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Chromatin Immunoprecipitation to Detect DNA Replication and Repair Factors

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Abstract

DNA replication is tightly coupled with DNA repair processes in order to preserve genomic integrity. During DNA replication, the replication fork encounters a variety of obstacles including DNA damage/adducts, secondary structures, and programmed fork-blocking sites, which are all difficult to replicate. The replication fork also collides with the transcription machinery, which shares the template DNA with the replisome complex. Under these conditions, replication forks stall, causing replication stress and/or fork collapse, ultimately leading to genomic instability. The mechanisms to overcome these replication problems remain elusive. Therefore, it is important to investigate how DNA repair and replication factors are recruited and coordinated at chromosomal regions that are difficult to replicate. In this chapter, we describe a chromatin immunoprecipitation method to locate proteins required for DNA repair during DNA replication in the fission yeast *Schizosaccharomyces pombe*. This method can also easily be adapted to study replisome components or chromatin-associated factors.

Keywords

DNA replication; DNA damage; DNA repair; Chromatin immunoprecipitation; ChIP; Protein–DNA cross-linking; Quantitative PCR; Difficult to replicate; Replication fork; Recombination; Rad52

1 Introduction

In every round of cell division, cells need to faithfully replicate and segregate their genetic material. However, the process of DNA replication itself is a major source of genomic instability [1–3]. Although a certain quota of genetic instability has naturally contributed to genetic variation, its exacerbation due to defects in DNA replication and/or repair processes is the main driver of cancer development, aging, and genetic diseases [2, 4].

Replication-dependent genomic instability can arise during replication initiation. A process called replication licensing controls replication initiation and ensures that the genetic material of the cell is replicated only once per cell cycle [5, 6]. Defects in replication licensing decrease the number of active origins and the rate of origin firing. These cause cells to accumulate DNA breaks and promote inappropriate activation of cell cycle checkpoint pathways, leading to genetic instability and cell death [7]. It is also known that overactivation of origin licensing causes genomic instability. Central to the replication licensing system is the regulation of the licensing factor Cdt1, which is required for the assembly of the pre-replication complex during initiation of DNA replication [8, 9]. If Cdt1 remains active after replication initiation due to the loss of the inhibitor geminin or defects

in Cdt1 degradation, cells undergo unscheduled firing of replication origins, leading to re-replication. As a consequence, cells accumulate multifork structures and DNA damage, which activate checkpoint pathways [8, 10, 11].

Genome instability can also arise during DNA replication. Defects in Okazaki fragment processing, dysfunction of replication and/or repair factors, and limited dNTP availability are some of the major causes of genomic instability during DNA synthesis. Faulty replisome progression increases the chances of replication fork stalling and collapse, which drive the formation of unusual DNA structures including ssDNA and double-strand breaks (DSBs) especially at difficult-to-replicate genomic regions. These DNA structures activate the homologous recombination (HR) pathway, which is critical for maintenance of genomic stability in cells undergoing active DNA replication, both in prokaryotic and eukaryotic organisms [1–3]. The HR pathway involves DSB resection to generate ssDNA followed by strand invasion, strand annealing, and resolution of intermediate DNA structures, in order to repair damaged DNA [12]. One of the major players of HR is Rad52, which is essential for the repair of DSB in yeast [13, 14]. Rad52 is an ssDNA-binding protein, and its main functions are to promote DNA annealing and to facilitate the recruitment of the Rad51 recombinase onto RPA-coated ssDNA in order to promote strand invasion [13, 15–17]. In mitotic cells, DSBs may not be a major driver of HR. Therefore, it has been suggested that Rad52 is involved in the repair of ssDNA gaps at collapsed replication forks and in telomere maintenance [18, 19]. In yeast, Rad52 DNA repair foci are sporadically seen due to spontaneous DNA damage in WT cells; however the number of Rad52 foci increases in mutants defective for fork stabilization and/or checkpoint activation [20–23].

