase Assays

1. Kinase buffer: 20 mM Tris–HCl, pH 8, 50 mM KCl, 7 mM $MgCl_2$, 5 mM 2-glycerophosphate, 100 μg/ml BSA, 10 μM ATP, 2 μCi (7.4×10^4 Bq) α-^{32}P-ATP.
2. 1 mg/ml GST-CTD.
3. 1 mg/ml myelin basic protein (MBP).

2.7 Other Equipment

1. Basic radiation safety equipment and Geiger counter.
2. Mini-PROTEAN II electrophoresis cell (Bio-Rad) or comparable mini-gel system.
3. Microcentrifuge, standard molecular biology equipment.

3 Methods

3.1 Production of and Handling of Recombinant Viruses

The viruses for expression of CDK7, MAT1 [17] and CycC [18] were gifts from Dr. D. Morgan and Dr. E. Lees, respectively. The virus for the expression of His_6-CDK9 and CycT1 was produced by pBAC4/CDK9/CycT1 plasmid [19] and the BacVector-3000 kit (Novagen) according to the instructions of the manufacturer (*see* **Note 3**). The virus for the expression of His_6-CycH was produced by cloning CycH into pBlueBac and the Bac-N-Blue transfection system (Invitrogen Life Technologies) (*see* **Note 3**). The recombinant virus was purified through three rounds of selection of blue plaques. His_6-CDK8 was produced by cloning CDK8 into pFastBacHTa and the BAC-to-BAC recombination system (Invitrogen Life Technologies) according to the instructions of the manufacturer (*see* **Note 3**). All three systems are reliable and produce viruses with equal success. Space limitations preclude extensive details on these procedures. However, we must note that in our hands Bac-to-Bac (Invitrogen Life Technologies) has been most user-friendly and least time-consuming.

3.2 Amplification of Recombinant Viruses

The initial preparations of recombinant baculovirus (termed Passage 1 virus stocks) typically contain low titer of the virus. For the viruses produced by the Bac-N-Blue system Passage 1 corresponds to the cell culture infected with a virus plaque that has been purified by three rounds of plaque selection. Passage 1 for the viruses produced by Bac-to-Bac or BacVector systems is the transfected Sf9 cultures themselves. High titer viral stocks are prepared from Passage 1 by two-step amplification as described below.

1. Infect 10 ml Sf9 cells at $0.1–0.3 \times 10^6$ cells/ml in a 25 cm^2 T-flask with 0.1 ml of virus Passage 1. The expected Multiplicity of Infection (MOI) is in the range of 0.05–1 (*see* **Note 4**).
2. Incubate for 4–6 days at 28 °C. At this point all cells should be in suspension (*see* **Note 5**).
3. Spin the culture at $275 \times g$ for 5 min in sterile 15 ml conical tubes and collect the supernatant. These supernatants contain $1 \times 10^6–1 \times 10^7$ plaque forming units per ml (pfu/ml).
4. Freeze 1 ml aliquots as Passage 2 and store at −80 °C (*see* **Note 6**).
5. Infect 75 ml Sf9 cells at $0.5–1 \times 10^6$ cells/ml in a 175 cm^2 flask (Sarsted) with 1 ml of the passage 2 stock. Incubate for 4 days

and collect the supernatant. These supernatants typically contain 1×10^8 pfu/ml.

6. Use the supernatants from **step 5** for large-scale protein expression. The viral stocks can be stored at 4 °C for several weeks.

3.3 Infection and Harvesting of Insect Cells

High levels of expression are achieved by the infection of high density Sf9 cells at high MOI. The gain of yields exceeds the concerns of superfluous virus recombination.

