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Chromatin Immunoprecipitation of Replication Factors Moving with the Replication Fork

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Summary

Replication of chromosomes involves a variety of replication proteins including DNA polymerases, DNA helicases, and other accessory factors. Many of these proteins are known to localize at replication forks and travel with them as components of the replisome complex. Other proteins do not move with replication forks but still play an essential role in DNA replication. Therefore, in order to understand the mechanisms of DNA replication and its controls, it is important to examine localization of each replication factor. Here we describe a chromatin immunoprecipitation (ChIP) method to locate a replication factor at the replication fork. Defining the localization of replication proteins should provide important insight into mechanistic understanding of the regulation of the DNA replication process.

Keywords

Replication fork; Replisome complex; Chromatin immunoprecipitation; Protein-DNA crosslinking

Introduction

Replication of eukaryotic chromosomes initiates at multiple sites referred to as replication origins. Initiation of DNA replication is directed by formation of the pre-replicative complex (pre-RC) at the origin of replication. The pre-RC includes a number of essential replication factors such as ORC (origin recognition complex), Cdc6, Cdt1, and MCM (minichromosome maintenance proteins). Among the components of the pre-RC, MCM is known to travel with replication forks, but other factors such as ORC stay at origins. To initiate DNA synthesis, additional factors are needed to facilitate unwinding of origins and generate replication forks. These factors include Mcm10, Cdc45, Sld3, Sld2/Drc1, and RPA prior to the loading of DNA polymerases, and PCNA and other accessory factors, which together form the replisome complex at the replication fork (1, 2).

Ancillary factors, which are not essential for DNA synthesis but nevertheless important for accuracy of DNA replication, also travel with moving replication forks. For example, in yeast, Mrc1 and the Swi1–Swi3/Tof1–Csm3 replication fork protection complex are known to play a critical role in the stabilization of stalled replication forks (3–7). These factors are also important for the activation of the DNA replication checkpoint to preserve genomic integrity (3–5, 7, 8). Thus, multiple factors are involved in DNA replication; however, their localizations differ from one protein to another. Therefore, it is essential to understand the location of each replication factor to understand the molecular mechanisms of DNA replication and its controls.

A number of approaches can be utilized to detect chromatin association of specific proteins. Western blotting is used to determine which proteins are present in crude chromatin preparations (9). Another method is the use of an in situ chromatin-binding assay that involves removal of cellular soluble proteins by Triton X-100 extraction of cells prior to microscopic analysis. In this assay, DNase treatment of cells confirms whether proteins persisting within the nucleus after Triton X-100 extraction actually represent chromatin binding of proteins (10). However, these approaches do not tell whether a protein of interest is localized at the replication fork nor do they tell whether the protein travels with moving replication forks. One possible approach to overcome this problem is to examine colocalization of a protein of interest with a known replication fork protein such as PCNA. Fork localization of the protein could be further confirmed by examining physical association of the protein with a known replication fork protein using immunoprecipitation (IP) or in vitro protein-binding assays. However, chromatin immunoprecipitation (ChIP) can persuasively determine the specific locations of proteins on chromosomes using formaldehyde to crosslink proteins to DNA, then performing PCR analysis of DNA precipitated with those proteins (11, 12). Therefore, in this chapter, we will describe chromatin immunoprecipitation (ChIP) of replication factors using the fission yeast *Schizosaccharomyces pombe* as a model organism.

2 Materials

2.1. Preparation and Synchronization of *S. pombe* Cells

1. YES (yeast extract and supplements medium): 5 g/L yeast extract, 30 g/L glucose, 187.5 mg/L leucine, 187.5 mg/L histidine, 187.5 mg/L adenine, and 100 mg/L uracil.
2. 25°C Water bath shaker.
3. 36°C Water bath shaker.
4. Ice bath.
5. Thermometer.
6. Light microscope.
7. Hemacytometer.