Yeast models have been of great importance to investigate the mechanisms of replication-dependent DNA damage and its repair pathways. However, many questions still remain unanswered, including: (1) where in the genome does replication stress occur? (2) what are the types of DNA damage generated at the replication fork during replication stress? (3) how is the damage repaired at the replication fork? Identifying the causes of genomic instability and gaining a clear understanding of how the repair pathways work during DNA replication to prevent replication-associated DNA damage is of critical importance to understand the basis of aging and cancer development.

This chapter describes a chromatin immunoprecipitation (ChIP) method to detect the binding of a protein of interest to a specific genomic region. Since its development in the mid-1980s [24, 25], many modifications have been made to improve the technique and to allow for its application on different model systems [26, 27]. We present an updated ChIP protocol that is well suited for the study of non-chromatin DNA binding proteins that interact with DNA at variable rates. We describe Rad52-ChIP as an example for locating DNA repair factors at difficult-to-replicate regions using the fission yeast *Schizosaccharomyces pombe*. Similar methods can be adapted for the study of replication proteins at specific chromosomal loci in other model systems.

2 Materials

2.1 Preparation 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, 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 Cross-Linking Cells

1. 2× Phosphate buffer saline (PBS): 274 mM NaCl, 5.4 mM KCl, 20 mM Na₂HPO₄, 3.6 mM KH₂PO₄. Adjust pH to 7.4 using 10 N HCl, then 1 N HCl.
2. 1× PBS buffer: dilute 2× PBS with H₂O.
3. 30 % paraformaldehyde (PFA) in 1× PBS (*see* **Notes 1 and 2**).
4. 50 mL conical tubes.
5. 10 mM dimethyl adipimidate (DMA) in 1× PBS containing 0.25 % DMSO (*see* **Notes 1 and 3**).
6. 1.5 mL screw-cap tubes.
7. Parafilm.

2.3 Primary Antibody-Dynabeads Coupling

1. Lysis Buffer L: 50 mM HEPES–KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8.0, 1 % Triton X-100, 0.1 % sodium deoxycholate (DOC), 0.1 mM *p*-4-amidinophenyl-methane sulfonyl fluoride hydrochloride monohydrate (*p*APMSF) and Roche Diagnostics Complete EDTA-free protease inhibitor cocktail (*see* **Note 1**).
2. Life Technologies Dynabeads-Protein G or -Protein A depending on the animal source of the antibody.
3. Life Technologies DynaMag-2 magnet.
4. Primary antibody (*see* **Note 4**).
5. Rotation wheel.

2.4 Preparation of Cell Extracts

1. Lysis Buffer L (*see* Subheading 2.3, **item 1**).

2. 0.5 mm glass beads.
3. FastPrep cell disruptor.
4. 26 G needle.
5. Sonicator (*see Note 5*).
6. 1.5 mL siliconized microcentrifuge tubes.

2.5 Chromatin Immunoprecipitation (ChIP)

1. 1.5 mL siliconized microcentrifuge tubes.
2. Lysis Buffer H: 50 mM HEPES–KOH, pH 7.5, 0.65 M NaCl, 1 mM EDTA, pH 8.0, 1 % Triton X-100, 0.1 % DOC, 0.1 mM *p*APMSF, Roche Complete EDTA-free protease inhibitor cocktail.
3. Wash buffer: 10 mM Tris–HCl, pH 8.0, 250 mM LiCl, 0.5 % NP-40, 0.5 % DOC, 1 mM EDTA, pH 8.0.
4. Tris–EDTA (TE) buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
5. Qiagen QIAquick PCR purification kit or Chelex 100 resin.
6. Lysis Buffer L (*see Subheading 2.3, item 1*).
7. Life Technologies DynaMag-2 magnet.

2.6 Removing Cross-Link

1. TES buffer: 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1 % SDS.
2. 65 °C water bath.
3. Life Technologies DynaMag-2 magnet.

2.7 DNA Extraction

1. 10 mM Tris–HCl, pH 7.4.
2. 20 mg/mL proteinase K (dissolved in water and stored at –20 °C).
3. Qiagen QIAquick PCR purification kit, or 10 % Bio-Rad Chelex 100 resin in sterile water.