1. Grow Sf9 cells to $1.5–2 \times 10^6$ cells/ml in several (5–8) 175 cm^2 T-flasks or in a roller-flask.
2. Infect each flask with 2 ml of passage 4 of each of the appropriate viruses (MOI = 4) and incubate for 48 h at 28 °C (*see* **Note 7**). All cells must be floating (100 % infection) 30 h post infection. If less than 100 % infection is observed, do NOT proceed. Significantly lower yields of recombinant protein should be expected.
3. Harvest the cells after 48 h by spinning at $275 \times g$ for 5 min at 4 °C.
4. Pour off the supernatant, loosen the pellet by gently shaking it, and wash the pellets with 15 ml of chilled phosphate buffered saline.

3.4 Preparation of Cell Lysate

Cell lysis and chromatography must be performed on ice with prechilled buffers. Samples should be constantly kept on ice or in a 4 °C cold rooms.

1. Resuspend the cells in 3 pellet volumes of Cell lysis buffer. At this point the cells can be immediately processed or frozen at –80 °C (*see* **Note 8**).
2. Transfer the cell lysate to a Dounce homogenizer and break cells by ten strokes. Check for cell lysis under a microscope.
3. Add imidazole and NaCl to a final concentration of 5 mM and 0.5 M, respectively. Homogenize by two additional strokes.
4. Transfer the cell lysate to a 50 ml screw cap tube. Rock for 30 min in a cold room.
5. Spin at $75{,}000 \times g$ at 4 °C in SW50.1 rotor for 30 min.
6. Transfer the supernatant to a fresh prechilled tube; add glycerol and $MgCl_2$ to final concentrations of 10 % and 3 mM, respectively (*see* **Note 9**).

3.5 Ni^{2+}-NTA Chromatography

The Ni^{2+}-NTA and Mono S chromatography steps should be performed without any freezing and thawing of the samples. Buffer exchange should be in desalting columns rather than by dialysis (*see* **Note 10**). Several 10 % SDS–polyacrylamide gels should be

prepared prior to the chromatography for quick analysis of the fractions.

1. Equilibrate 1 ml Ni^{2+}-NTA agarose beads for 30 min with 10 ml of Equilibration buffer in a 50 ml screw cap tube. Swirl occasionally.
2. Spin the beads at 2000 rpm for 2 min and aspire the supernatant.
3. Add the cleared cell extract from 1 l of infected cells. Rock the tube for 1 h in a cold room.
4. Pellet the beads at 2000 rpm for 2 min, collect the supernatant (flow-through) and store at −80 °C. If there is any problem with the binding of proteins to the beads, the supernatant can be reused.
5. Add 10 ml of Ni-NTA Elution buffer supplemented with 5 mM imidazole. Swirl gently and transfer to a 15 ml disposable Bio-Rad column.
6. Wash the column with five aliquots of 2 ml Ni-NTA Elution buffer supplemented with 5 mM imidazole.
7. Elute the proteins as 5 × 1 ml fractions of each of the following buffers:
   - Ni-NTA Elution buffer, 15 mM imidazole.
   - Ni-NTA Elution buffer, 25 mM imidazole.
   - Ni-NTA Elution buffer, 100 mM imidazole.
   - Ni-NTA Elution buffer, 400 mM imidazole.
8. Run 10 µl of each fraction on 10 % SDS-PAGE and stain with Coomassie Brilliant Blue (*see* **Notes 11** and **12** and Fig. 2).
9. Pool the fractions that contain the proteins of interest and proceed to MonoS chromatography.

3.6 Mono S Chromatography

1. Equilibrate several disposable PD10 column with S-buffer/80 mM NaCl.
2. Attach a 5 ml Econo-Pac Mono S cartridges to a BioLogic DuoFlow system (*see* **Note 13**). Set the flow rate at 1 ml/min. Equilibrate as follows:
   - 10 ml of S-buffer with no NaCl.
   - 10 ml of 1 M NaCl in buffer A.
   - 5 ml of gradient 1 M–80 mM NaCl in buffer A.
   - 15 ml of 80 mM NaCl in buffer A.
3. In the meanwhile, exchange the buffer of the pooled Ni^{2+}-NTA chromatography fractions that contain the recombinant kinases. Process no more than 3 ml per column.