2.2. Crosslinking Cells

1. 10 mM Dimethyl adipimidate (DMA) in 1× PBS containing 0.25% DMSO (*see* Note 1).
2. 37% Formaldehyde (we purchased 25-mL bottles to keep formaldehyde fresh).
3. 2.5 M Glycine.
4. 1× Tris-buffered saline (TBS).
5. Filtering apparatus with vacuum manifolds (Analytical funnel filter holder PFC0004703, GE Osmonics Labstore, Minnetonka, MN; 3-place filter PVC vacuum manifolds XX2604735, Millipore, Billerica, MA; membrane filters, 0.45 µm, Millipore) (*see* Note 2).
6. 1.5-mL Screw-top tubes.

¹Theses reagents should be prepared on the day of the experiments.

²A filtering apparatus allows us to save time by reducing the frequency of centrifugation.

2.3. Preparation of Cell Extracts

1. Lysis Buffer L: 50 mM HEPES–KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 0.2 mM *p*-4-amidinophenyl-methane sulfonyl fluoride hydrochloride monohydrate (*p*APMSF) and Complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) (*see* Note 1).
2. 0.5-mm Glass beads.
3. FastPrep cell disrupter (Qbiogene, Irvine, CA).
4. 26-Gauge needle.
5. Misonix sonicator 3000 with a 14 cm-Cup Horn (Misonix, Farmingdale, NY) (*see* Note 3).
6. 1.5-mL Siliconized tubes.
7. Protein Assay Dye Reagent Concentrate (BioRad, Hercules, CA).
8. TES: 50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 1% SDS.

2.4. Immunoprecipitation

1. Anti-FLAG M2-agarose (Sigma-Aldrich).
2. Lysis Buffer L (*see* Subheading 2.3).
3. Lysis Buffer H: 50 mM HEPES–KOH, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 0.2 mM *p*APMSF and Complete EDTA-free protease inhibitor cocktail (Roche) (*see* Note 1).
4. DOC buffer: 10 mM Tris–HCl, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, and 1 mM EDTA (*see* Note 1).
5. TE: 10 mM Tris–HCl, pH 8.0 and 1 mM EDTA.

2.5. Removing Crosslink

1. TES (*see* Subheading 2.3).
2. 2× SDS–PAGE sample buffer: 100 mM Tris–HCl, pH 8.0, 4% SDS, 4 mM EDTA, 20% glycerol, 0.005% bromophenol blue, and 10% mercaptoethanol.

2.6. DNA Extraction

1. 10 mM Tris-HCl, pH 7.4.
2. 20 mg/mL Fungal proteinase K (Invitrogen, Carlsbad, CA) (dissolved in water and stored at –20°C).
3. 10 mg/mL Yeast tRNA from baker's yeast (Roche) (dissolved in water and stored at –20°C).
4. QIAquick PCR purification kit (Qiagen, Chatsworth, CA).

³We use a sonicator with a cup horn filled with ice water to process multiple samples at a time. We use a handmade Styrofoam float that fits into the 14-mm diameter cup horn. This float has holes for microcentrifuge tubes at the edge so that the distances between the sonicator probe and each tube is the same. This allows us to process each sample with equal sonication strength.

2.7. PCR

1. EXtaq DNA polymerase (TaKaRa, Ohtsu, Japan).
2. 10× EXtaq buffer (TaKaRa).
3. 2.5 mM dNTP.
4. Primers for PCR amplification of the *ori2004* and *ori3002* regions (13, 14) (Fig. 1)