2.8 Competitive PCR Reaction

1. TaKaRa EXtaq DNA polymerase.
2. 10× EXtaq buffer (TaKaRa).
3. 2.5 mM dNTP mix.
4. 10 µM target primer mix (*see Note 6*).
5. 10 µM control primer mix (*see Note 6*).
6. PCR machine.

2.9 Gel Running and Scanning

1. 50× TAE buffer: 2 M Tris, 1 M acetic acid, 50 mM EDTA.
2. 4 % polyacrylamide in 1× TAE buffer. For 1 mini-gel mix 1,250 µL 40 % acrylamide, 250 µL 50× TAE, 125 µL 10 % ammonium persulfate (APS), 6.25 µL TEMED, water to 12.5 mL.
3. 5× Orange G dye DNA loading buffer: 7.5 mL glycerol, 100 mg Orange G dye (Sigma), water to 50 mL.
4. SYBR green solution: Life Technology SYBR Green I diluted 1:10,000 in 1× TAE buffer (prepared from 50× stock solution, **item 1** above).
5. Bio-Rad Minigel System.
6. Phosphorimager scanner.
7. ImageJ software.
8. 100 bp DNA ladder.
9. Loading tips.

2.10 SYBR Green-Based Real- Time PCR Analysis

1. Bio-Rad iQ SYBR Green Supermix (uses hot-start Taq DNA polymerase).
2. 10 µM forward primer (*see Note 7*).
3. 10 µM reverse primer (*see Note 7*).
4. Quantitative PCR machine.

3 Methods

To determine the recruitment of homologous recombination proteins at natural replication barriers, we monitored chromatin binding of Rad52 in *S. pombe* (Fig. 1). In order to facilitate the immunoprecipitation of Rad52, we previously engineered *S. pombe* cells to express Rad52 fused to 12 tandem copies of the Pk epitope (12Pk) at the C-terminus [28]. The Pk (also called V5) is a short amino acid epitope with the sequence, GKPIPNPLLGLDST, originally found in the paramyxovirus SV5 proteins P and V [29]. The availability of vectors for Pk-epitope tagging in *S. cerevisiae* and *S. pombe* [28, 30], as well as commercially available anti-Pk antibodies, allow for straightforward and efficient detection and purification of proteins. Other epitope tags such as 5FLAG, 13Myc, 3HA, GFP, and TAP can also be used for ChIP, as described for the 5FLAG tag in our previous protocol [27]. Representative results of ChIP assays for the Rad52-12Pk protein are shown in Fig. 1. For interpretation of the results, *see figure legends*.

The synchronization of cell cultures can also provide significant information on the kinetics and diversity of DNA repair pathways throughout the different cell cycle stages. For this purpose, we also describe a method that utilizes the *cdc25-22* genetic background [31] to reversibly arrest cells at the G2-M boundary at a restrictive temperature, 36 °C. Then, cells are released into the cell cycle at a permissive temperature, 25 °C. To monitor cell cycle

progression, one can evaluate the septation index by quantifying the appearance of the division plate (septum) in the cells. In fission yeast, septation coincides with S phase (Fig. 2a) and is easily detected by bright field microscopy. Representative results of septation indices are shown in Fig. 2c.

3.1 Preparation of *S. pombe* Cells

3.1.1 Preparation of Asynchronous *S. pombe* Cell Culture

1. Inoculate pre-cultures of 10 mL YES and grow for ~8 h at 32 °C (*see* **Notes 8 and 9**).
2. Once the OD₆₀₀ is ~0.5, dilute cells into 50 mL YES with a final OD₆₀₀ of 0.05 and incubate overnight at 32 °C until they reach a final OD₆₀₀ of ~0.4.

3.1.2 Preparation and Synchronization of *S. pombe* Cell Culture

1. Inoculate 50 mL YES with *cdc25-22* mutant 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 (*see* **Note 9**).
2. The final OD₆₀₀ target the next day is ~0.3. Shift cells to a 36 °C water bath shaker, and grow cells for 3 h to arrest cells at the G2-M boundary (*see* **Note 10**).
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, transfer the flask to a 25 °C water bath.
4. Collect and cross-link cells every 20 min (0 min to 200 min) for ChIP as described in Subheading 3.2 (*see* **Note 11**).
5. At the time of cell collection, use 10 µL of cell culture to monitor septation using a hemacytometer. At least 200 cells should be checked. Figure 2 shows how to monitor and count septation of cells. This can be done while cell culture for ChIP is being treated with cross-linking agents (*see* Subheading 3.2).