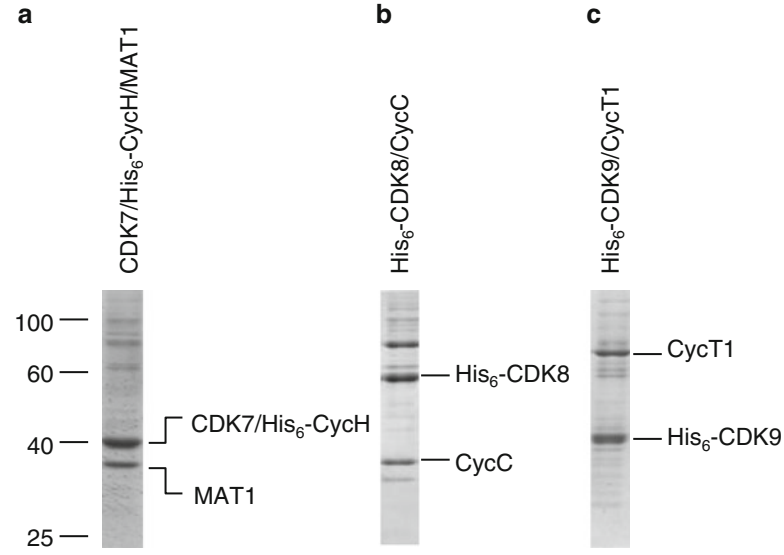

Fig. 2 Analysis of the recombinant peptides by SDS-PAGE. Coomassie stained gels from the peak fractions from the Ni^{2+}-NTA agarose chromatography are shown. (**a**) CDK7/His_6-CycH/MAT1; (**b**) His_6-CDK8/CycC; (**c**) His_6-CDK9/CycT1. The mobility of the molecular size standards is shown on the *left*. The position of the recombinant proteins is shown to the *right* of each lane. Their identity has been confirmed by Western blot (not shown)

4. Immediately load the buffer-exchanged fractions on the MonoS column at 1 ml/min. Collect the flow-through and save.
5. Wash the column with 5 ml of S-buffer/80 mM NaCl.
6. Elute with a 20 ml linear gradient of 80–500 mM NaCl in S-buffer A. Collect 1 ml fractions in 1.5 ml tubes that contain 100 µl of 80 % glycerol.
7. Vortex all fractions. Remove two 10 µl aliquots from each fraction for analysis by SDS-PAA electrophoresis and for kinase assays. Immediately freeze all fractions and aliquots at −80 °C. You can run the gels and stain/destain on the next day (*see* **Note 14**).

3.7 Expression and Purification of GST-CTD (1–52)

1. Transform pGEX-$CTD_{(1–52)}$ [20] in *E. coli BL21(DE3)lysS* cells and select colonies on LB/amp plates.
2. Inoculate 3 ml LB/amp with a single colony. Grow overnight at 37 °C.
3. Transfer the overnight culture to 400 ml LB/amp in a 2 l flask. Grow with vigorous shaking at 37 °C to $OD_{600} = 0.6$. bear in mind that the doubling time of this culture is about 20 min.