P1	<i>ori2004</i> F: 5'-ATGGTAGATGGAGAAACGGG-3'
P2	<i>ori2004</i> R: 5'-CACGGCATCTTTCTTCACGA-3'
P3	<i>ori2004</i> -14kbF: 5'-ATGTATAGCTGGAACGCCTG-3'
P4	<i>ori2004</i> -14kbR: 5'-TTCCTCAAATCACCCACGT-3'
P5	<i>ori2004</i> -30kbF: 5'-TCGAAGATCCTACCGCTTTC-3'
P6	<i>ori2004</i> -30kbR: 5'-GATTCACATAACCCGCTAGC-3'
P7	<i>ori3002</i> F: 5'-TTGGCGCTAAACAATCTCTG-3'
P8	<i>ori3002</i> R: 5'-TCCTTGTCGAACTCAATTGC-3'
P9	<i>ori3002</i> -10kbF: 5'-GAATCTTCAGACCTTGCAGC-3'
P10	<i>ori3002</i> -10kbR: 5'-ACGGACTCCAACAACCATTTG-3'
P11	<i>ori3002</i> -19kbF: 5'-TTGCTGGCAGGGGTGAGTTG-3'
P12	<i>ori3002</i> -19kbR: 5'-TATGCTCTTTTCCCTCCACC-3'

3. Methods

To determine relocation of replication fork proteins on a chromosome as the replication fork progresses, we monitor chromatin binding of proteins at the well-characterized replication origin 2004 (*ori2004*) and at two positions 14- and 30-kb away on *S. pombe* chromosome II (Fig. 1a) (13). To generalize protein binding to other origin regions, we also evaluate chromatin binding at *ori3002*, another active replication origin on chromosome III (Fig. 1b) (14).

In this chapter, we show examples of ChIP analyses that monitor Rad11 and Orp1. Rad11 is an *S. pombe* RPA homolog that is closely associated with the replication fork (5). Orp1 (homologous to *S. cerevisiae* Orc1) is a component of the origin recognition complex that associates with the replication origin throughout the cell cycle (13). *S. pombe* cells are engineered to express Rad11-3FLAG (5) or Orp1-5FLAG proteins (13) to facilitate efficient immunoprecipitation of these proteins (see Note 4). These proteins are confirmed to be functional because strains carrying *rad11-3FLAG* or *orp1-5FLAG* grow at the wild-type level and have no apparent growth defect or significant sensitivity to DNA damaging agents such as HU and MMS (5, 13). Representative results of ChIP assays are shown in Fig. 3a. For interpretation of the results, see figure legends.

ChIP of replication fork proteins also involves synchronization of cells since we monitor protein relocation on a chromosome as DNA replication progresses. For this purpose, we utilize the *cdc25-22* genetic background (15) to reversibly arrest cells at the G2-M boundary at a restrictive temperature 36°C. Then, cells are released into the cell cycle at the

⁴3FLAG and 5FLAG contains three and five tandem repeats of the FLAG epitope, respectively. Other tags such as 13xMyc, 3xHA, GFP, and TAP can also be used for ChIP.

permissive temperature, 25°C. It is also important to examine septation, which in fission yeast occurs in S phase (Fig. 2a), to monitor cell cycle progression. Representative results of septation indices are shown in Fig. 3b.

3.1. Preparation and Synchronization of *S. pombe* Cells

1. Inoculate 50 mL YES with *cdc25-22* cells and grow overnight at 25°C. The next day, when OD₆₀₀ is ~0.5, dilute cells into 200 mL YES with a final OD₆₀₀ of 0.1, incubate at 25°C for about 6 h until OD₆₀₀ reaches ~0.2, dilute cells into 600 mL YES, and incubate overnight at 25°C.
2. The final OD₆₀₀ target the next day is ~0.3. It is important to use midlog phase cells for efficient synchronization of cells. Shift cells to a 36°C water bath shaker, and grow cells for 4 h to arrest cells at the G2-M boundary (*see* Note 5).
3. To quickly cool down the cell culture, place the flask in an ice bath while shaking the flask by hand, and monitor the temperature of cell culture using a clean thermometer. When the temperature reaches 27°C, move the flask to a 25°C water bath, and grow cells.
4. Collect and crosslink cells every 20 min (0–200 min) for ChIP as described in Subheading 3.2.
5. It is important to monitor septation, which coincides with the onset of S-phase (Fig. 2), to confirm cell cycle synchrony of the culture. Septation of *S. pombe* cells can be easily monitored using a light microscope. At the time of cell collection, use 10 µL of cell culture to monitor septation using a hemacytometer. Two hundred cells should be checked. Fig. 2 shows how to monitor and count septation of cells. This can be done while cell culture for ChIP is being treated with crosslinking agents (*see* Subheading 3.2).