3.2 Cross-Linking Cells

Cross-linking proteins to DNA is a critical step in the ChIP assay. The conditions used in this protocol ensure strong cross-linking. The use of paraformaldehyde preserves protein–DNA interactions and is suitable for proteins that bind DNA directly. Because many repair factors are recruited to the DNA indirectly through association with other DNA-binding proteins, the addition of a protein–protein cross-linking agent, such as dimethyl adipimidate (DMA), enables efficient precipitation of more unstable protein–DNA complexes [32]. Depending on the characteristics of the target protein, researchers are recommended to optimize the cross-linking conditions. Decreasing the percentage of formaldehyde and/or skipping the DMA cross-linking steps should result in the selective preservation of tight protein–DNA complexes. Such a modification is useful if the protein of interest is directly or closely associated with DNA.

1. Transfer 45 mL of the culture to a 250-mL flask and incubate for 5 min at 18 °C while shaking (*see Note 12*).
2. Add 5 mL of 30 % PFA solution and incubate for 30 min at 18 °C while shaking.
3. Keep samples on ice. Centrifuge in a 50-mL tube at $1,000 \times g$ for 5 min at 4 °C to collect cells.
4. Remove the supernatant completely by decanting.
5. Wash the cell pellets with 20 mL of cold 1× PBS and centrifuge at $1,000 \times g$ for 5 min at 4 °C.
6. Repeat the wash step (**step 5**). After this, the cell pellets can be kept at room temperature.
7. When not using DMA, proceed to **step 11**. If using DMA, add 10 mL of 10 mM DMA solution to the pellet and vortex to release cells into solution.
8. Seal the tubes with Parafilm and rotate the samples at room temperature for 45 min.
9. Centrifuge at $1,000 \times g$ for 5 min at 4 °C. Remove supernatant and put the samples back on ice.
10. Wash cells with 20 mL of cold 1× PBS. Centrifuge at $1,000 \times g$ for 5 min at 4 °C. Remove the supernatant.
11. Resuspend the pellet in 1 mL of 1× PBS, and transfer to a 1.5-mL screw-cap tube.
12. Quickly pellet cells at $9,000 \times g$ for 10 s using a microfuge, discard supernatant, and immediately freeze the cell pellet at −80 °C.

3.3 Primary Antibody–Dynabeads Coupling

Dynabeads provide a magnet-based separation method and are used for a wide range of research applications [33–35]. Dynabeads are polymer-coated superparamagnetic particles that allow for the coupling of biologically relevant molecules such as antibodies. Compared to other protocols that use agarose or sepharose beads, Dynabeads cut down on the use of column and centrifugation steps. This permits a simple and efficient method for the separation of the molecules of interest, thereby allowing for highly reproducible ChIP results. Moreover, the use of the magnet to generate a bead pellet minimizes sample-handling errors by preventing suction of the bead slurry or eliminating residual buffer left in the sample tube during the washing steps. In this section, we describe a method to couple primary antibodies to Dynabeads for chromatin immunoprecipitation. All steps should be performed in a cold room at 4 °C.

1. Transfer 15 µL of Dynabeads (per sample) to a 1.5 mL microcentrifuge tube (*see Note 13*).
2. Place the tubes on DynaMag for 1 min or until the supernatant becomes clear. Remove the supernatant by aspiration.
3. Wash the beads by resuspending with the pipette in 200 µL Lysis buffer L without making bubbles.

4. Repeat washing steps (**steps 2 and 3**) two more times.
5. Place the tubes on DynaMag as described in **step 2**, and remove supernatant completely by aspiration. Resuspend the beads in 50 μ L of fresh cold Lysis buffer L (per sample), and add 3–5 μ L of primary antibody (per sample) (*see Note 4*).
6. Incubate for 4–6 h with rotation at 4 °C.