4. Transfer the culture to a 30 °C shaker. Add IPTG powder to 1 mM final concentration. Incubate with vigorous shaking for 3 h.
5. Transfer cells in two 250 ml centrifuge bottles and pellet cells at 5000 rpm in a SLA1500 rotor. Pour off supernatant. At this point you can proceed or freeze pellets at –80°C and process them later.
6. All subsequent steps are performed on ice with prechilled buffers. Resuspend the cells in 25 ml TEN buffer supplemented with protease inhibitors and fresh PMSF. Vortex to get an even suspension and transfer to a 35 ml centrifuge tube.
7. Sonicate with five bursts of 10 s at 30 % output with Misonix medium tip (*see* **Note 14**).
8. Rock for 30 min in a cold room. Spin the extract for 10 min at 15,000 rpm in a SA300 rotor.
9. In the meanwhile, mix 0.5 ml of Glutathione-Agarose beads with 25 ml of TEN buffer in a 50 ml screw cap tube. Rock for 5 min and pellet for 3 min at 1000 rpm in a bench top centrifuge. Pour off the buffer.
10. Using a 10 ml pipette, carefully remove the supernatant from **step 8** and add to the equilibrated Glutathione-Agarose beads. Add Triton X100 to 0.2 % and rock for 30 min in a cold room. Spin 3 min at 1000 rpm and remove the supernatant.
11. Wash beads three times with 25 ml of TEN buffer/0.2 % TX100. Transfer to a disposable 10 ml column.
12. Wash two times with 3 ml TEN buffer plus 0.2 % TX100.
13. Elute with ten aliquots of 1 ml Kinase buffer (*see* Subheading 2.6) supplemented with 10 mM Glutathione. Detect the peak of proteins by a standard Bradford assay.
14. Pool the fractions. Use PD10 columns to immediately exchange into Kinase buffer (*see* Subheading 3.8). Prepare 250 and 50 µl aliquots and freeze at –80 °C.
15. Run 10 µl on 10 % SDS-PAGE gel to assess the quality of the purified protein.

3.8 Kinase Assays

1. Use one of the 10 µl aliquots from Subheading 3.6 in SDS-PAGE gels to identify the fractions containing recombinant kinase (*see* **Note 15**).
2. After selecting the peak of kinase fractions, thaw the other 10 µl aliquots and use 1 and 5 µl in kinase assays.
3. The kinase assays are performed in 20 µl containing 20 mM Tris–HCl, pH 8, 50 mM KCl, 7 mM $MgCl_2$, 5 mM 2-glycerophosphate, 100 µg/ml BSA, 10 µM ATP, 2 µCi α-3^2P-ATP (ICN), 40 µg/ml GST-CTD or MBP for 30 min at 30 °C.

4. Terminate reactions by the addition of 5 μl 5× SDS-PAGE loading buffer and boil for 5 min. Load 12 μl on SDS-PAGE gels and run until the blue line of Bromo-Phenol-Blue dye leaves the gel. Use a Geiger counter to confirm that the unincorporated radioactive α-^{32}P-ATP has left the gel.
5. Discard the tank buffer according to the safety regulations of your institution.
6. Put the gel in 100 ml of 10 % methanol/10 % acetic acid and rock for 20 min. Repeat until no trace of radioactivity is detected in the wash buffer.
7. Dry the gel and expose to X-ray film or a Phosphorimager screen (if available).
8. If desired, the activity of the enzymes can be determined as incorporation of pmols ATP/min/mg of GST-CTD(1–52) or MBP (*see* **Note 16**).

3.9 Important Technical Considerations

1. *Expected yields of recombinant kinases. Infection with individual viruses versus co-infection.* Using the procedure outlined in Fig. 1, we purify between 115 μg and 1 mg of recombinant kinase from 1 l of co-infected Sf9 cells. The lowest yields are achieved with CDK7/His$_6$-CycH/MAT1. Nevertheless, the levels of co-expressed proteins are still slightly higher as compared to the levels produced by infection with individual CDK7, His$_6$-CycH, and MAT1 viruses. We suspect that co-expression and the formation of complexes in vivo stabilizes the individual peptides and so increases the yields. We must note that individual expression of cyclins and catalytic subunits and their subsequent assembly in vitro has been successfully used in the past [5, 18, 21–25]. We recommend co-expression as the more economical and efficient approach.
2. *Activity of the kinases.* The activities of the purified kinases have been assessed using GST-CTD as substrate [6]. In a standard kinase reaction, the CDK7/His$_6$-CycH/MAT1 preparations transferred about 1.5 nmol ATP/min/mg protein; the His$_6$-CDK8/CycC preparations transferred 0.1 nmol ATP/min/mg protein; His$_6$-CDK9/CycT1 transferred about 4 nmol ATP/min/mg protein. The kinases displayed distinct patterns of CTD phosphorylation that were reported elsewhere [6]. All kinases also efficiently phosphorylate MBP. In summary, the proposed expression and purification method produces highly active recombinant kinases with distinct substrate specificities.
3. *Background kinase activities.* The most haunting issue with in vitro kinase assays is the loose specificity of the enzymes and the adequacy of the observed phosphorylation events. Many CDKs phosphorylate a wide range of substrates in vitro,