3.2. Crosslinking Cells

To preserve protein-DNA interaction, cells should be treated with formaldehyde to promote protein-DNA crosslinking prior to immunoprecipitation. This method may be appropriate if the protein of interest is directly or closely associated with DNA.

It is possible that a protein of interest associates indirectly with DNA through other proteins. In such a case, it may be appropriate to promote protein-protein crosslinking prior to protein-DNA crosslinking. DMA can be used to facilitate protein-protein crosslinking (16).

1. When not using DMA, transfer 45 mL of the cell culture into a 50-mL conical tube, and proceed to step 8. When using DMA, pour 45 mL of cell culture into a filtering apparatus to trap cells on a membrane filter, and proceed to step 2.
2. Wash cells twice on the membrane with 20 mL of TBS.
3. Remove the membrane from the apparatus and place it in a new 50-mL conical tube.
4. Add 10 mL of 10 mM DMA, vortex to release cells into solution, discard the membrane, and gently shake the tube for 45 min at the room temperature.
5. Wash cells on the filter with 20 mL of ice-cold TBS as described earlier.

⁵*S. pombe* cells arrested at the G2-M boundary show a highly elongated phenotype without septum (*see* 0 min of septation indices of Fig. 3b).

6. Remove the membrane from the apparatus and place it in a new 50-mL conical tube.
7. Add 45 mL of ice-cold TBS, vortex to release cells from the membrane, and discard the membrane.
8. Add 1.25 mL of 37% formaldehyde (final concentration is 1%) and gently shake the tube for 20 min at the room temperature.
9. To quench cells, add 2.5 mL of 2.5 M glycine (final concentration is 125 mM) and gently shake the tube for 10 min.
10. To wash cells, pour the cell culture into a filtering apparatus to trap cells on a membrane filter. Wash cells on the membrane with 20 mL of ice-cold TBS.
11. Remove the membrane from the apparatus, and place it in a new 50-mL conical tube.
12. Add 20 mL of ice-cold TBS, and vortex to release cells from the membrane.
13. Discard the membrane, and centrifuge at $1,000 \times g$ for 3 min at 4°C to collect cells at the bottom of the tube.
14. Discard supernatant, resuspend cells in 1 mL of ice-cold TBS, and transfer cells to a 1.5-mL screw-top tube (*see Note 6*).
15. Quickly pellet cells at $9,000 \times g$ for 10 s using a microcentrifuge, discard supernatant, and immediately freeze pellet at -80°C . Proceed to Subheading 3.3.

3.3. Preparation of Cell Extracts

1. Thaw cell pellets on ice.
2. Add 400 μL of ice-cold Lysis Buffer L to the cell pellet.
3. Add cold glass beads until they reach the surface of buffer.
4. Break cells by a FastPrep cell disruptor at 4°C (output 6, 20 s, 2 cycles, 2-min interval between cycles) (*see Note 7*).
5. To recover cell lysate, pierce the bottom of the tube with a heated needle (26-gauge), and place it inside a new 1.5-mL microcentrifuge tube. Centrifuge at $800 \times g$ using a micro-centrifuge for 30 s to collect cell lysate in the new tube.
6. Discard the tube containing glass beads, and vortex the new tube to resuspend the content.
7. Place all sample tubes in a special float (*see Note 3*) for the Misonix sonicator 3000, and put the float in the cup horn filled with ice water.
8. Sonicate samples: output 10; process time, 4 min; on time, 20 s; off time 1 min 30 s (*see Note 8*).
9. Add 200 μL of ice-cold Lysis Buffer L to the sonicated samples, centrifuge at $16,000 \times g$ for 5 min at 4°C . Transfer supernatant to a new tube, centrifuge again at $16,000 \times g$ for 10 min at 4°C to eliminate cell debris.