3.4 Preparation of Cell Extracts

1. Thaw cell pellets on ice.
2. Add 400 μ L of ice-cold Lysis Buffer L to the cell pellet (*see Note 14*).
3. Add 1.9 g of cold glass beads per tube (*see Note 15*).
4. Break cells using a FastPrep cell disruptor at 4 °C. For Qbiogene FastPrep cell disruptor that we use, the conditions are the following: output 6, 20 s, two cycles, 2 min interval between cycles (*see Note 16*).
5. To recover cell lysate, pierce the bottom of the tube with a heated needle (26-G), and place it inside a new 1.5-mL microcentrifuge tube. Centrifuge at $800 \times g$ using a microfuge for 30 s at 4 °C to collect cell lysate in the new tube.
6. Discard the tube containing glass beads and resuspend the content without making any bubbles.
7. Place all sample tubes in a special float (*see Notes 5 and 17*) for the sonicator, and put the float in the cup horn filled with ice-cold water.
8. Sonicate samples. For Misonix Sonicator 3000, the conditions are the following: output, 10; process time, 4 min; ON time, 20 s; OFF time 1 min 30 s (*see Note 18*).
9. Add 200 μ L of ice-cold Lysis Buffer L to the sonicated samples.
10. Centrifuge at $16,000 \times g$ for 10 min at 4 °C.
11. Transfer supernatant (cell extract) to a new tube and keep tubes on ice.

3.5 Chromatin Immunoprecipitation (ChIP)

To reduce the nonspecific association of proteins with Dynabeads, it is important to incubate cell extracts with Dynabeads that have not coupled with antibodies. Such pre-incubation of the cell extract with Dynabeads prior to immunoprecipitation significantly reduces nonspecific protein binding to antibody-coupled Dynabeads.

1. Transfer Dynabeads (15 μ L per sample) to a 1.5-mL microcentrifuge tube. Collect Dynabeads using the DynaMag as described in **step 2** of Subheading 3.3 and remove buffer completely (*see Note 13*).
2. Wash the beads by adding 500 μ L of lysis buffer L (Subheading 2.3, **item 1**) to the beads and collect beads using DynaMag. Remove the buffer completely.
3. Repeat **step 2** and after removing the buffer, resuspend the beads in Lysis buffer L (50 μ L per sample).

4. Add the washed beads (50 μ L) to the supernatant prepared in Subheading 3.4 and incubate for 1 h with rotation at 4 $^{\circ}$ C.
5. Centrifuge at full speed in a microfuge for 10 min at 4 $^{\circ}$ C and transfer 510 μ L of supernatant to a clean 1.5-mL microcentrifuge tube (*see Note 19*).
6. Save 10 μ L of the solution in a 1.5-mL microcentrifuge tube on ice. This will be the whole cell extract (WCE) sample. Samples can be stored at 4 $^{\circ}$ C.
7. Add 50 μ L of the antibody-bound Dynabeads solution prepared in Subheading 3.3 to the remaining supernatant (500 μ L) and incubate overnight with rotation at 4 $^{\circ}$ C.
8. Spin down the tubes to remove any liquid trapped in the tube cap.
9. Place the tubes in the DynaMag until the pellet is formed (approximately 1 min or until the supernatant becomes clear). Remove the solution without disturbing the pellet.
10. Wash the Dynabeads (add 800 μ L of buffer, incubate 15 min with rotation at 4 $^{\circ}$ C, spin down the tubes, place them in the DynaMag until the pellet is formed and discard supernatant) as follows:
 - a. Wash two times with ice-cold Lysis Buffer L.
 - b. Wash one time with ice-cold Lysis Buffer H.
 - c. Wash one time with ice-cold Wash buffer.
 - d. Wash one time with ice-cold TE buffer and transfer the beads to a new 1.5-mL microcentrifuge tube.
11. Place tubes in the DynaMag until the pellet is formed and aspirate the supernatant.
12. When extracting DNA with QIAquick PCR purification kit, proceed to Subheading 3.6.
13. When extracting DNA using Chelex 100 resin, skip Subheading 3.6 and proceed to Subheading 3.7.2.