including non-physiological "generic" substrates such as MBP, Histone H1, the carboxy-terminal domain of RNA pol II. Even worse, these "generic" substrates are also targeted by many of the contaminating kinases in the CDK fraction or in the substrate preparation itself. Hence, two important tests must be performed to determine background kinase activities for each substrate that will be used. The first one is a kinase assay(s) with parallel "blank" Ni-NTA and MonoS chromatography fractions from uninfected Sf9 cell cultures. The second important test is to perform kinase reactions with the substrate preparations only. If any of these tests show kinase activity towards the substrate of interest, additional purification of both the substrate and the kinase is needed.

4. *Autocatalytic activities.* Autocatalytic activities have been reported for many kinases including CDKs. These vary in magnitude relative to the substrates. In our hands, very low autocatalytic activity has been observed for CDK7/His_6-CycH/MAT1 (*see* Fig. 3). There is a clear auto-phosphorylation of His_6-CDK9 by His_6-CDK9/CycT1 (*see* Fig. 3). This could be a genuine auto-phosphorylation event or an artifact

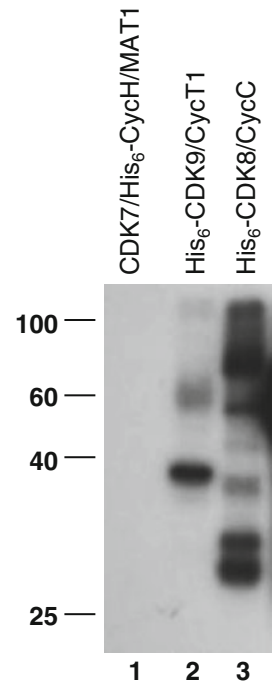

Fig. 3 Autocatalytic activity of the recombinant kinases. Peak fractions from the MonoS chromatography of the kinases shown on the top have been used in kinase reactions with no substrate. The reactions were run on SDS-PAGE gels and exposed for 2 days on X-ray films. The mobility of the molecular size standards is shown on the *left*

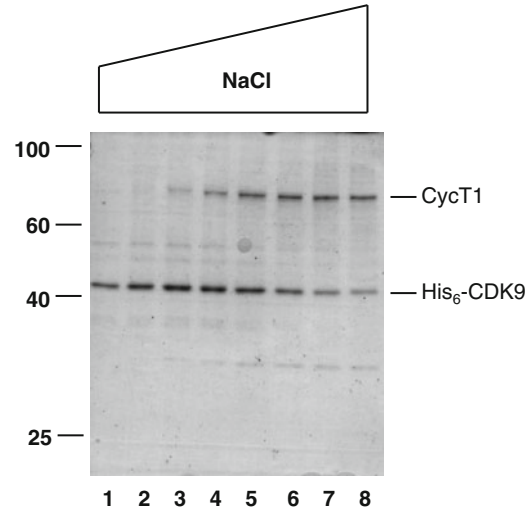

Fig. 4 Purification of His_6-CDK9/CycT1 on MonoS column. Pooled His_6-CDK9/CycT1 fractions from the Ni^{2+}-NTA chromatography were loaded on a 5 ml Mono S cartridge and eluted by linear 80–500 NaCl gradient. The 300–500 mM NaCl range is shown. Note the earlier elution of His_6-CDK9 monomers

because of the excess His_6-CDK9 in the prep (*see* **Note 15** and *see* Fig. 4). His_6-CDK8/CycC shows a broader pattern of auto-phosphorylation signals that include both His_6-CDK8 and CycC, but also other contaminating peptides (*see* Fig. 3).