⁶It is important to use tubes that fit properly in the FastPrep cell disruptor.

⁷It is important to monitor cell disruption under the microscope. More than 90% of cells should be disrupted.

⁸This condition allows us to shear DNA into 500- to 700-bp fragments. The condition should be optimized in individual laboratories by extracting and monitoring DNA after sonication.

10. Transfer supernatant (cell extract) to a new 1.5-mL siliconized tube (*see* Note 9).
11. Adjust protein concentration to the lowest concentrated sample using BioRad Protein Assay Dye Reagent Concentrate (*see* Note 10).
12. Transfer 5 μ L of each extract to a new tube to use for the input control. Add 35 μ L of TES and incubate at 65°C for 12–16 h to reverse crosslinking (*see* Note 11).
13. Perform immunoprecipitation with the rest of the cell extracts.

3.4. Immunoprecipitation

1. Before immunoprecipitation, prewash Anti-FLAG M2 agarose beads (FL beads). Forty microliters (50% slurry, 20 μ L bed volume) of FL beads is required for each sample.
2. To wash beads, transfer the required amount of FL beads to a 1.5-mL microcentrifuge tube, centrifuge at $4,500 \times g$ for 10 s, aspirate supernatant, and resuspend beads in 1 mL of ice-cold Lysis Buffer L. Wash beads two more times, then resuspend beads in ice-cold Lysis Buffer L to obtain a 50% slurry of FL beads. Keep on ice until needed.
3. Add 40 μ L of the prewashed FL beads to the cell extract obtained in Subheading 3.3. Rotate samples for 1.5 h at 4°C.
4. Wash FL beads (spin down beads at $4,500 \times g$ for 10 s, discard supernatant, add 1 mL of buffer to beads, invert tube 10 times to mix the content) as follows:
 - (a) Wash 3 times with ice-cold Lysis Buffer L
 - (b) Wash 2 times with ice-cold Lysis Buffer H
 - (c) Wash 2 times with ice-cold DOC buffer
 - (d) Transfer the slurry to a new 1.5-mL microcentrifuge tube
 - (e) Wash 1 time with ice-cold TE buffer and discard supernatant (*see* Note 12).
5. Samples (FL beads) can be now handled at the room temperature. Proceed to Subheading 3.5.

3.5. Removing Crosslink

1. Add 52 μ L of TES to the FL beads and incubate for 30 min at 65°C. Mix occasionally by tapping tubes.
2. Centrifuge at $4,500 \times g$ for 10 s to collect beads to the bottom of the tube.
3. To prepare protein samples for immunoprecipitation, transfer 10 μ L of supernatant to a new 1.5-mL microcentrifuge tube. Add 10 μ L of 2 $\times$ SDS-PAGE loading buffer and incubate at 65°C for 12–16 h. Store samples at –20°C (*see* Note 13).

⁹We use siliconized tubes at this step to reduce background of PCR amplification.

¹⁰When DMA is used for crosslinking, protein assays should be performed immediately after FastPrep-cell disruption (*see* Subheading 3.3, step 5). Then centrifuge samples to collect the chromatin-containing fraction as a pellet. Resuspend pellet in 400 μ L of ice-cold Lysis Buffer L, and sonicate the sample. This would also reduce nonspecific amplification of DNA. These procedures can be also applied when not using DMA.

¹¹This serves as a control to evaluate that each PCR primer set amplifies DNA equally in all samples.

¹²Caution is required since bead pellets are loose.