3.6 Removing Cross-Link

1. Add 65 μ L of TES buffer to the beads and incubate at 65 $^{\circ}$ C for 15 min with shaking (*see Note 20*).
2. Spin down the tubes at $200 \times g$ for 30 s in a microfuge to collect the solution to the bottom of the tube and then place the tube in the DynaMag.
3. Let it stand until a tight pellet forms (approximately 1 min or until the supernatant becomes clear).
4. Transfer 60 μ L of supernatant to a new 1.5 mL microcentrifuge tube.
5. Repeat **steps 1–3** and pool both fractions to the same tube (120 μ L total: ChIP sample).

6. Remove WCE samples from the storage (*see* Subheading 3.5, **step 6**) and add 110 μL of TES buffer (120 μL total: WCE sample).
7. Incubate the ChIP and WCE tubes overnight in a water bath at 65 °C.

3.7 DNA Extraction

3.7.1 DNA Extraction Using QIAquick PCR Purification Kit

1. After incubation, prepare DNA extraction buffer (mix 120 μL of Tris–HCl, pH 7.4 and 3 μL of 20 mg/mL fungal proteinase K for one sample).
2. Add 120 μL of DNA extraction buffer to each sample (ChIP and WCE).
3. Incubate at 37 °C for 5 h with regular shaking.
4. Purify DNA using QIAquick PCR purification kit and following the protocol provided by the manufacturer. For the last step of DNA purification, use 50 μL of TE buffer. Store purified DNA samples at –20 °C until needed for PCR or high-throughput DNA sequencing techniques.

3.7.2 DNA Extraction Using Chelex 100 Resin—Chelex 100 resin allows for inexpensive and efficient DNA extraction [36]. This section also includes steps for cross-link removal.

1. Add 100 μL of Chelex 100 resin (10 % slurry in sterile H_2O) to the washed beads (ChIP samples, *see* Subheading 3.5, **step 11**) and the input samples (WCE samples, *see* Subheading 3.5, **step 6**), mix well by vortex, and boil the samples for 15 min, in order to remove cross-link and extract DNA.
2. Cool to room temperature, add 1 μL of 20 mg/mL proteinase K, and incubate at 55 °C for 30 min. Mix samples occasionally.
3. Add 100 μL of H_2O and boil the samples for 10 min to inactivate proteinase K.
4. Centrifuge at the maximum speed in a microfuge at 4 °C for 1 min.
5. Carefully transfer 120 μL of supernatant (extracted DNA) to a new 1.5-mL microcentrifuge tube. Store extracted DNA at –20 °C until needed for PCR analysis (*see* **Note 21**).

3.8 Competitive PCR Reaction

1. Mix the following (for each sample) (*see* **Note 22**):
 - a. 1 μL of template DNA (input or ChIP DNA) (*see* **Note 23**).
 - b. 12.2 μL of H_2O .
 - c. 1.5 μL of target primer mix (10 μM) (*see* **Note 6**).
 - d. 1.5 μL of control primer mix (10 μM) (*see* **Note 6**).
 - e. 1.6 μL of 2.5 mM dNTP mix.
 - f. 2 μL 10 $\times$ EXtaq buffer.

2. Perform PCR using the following program.
 - a. 95 °C for 2 min.
 - b. 95 °C for 10 s.
 - c. 55–60 °C for 30 s.
 - d. 72 °C for 30 s (perform SYBR Green detection).
 - e. Repeat above **steps (b)–(d)** for 39 cycles.
 - f. 72 °C for 7 min.
 - g. (Optional) Melting curve analysis: 55–95 °C.
3. Obtain cycle threshold (Ct) values for each sample.
4. Calculate % precipitated DNA using Ct values between WCE and ChIP samples using the following equations:
 - a. Calculate dilution factor. This is based on the amount of DNA samples used for PCR. If input DNA is diluted by 100 times, the dilution factor is 100 (*see Note 23*).
 - b. Calculate Ratio between the amounts of WCE used for input and ChIP samples. In this protocol, we used 10 µL and 500 µL of WCE for input and ChIP samples, respectively (*see Subheading 3.5*). Thus, the ratio is $500/10 = 50$.
 - c. Calculate raw % precipitated DNA from input and ChIP samples prepared from cells expressing a tagged version of the protein of interest.