The auto-phosphorylation signals in His_6-CDK8/CycC and His_6-CDK9/CycT1 preps have been detected upon long exposures of the gels. However, in cases of moderate phosphorylation of a substrate these auto-phosphorylation signals can impede the analysis of the data. In this situation a substrate molecule with an affinity tag can be useful. *See* **step 6** (GST-fusion proteins).

5. *Choice of substrate. Use of synthetic peptides.* If a contaminating kinase is detected in the substrate preparation, one can use short synthetic peptides (20 amino acids or so) that represent the phosphorylation site of interest. While most of the times these serve the purpose of measuring the activity of the CDK, they should be used with caution as they are not a true representative of the whole substrate.
6. *GST-fusion proteins.* We have found that, whenever possible, the best option for substrate is the natural target of the CDK, which is expressed as a GST fusion protein in *E. coli* (*BL21D*) cells. A variety of pGEX vectors can be used. Many GST-fusion proteins are soluble; they are produced at significant levels and can be easily purified by a one-step affinity chromatography on Glutathione-Agarose geads. Importantly, in our hands such preps are essentially free of contaminating kinase.

A disadvantage of this system is that sometimes the proteins are partially degraded. In this situation a gel filtration column that can enrich the preparation in full-length peptides should be considered.

GST-fusion proteins provide an important advantage in cases where the kinases display autocatalytic activities. In these situations the substrate can be pulled out of the kinase reaction by Glutathione-Agarose beads before analysis by SDS-PAGE [6]. Even if weak, only the signals from the phosphorylation of the substrate will be detected.

4 Notes

1. Comprehensive information on growth and maintenance of Sf9 cells plus extensive discussion on baculovirus expression vectors can be found in *Baculovirus and Insect Cell Expression Protocols* [26] and in *Baculovirus Expression Protocols* [27].
2. Commercial protease inhibitor cocktails can be used instead of the listed inhibitors.
3. We have used Bac-N-Blue (Invitrogen Life Technologies), Bac-to-Bac (Invitrogen Life Technologies), and BacVector-3000 system (Novagen) with equal success. We do not provide details on these procedures as point-by-point protocols are available on the manufacturers' websites. However, we mention some notes of consideration.

   In the Bac-N-Blue system a pBlueBac plasmid (it carries a cassette of the cloned gene of interest plus *LacZ*) is co-transfected with linearized *AcNPV* baculovirus DNA in Sf9 cells. The assembly of recombinant virus takes place in Sf9 cells to produce blue plaques while reconstituted wild type baculovirus produces translucent plaques. Normally, three rounds of purification of blue plaques are needed. This is a cumbersome, costly and time-consuming process that requires certain skills from the operator. Once established, the purified stock of the recombinant virus can be propagated and frozen stocks can last for years. The stock can be retested for the formation of only blue plaques to affirm its purity.

   The BacVector system (Novagen) uses a similar approach, but very efficiently suppresses the production of non-recombinant virus and confers more than 95 % of recombinant virus. Hence, no plaque purification is normally needed. The disadvantage of this convenient shortcut is that in a long run the contaminating non-recombinant viruses can outgrow the virus of interest. We recommend that the original preparation of recombinant virus is propagated to passage 2 and this stock is used for the expression of the protein. If more protein is

required, a fresh transfection with the plasmid and the pricey BacVector DNA should be performed.

