

Chromatin Fractionation Assay in Fission Yeast

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[Abstract] Protein recruitment onto chromatin is a critical process for DNA metabolism, including DNA replication, DNA repair and DNA recombination. Especially, DNA modification enzymes and checkpoint proteins are loaded onto DNA damage sites in a context-dependent manner. In our recent study (Kunoh and Habu, 2014), the chromatin association of Pcf1, a large subunit of Chromatin Assembly Factor-1 (CAF-1), was monitored after exposure of cells to hydroxyurea which slowed down the DNA replication. Results of the chromatin fractionation assay provided evidence that Pcf1 was recruited to chromatin upon DNA replication stress. A similar procedure revealed the chromatin association of Orp1, Mcm proteins, and Swi6 (Sadaie *et al.*, 2008; Ogawa *et al.*, 1999). This assay allows us to fractionate chromatin-bound and -unbound proteins from living cells. The following immunoblot of the respective fractions provides the information concerning the chromatin binding status of our target proteins.

Materials and Reagents

1. Yeast strain (*Schizosaccharomyces pombe*)
2. Flask (IWAKI PUMPS, catalog number: 4980FK500)
3. Conical tube (BD Biosciences, Falcon®, catalog number: 2070)
4. 1.5 ml microcentrifuge tube (Eppendorf, catalog number: 022364111)
5. Lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich, catalog number: L1412)
6. Zymolyase 100T (Seikagaku Corporation, catalog number: 120493-1)
7. Complete Mini (Roche Diagnostics, catalog number: 11836153001)
8. Anti-GFP antibody (Roche Diagnostics, catalog number: 11814460001)
9. Anti-histone H3 antibody (Millipore, Upstate Biotechnology, catalog number: 05-499)
10. Anti-alpha-tubulin antibody (generously provided by Dr. A. Baines)
Note: Commercially available antibodies against alpha-tubulin (such as Abcam, catalog number: ab6161) can be used.
11. Goat HRP conjugated-anti-mouse antibody (Life Technologies, Biosource, catalog number: A10551)
12. YES liquid medium (see Recipes)

13. STOP buffer (see Recipes)
14. PEMS buffer (see Recipes)
15. 2x HBS buffer (see Recipes)
16. Lysis buffer (see Recipes)
17. 2x Laemmli protein sample buffer (see Recipes)

Equipment

1. Air- (Tykyo ikakikai, Eyela, model: FMC-1000) or water bath- (Taitec, model: MM-10) incubator shaker
2. Centrifuges equipped with 50 ml tubes (Tomy Digital Biology, model: AX-501) and 1.5 ml microtubes (Tomy Digital Biology, model: MX-107)
3. Heat block (Taitec, model: DTU-1BN) or water bath (Taitec, model: EXN-B)
4. Light microscope (Nikon Corporation, model: Eclipse E200)
5. Electrophoresis apparatus (Bio-Rad Laboratories, catalog numbers: 165-8002JA and 164-5052)
6. Transfer unit (Bio-Rad Laboratories, catalog numbers: 170-3930JA and 170-3935JA)

Procedure

1. Culture fission yeast cells in 200 ml of YES medium in a 500 ml flask at 26 °C.
Note: Stop culturing during the mid-log phase ($OD_{595} = \sim 1.0$).
2. Prepare approximately 2.5×10^8 cells by dilution of inoculum to 25 ml at $OD_{595} = 0.5$.
3. Harvest the cells by centrifugation at $400 \times g$ for 5 min at 4 °C.
4. Resuspend the cell pellet in 1 ml of ice-cold STOP buffer and transfer the cell suspension to a 1.5 ml microtube.
5. Centrifuge the cell suspension at $400 \times g$ for 5 min at 4 °C and pour out the supernatant (STOP buffer) by aspiration.
6. Place the microtube containing the cell pellet on ice for 5 min.
7. Resuspend the cell pellet in 1 ml of PEMS buffer dissolving 1 mg/ml lysing enzymes and 1 mg/ml Zymolyase 100T.
8. Incubate the cell suspension for 20 min at 37 °C for making spheroplasts which readily burst for enabling to fractionate chromatin-bound and -unbound proteins at the later step.
Note: To test whether these lysing enzymes work adequately, take a small volume of the cell suspension, add the equal volume of 10% SDS, and then monitor yeast lysis progression by light microscopy. If lysing is successful, almost all cells (spheroplasts) are expected to burst in the presence of SDS.