$$\text{Raw \% precipitated DNA} = 100 \times 2^{\text{Input Ct} - \text{ChIP Ct}} / [\text{Dilution factor} \times \text{Ratio between WCE and ChIP}]$$
 (*see Note 27*).
 - d. Calculate raw % background precipitation from input and ChIP samples prepared from cells without expression of a tagged version of the protein of interest.

$$\text{Raw \% background precipitation} = 100 \times 2^{\text{Input Ct} - \text{ChIP Ct}} / [\text{Dilution factor} \times \text{Ratio between WCE and ChIP}]$$
 - e. Background corrected % precipitated DNA = Raw % precipitated DNA – Raw % background precipitation.

4 Notes

1. These reagents should be prepared freshly on the day of the experiments.
2. Paraformaldehyde should be weighed and handled carefully inside a fume hood. Use a protective mask to prevent inhalation. Dissolve an appropriate amount of paraformaldehyde in 1× PBS in a 50-mL conical tube. Heat to 65 °C in a water bath and add 10 N NaOH, then 1 N NaOH drop wise until the solution becomes clear. During this step, shake the tube continuously to allow paraformaldehyde to

dissolve into solution. It may take 10–20 min. Cool to room temperature. Adjust the pH to 7.4 with 10 N NaOH or 1 N HCl and then adjust the volume with 1× PBS. Wrap the tube with Parafilm to prevent leakage and/or vapors.

3. The DMA solution can be prepared during the incubation of the cultures with PFA. First, dissolve the DMA in DMSO with a fraction of the total PBS volume. Once in solution, transfer to a 50-mL conical tube with the remaining PBS volume and mix well.
4. In this chapter, we show ChIP of Rad52 fused to 12 tandem copies of the Pk epitope as an example (Fig. 1). We commonly use monoclonal anti-V5 (Pk) antibody (Sigma, V8012) to precipitate Rad52-Pk. However, antibodies against other epitope tags can also be used when those tags are introduced into the protein of interest. We prefer tag antibodies because they are often clean and commercially available (*see Note 9*). However, antibodies directly against the protein of interest are also useful if they efficiently precipitate the protein. As a control, it is also recommended to perform ChIP with IgG from the same animal source of the primary antibody. IgG is used to monitor background precipitation of DNA (*see Note 9*).
5. We use a Misonix Sonicator 3000 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 are all the same. This allows us to process each sample with equal sonication strength.
6. Example PCR primer mixes for control and target regions analyzed in Fig. 1 are: Gene-free region [40] (GFR, control primer mix) GFR-F: 5'- GCA TCG TTT TTC GCA CAA TA -3' and GFR-R: 5'- CAT GGC ATG GCA TTT TGT TA-3'; *rec8* (target primer mix), *rec8*-F: 5'- TTC GAG CTT TGT TTA CGA GGA-3' and *rec8*-R: 5'- ATT GCA ATG GAA CGG GTA AC-3'; tRNA^{ser-met} (target primer mix) are tRNA^{ser-met}-F: 5'- AGG TTC AAA TCC TGC TGG TG-3' and tRNA^{ser-met}-R: 5'- TCG CAG TAG TTG CAA TGT CG-3'.
7. Primers should be designed to amplify chromosome regions of interest. For SYBR-green based quantitative PCR, recommended amplicon sizes are between 80 and 200 bp.
8. Starting OD₆₀₀ should be below 0.1. Prepare the pre-cultures early in the morning and let them incubate throughout the day (~8 h). If cells grow at a normal speed, the pre-cultures should be ready in the afternoon.
9. When using antibodies against epitope tags and cells expressing a tagged protein, it is important to grow cells that do not express the tagged protein. These cells are used as a control to monitor background precipitation of DNA associated with antibodies against tags. When using antibodies directly against the protein of interest, we normally use IgG for ChIP analysis as a control to monitor background DNA precipitation (*see Note 4*).