The Bac-to-Bac system produces the recombinant baculoviral DNA by transposition in *E. coli* cells (*DH10Bac*) carrying the baculovirus genome as a bacmid (Bmon14272). The recombinant bacmid DNA is isolated from *E. coli* and is readily transfected into Sf9 cells. The produced viral stock is propagated to passage 2 and used for infection and expression of proteins. Again, there is no need for purification or selection of plaques. Importantly, more virus can be easily obtained by re-transfection with the bacmid. In our hands this is the most reliable and easy to handle procedure.

4. It is important to use low MOI during the amplification of the virus. This means that you need to have more cells than viral particles. Higher MOI generates the risk of multiple viruses infecting the same cell and recombining with each other.
5. Sf9 cells grow as monolayer, but lose adherence and change morphology upon infection. More details can be found in [26].
6. These small aliquots are handy if the expression of the proteins needs to be repeated. Their titer can decrease because of the freezing; however, they still can be used to directly proceed to the preparation of high titer viral stock.
7. In our hands MOI = 4 generates the highest level of expression for all the CDKs. Further increase in MOI does not yield more protein.
8. The cells should be quickly frozen using several small aliquots rather than one large aliquot. When thawing, gently swirl the tubes in ice bath until homogeneous suspension is produced. Do not leave on ice for prolonged periods of time.
9. You can freeze the extract or proceed directly to chromatography. If planning to load on N^{2+}-NTA agarose beads on the same day, do not add glycerol.
10. Freezing and thawing of proteins leads to denaturing and the formation of precipitates. Similarly, the dialysis of the Ni-NTA purified proteins causes extensive precipitation and significant loss of recombinant kinases. All these precipitates must be removed by high speed centrifugation. If not, such "cloudy" samples can clog the columns and compromise the purification. These complications and the loss of material can be avoided if the two chromatographies are performed in one long day.
11. The recombinant kinases typically elute in the 25 and 100 mM imidazole fractions. His_6-CycH and CDK7 co-migrate as a single band of 40 kDa while MAT1 is a single band of 36 kDa (*see* Fig. 2a). His_6-CDK8, CycC, and His_6-CDK9 and CycT1 are approximately 53 kDa, 36 kDa, 43 kDa, and 87 kDa, respectively (*see* Fig. 2b, c).

12. Stain with Coomassie Brilliant Blue for about 15 min on a rocking platform. Quick destaining of the gels can be achieved by adding a couple of Kimwipes (or other sturdy paper wipes) to the destaining container. We certainly prefer this fast and simple method of detection. Western blot or kinase assays could be more informative, but are significantly slower and can be very tricky because of the risk of using significant excess of kinase or antigen.
13. Any low-pressure gradient-making chromatography system can be used.
14. Avoid extensive sonication. Sonication breaks some of the cells and releases lysozyme, which continues to degrade the cell walls. If sonication is overdone, the cellular DNA is sheared in large pieces. This DNA is going to increase the viscosity of the extract and interfere with the subsequent affinity chromatography.
15. Sometimes the cyclin and the catalytic unit of the kinase are expressed at different levels. Purification by Ni-NTA agarose or other affinity resins will not reveal the unbalanced abundance of the peptides. The subsequent purification on MonoS (or other ion-exchange resins) can reveal fractions that contain the tagged subunit only. One such example is shown in Fig. 4. The tagged CDK9 elutes as an earlier peak as compared to the untagged CycT1, but then the peak trails and coincides with CycT1. The earlier CDK9 fractions contain significantly lower kinase activity as compared to the fractions that contain both subunits of the kinase (not shown). We have similar unbalanced expression in the case of His_6-CDK8/CycC (not shown).
16. The assessment of incorporation of ATP/min/mg of substrate gives a good idea of the activity of the kinase and can serve as cross-reference for different preparations. However, the measurement is inaccurate as very low proportion of the total ATP is incorporated. If such measurements are obligatory, details on the procedure can be found in [28].

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