¹³Use these protein samples for Western blotting to monitor the efficiency of immunoprecipitation. Generally, the efficiency is lower when using crosslinked cells.

4. For ChIP analysis, transfer the rest of supernatant (40 μ L) to a new 1.5-mL microcentrifuge tube, and incubate at 65°C for 12–16 h. Then proceed to Subheading 3.6.

3.6. DNA Extraction

1. Prepare DNA extraction buffer (mix 160 μ L of 10 mM Tris–HCl, pH 7.4, 1 μ L of 20 mg/mL proteinase K and 4 μ L of 10 mg/mL yeast tRNA for one ChIP sample).
2. Add 165 μ L of DNA extraction buffer to the sample prepared as described in Subheading 3.5, step 4. For the input control, also add 165 μ L of DNA extraction buffer to the sample prepared as described in Subheading 3.3, step 12.
3. Incubate for 2 h at 50°C.
4. Purify DNA using Qiagen's PCR purification kit and following the protocol provided by the manufacturer. For the last step of DNA purification, use 30 μ L of elution buffer for the ChIP samples and 50 μ L of elution buffer for the input control samples. Store purified DNA samples at –20°C until needed for PCR.

3.7. PCR

1. Mix the following: 1 μ L of template DNA (input control or ChIP sample), 1.5 μ L of each of the primers (3 pmol/ μ L), 2.4 μ L of dNTP mix (2.5 mM each), 3.0 μ L 10 $\times$ EXtaq buffer, 0.15 μ L of EXtaq polymerase, and 14.45 μ L of H₂O (*see* Note 14).
2. Perform PCR at the following cycles (*see* Note 15):
 - (a) 94°C for 10 min.
 - (b) 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s (25–35 cycles)
 - (c) 72°C for 7 min.
3. Run PCR samples on 1.5–3% agarose gel to separate PCR products.

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¹⁴We include three primer sets in one single PCR to evaluate relocation of proteins on a chromosome. For example, we use six primers for *ori2004* (P1–P6) described in Subheading 2.7, item 4 to monitor the *ori2004* region. Optimization of primer concentration is needed to obtain equal amplification of three PCR products.

¹⁵It is important to optimize the number of PCR cycles to ensure that the amount of DNA product remains in the logarithmic range of the amplification curve. This allows us to make reliable semiquantified comparisons.

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Fig. 1. Diagrams of the *ori2004* (a) and *ori3002* (b) regions on *S. pombe* chromosomes II and III, respectively. Primers used for PCR are shown. See Subheading 2.7 for primer sequences.

Fig. 2.

(a) *S. pombe* cell cycle. Morphological changes and cell cycle progression are shown. The cell cycle position can be estimated by analyzing cell length, nuclei number, and position; and the presence of a division plate (septum). (b) Morphological changes during septation and a method of cell number counting are shown. To obtain a septation index, the number of septated cells was divided over the total cell number.

Fig. 3.

(a) ChIP assays of Rad11-3FLAG or Orp1-5FLAG were performed at *ori2004* and at sites located 14- or 30-kb away from this origin. *cdc25-22* cells were synchronized at the G2-M boundary by incubation at 36°C for 4 h and then released into fresh YES medium at 25°C. ChIP assays were performed at the indicated times using cells crosslinked only with formaldehyde. Rad11-3FLAG associated with *ori2004* at the onset of S-phase when the septation index was about to increase. This origin association peaks at 60 min. Rad11-3FLAG most strongly associated with the 14-kb position between 80 and 100 min and with the 30-kb positions at 120 min, indicating that Rad11-3FLAG relocated from *ori2004* to the 14- and 30-kb positions during the course of experiments. On the other hand, Orp1-5FLAG associated with *ori2004* throughout the cell cycle and did not relocate to distant positions. ChIP of input samples shows that three PCR products at *ori2004* amplify equally with the primers. (b) An increase in the septation index indicates the onset of S-phase.