9. Centrifuge the spheroplast suspension at 400 x g for 5 min at 4 °C. Pour out the supernatant (PEMS buffer) carefully by aspiration.
10. Wash the spheroplast pellet twice by resuspending in 1 ml of 1.2 M sorbitol to avoid bursting of spheroplasts, followed by centrifugation at 400 x g for 5 min at 4 °C.
11. Lyse the spheroplasts by resuspending in 0.5 ml of ice-cold lysis buffer, followed by incubation on ice for 5 min.
12. Take 1/10 aliquot of the resulting lysate and save it as the whole cell extract (optional).
Note: To check the protein expression of our interest, we recommend an immunoblot of the whole cell extract with the other samples.
13. Centrifuge the rest of the lysate at 22,000 x g for 15 min at 4 °C.
14. Save the supernatant as the chromatin-unbound fraction.
15. Wash the pellets twice by resuspending in 1 ml of lysis buffer, followed by centrifugation at 22,000 x g for 5 min at 4 °C.
16. Resuspend the pellet in 0.45 ml of lysis buffer and save it as the chromatin-bound fraction.
17. Add an equal volume of 2x Laemmli protein sample buffer to all of the saved fractions.
18. Apply an equal volume of these fractions to the 8% SDS-PAGE gel and immunoblot using antibodies against histone H3 (1/5,000 dilution), alpha-tubulin (1/5,000 dilution) and specific protein(s).
Note: We detected the chromatin association of Pcf1-GFP protein by using the anti-GFP antibody (1/1,000 dilution). The secondary antibody was diluted at 1/10,000.

Representative data

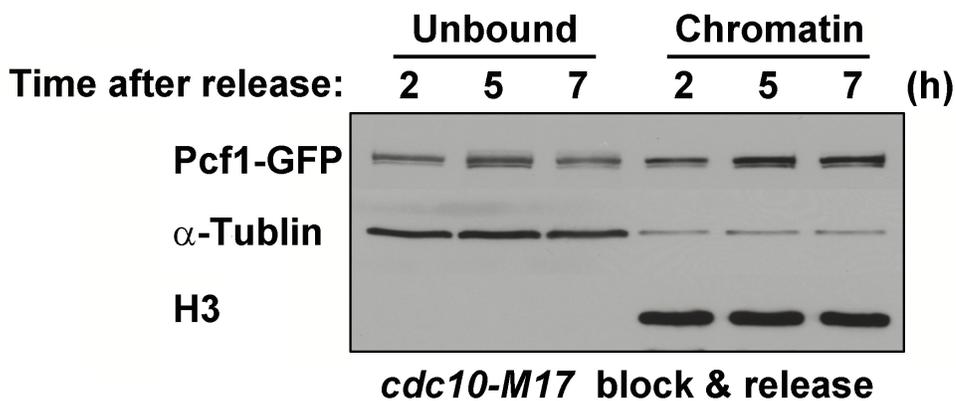


Figure 1. The *cdc10-M17* mutant cells were grown in YES medium at 26 °C, synchronized to G1 phase by incubation for 4 h at 36 °C, and returned to 26 °C. Released cells into S phase were harvested at indicated time(s) and subjected to chromatin fractionation assay to monitor the chromatin association of Pcf1-GFP.

Notes

1. The proper performance of the assay can be easily confirmed by immunoblots using anti-histone H3 and anti-alpha-tubulin antibodies, since histone H3 and alpha-tubulin are fractionated to the chromatin-bound and -unbound fractions, respectively. In some cases as shown in Figure 1, alpha-tubulin was fractionated into the chromatin-bound fraction. This contamination was probably due to low efficiency of cell lysis or insufficient washout of the chromatin-unbound proteins. Therefore, we recommend the reader(s) to check the former possibility by monitoring yeast lysis progression in the presence of SDS and adjusting cell number, concentration of lysing enzymes, and/or reaction time, if necessary. For the latter possibility, additional wash of the pellets should be done to remove the chromatin-unbound proteins completely.

Recipes

1. YES liquid medium
 - 5 g/L Bacto yeast extract
 - 30 g/L glucose
 - 225 mg/L adenine, histidine, leucine, uracil and lysine hydrochloride
2. STOP buffer
 - 150 mM NaCl
 - 50 mM NaF
 - 10 mM EDTA
 - 1 mM NaN₃
 - pH 8.0
3. PEMS buffer
 - 100 mM PIPES
 - 50 mM EDTA
 - 10 mM MgSO₄
 - 1.2 M sorbitol
 - pH 6.9
4. 2x HBS buffer
 - 50 mM MOPS
 - 120 mM beta-glycerophosphate
 - 30 mM MgCl₂
 - 30 mM EGTA
 - 30 mM p-nitrophenylphosphate

- 0.2 mM Na₃VO₄
 pH 7.2
5. Lysis buffer
 2x HBS buffer 5 ml
 10 % Triton X-100 0.5 ml
 2 M sorbitol 2 ml
 100 mM dithiothreitol (DTT) 0.1 ml
 0.1 mM phenylmethanesulfonyl fluoride (PMSF) 0.1 ml
 Complete mini 1 tablet
 Fill up to 10 ml with distilled water
6. 2x Laemmli protein sample buffer
 0.1 M Tris-HCl
 4 % sodium dodecyl sulfate (SDS)
 20 % glycerol
 0.2 % bromophenolblue (BPB)
 20 % 2-mercaptoethanol (2-ME)
 pH 6.8

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