10. It is important to use mid-log phase cells for efficient synchronization. *S. pombe* cells arrested at the G2-M boundary show an elongated phenotype without septum (see 0 min of septation indices in Fig. 2c).
11. When synchronizing cells, it is preferable to skip the incubation at 18 °C described in Subheading 3.2, **step 1** and move directly to Subheading 3.2, **step 2**.
12. To ensure that the temperature in the shaker remains constant, place it in a 4 °C cold room. Incubation at 18 °C is done to slow down the speed of metabolic processes in the cell. This step can enhance the binding of proteins that have fluctuating interactions with the DNA. Depending on the protein of interest, incubation times can range from 5 min up to 2 h.
13. Before transferring the Dynabeads from the original container to the 1.5 mL tube, make sure to resuspend them thoroughly by pipetting up and down.
14. It is not necessary to resuspend the cell pellet.
15. Using lint-free tissue, remove beads stuck at the tube opening as they may prevent the tube from closing properly and cause leaking in the following steps. Make sure the tubes are closed tightly.
16. It is important to monitor cell disruption under the microscope. More than 90 % of cells should be disrupted.
17. Add ice to the water in the cup horn. However, ice needs to be removed from the cup during the sonication process. Add and remove ice regularly to keep the water cold during the process.
18. This condition allows us to shear DNA into 500- to 700-base pair fragments. The condition should be optimized in individual laboratories by extracting and monitoring DNA fragment lengths after sonication.
19. We use centrifugation to remove all remaining cellular debris. This is not possible when using the DynaMag.
20. It can also be done using a heat block or in a water bath with no shaking.
21. Chelex 100 resin interferes with PCR reactions. Therefore, it is important to avoid transferring Chelex 100 to the new tube.
22. We include two primer sets in one single PCR reaction to evaluate the enrichment of proteins at a site of interest relative to a control region. For example, for Rad52-12Pk studies, we use control primers spanning a gene-free region (GFR) [40] that display no potential obstacles or fragile sites (Gadaleta and Noguchi, unpublished observation). For best results, design control and target primer sets to generate amplicons with a size difference of ~50 bp, in order to separate target and control amplicons by electrophoresis. Optimization of primer concentration is needed to obtain equal amplification of control PCR products in all samples studied.

23. It might be necessary to optimize the dilutions of ChIP and input DNA samples to obtain optimal results. In general, input DNA samples should be diluted further than ChIP DNA, in order to prevent saturation of PCR products.
24. It 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 achieve reliable semi-quantitative comparisons.
25. The volume must be enough to cover the gel. Ensure the container and the solutions are protected from light.
26. The gel is very fragile. To protect the gel from breaking, wet the scanner surface with a small amount of water before placing the gels and accommodate them carefully.
27. The amplification efficiency of PCR primer sets can be determined experimentally using a standard curve. A variety of efficiency calculators are freely available online. If PCR amplification efficiency is 100 %, use a value of 2 ($E = 2$).

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Fig. 1.

Transcription–replication collisions are known to generate genomic instability [41]. ChIP assay of Rad52-12P_k was performed at a control gene-free-region (GFR), and two target sites: *rec8* and tRNA^{ser-met} coding regions. Asynchronous wild-type cells were grown at 32 °C. ChIP assays were performed using cells cross-linked with formaldehyde and DMA. Rad52-12P_k weakly associated with the GFR region and similar results were obtained at *rec8*, a gene coding for a meiotic cohesin subunit only expressed in meiotic cells [42]. Rad52-12P_k strongly associated with the highly transcribed tRNA^{ser-met} coding region. This is consistent with the previously observed increase in mutation and recombination rates at transcriptionally active sites [43]

Fig. 2.

(a) Morphological changes and cell cycle progression of *S. pombe* are shown. The cell cycle position can be estimated by examining cell length, the number and position of nuclei, and the presence of septum. (b) Morphological changes during septation and a method to count the number of cells are shown. To obtain the septation index, the number of septated cells (*asterisk*) is divided by the total number of cells. (c) A representative result of septation index analysis is shown. An increase in septation levels coincides with the onset of S-phase