Chromatin Immunoprecipitation to Investigate Origin Association of Replication Factors in Mammalian Cells

Adam R. Leman and Eishi Noguchi

Abstract

A variety of DNA-binding proteins regulate DNA transactions including DNA replication and DNA damage response. To initiate DNA replication in S phase of the cell cycle, numerous replication proteins must be recruited to the replication origin in order to unwind and synthesize DNA. Some replication factors stay at the origin, while replisome components move with the replication fork. When the replisome encounters DNA damage or other issues during DNA replication, the replication fork stalls and accumulates single-stranded DNA that triggers the ATR-dependent replication checkpoint, in order to slow down S phase and arrest the cell cycle at the G2–M transition. It is also possible that replication forks collapse, leading to double-strand breaks that recruit various DNA damage response proteins to activate cell cycle checkpoints and DNA repair pathways. Therefore, defining the localization of DNA transaction factors during the cell cycle should provide important insights into mechanistic understanding of DNA replication and its related processes. In this chapter, we describe a chromatin immunoprecipitation method to locate replisome components at replication origins in human cells.

Key words Replication fork, Origin, Chromatin immunoprecipitation, ChIP, Replisome, Timeless, Cross-link, Fork protection complex, c-Myc origin

1 Introduction

Eukaryotic DNA replication begins at multiple replication origins, which are marked by the origin recognition complex (ORC). During late mitosis and G1 phase, Cdc6 and Cdt1 associate with origins and recruit the MCM (minichromosome maintenance proteins) DNA helicase complex, in order to assemble the pre-replication complex (pre-RC). However, to initiate DNA synthesis, additional factors are recruited at the origin for the unwinding of duplex DNA. These factors include Cdc45 and GINS, both of which associate with MCM to form the active DNA helicase known as the CMG complex. Once duplex DNA is unwound, the CMG complex moves with the replication fork together with DNA polymerases and other accessory factors, forming the replisome

complex, which is responsible for elongating DNA chains (reviewed in [1]). As DNA replication proceeds, the replisome complex may encounter a variety of obstacles including damaged template DNA, DNA secondary structures, and DNA binding proteins. Under these conditions, the replisome stalls, leading to accumulation of single-stranded DNA (ssDNA) at the replication forks and activation of the ATR-dependent replication checkpoint (reviewed in [2]). It is also possible that the replication fork collapses, generating DNA double-strand breaks (DSBs), which then induce a signal transduction cascade to activate the ATM-dependent DNA damage checkpoint. To properly activate these checkpoint pathways, which are essential for preservation of genomic integrity, numerous checkpoint proteins are recruited to stalled replication forks or at DSBs. These proteins sense the damage and send the checkpoint signal to arrest the cell cycle and coordinate with DNA repair pathways (reviewed in [3]). Therefore, to mechanistically understand DNA replication and DNA damage response mechanisms, it is important to investigate cell-cycle dependent localization of DNA transacting factors at specific sites of chromosomes. In this chapter, we describe a chromatin immunoprecipitation method to analyze origin association of replisome components during the cell cycle. Similar methods can also be used to investigate the localization of various DNA-binding factors, including damage response proteins and transcription factors, at specific chromosome loci.

2 Materials

2.1 Synchronization of HeLa Cells by a Double-Thymidine Block

1. HeLa cells cultured in growth medium in a 5 % CO₂ atmosphere at 37 °C.
2. Growth medium for HeLa cells: RPMI 1640 medium supplemented with 10 % calf serum, 10 mM HEPES–KOH pH 7.5, 100 units/mL penicillin, 100 µg/mL streptomycin.
3. 15-cm cell culture dishes.
4. 100 mM thymidine: Filter-sterilize with a 0.22 µm pore size membrane and store at –20 °C.
5. 1× PBS: 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4.

2.2 Cross-linking Cells

1. 37 % formaldehyde.
2. 2.5 M glycine.
3. 1× PBS.
4. 15-mL conical tubes.

2.3 Cell Extract Preparation

1. ChIP lysis buffer: 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 % NP-40, 0.1 % Triton X-100, 0.2 mM *p*-APMSF (4-amidinobenzylsulfonyl fluoride hydrochloride),

Roche Complete EDTA-free protease inhibitor cocktail. Prepare fresh and keep on ice.

2. 1.5-mL microcentrifuge tubes.
3. Misonix sonicator 3000 (Misonix).
4. 100-mL glass beaker.
5. Floating tube rack that fits in a 100-mL glass beaker (*see Note 1*).
6. Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad).

2.4 Immuno-precipitation

1. Protein A-Sepharose beads.
2. Anti-Timeless antibody or antibodies against other replisome components (*see Note 2*).

2.5 DNA Extraction

1. Chelex 100 resin (Bio-Rad).
2. 10 mg/mL proteinase K.

2.6 SYBR Green-Based Real-Time PCR Analysis

1. iQ SYBR Green Supermix (uses hot-start iTaq DNA polymerase, Bio-Rad, 170-8880).
2. 10 μ M forward primer (*see Note 3*).
3. 10 μ M reverse primer (*see Note 3*).

3 Methods

PCNA associates with chromatin and moves with the replication fork during S phase [4]. Therefore, S-phase cells can be identified as PCNA-positive cells in immunofluorescence microscopy. We also showed that the Timeless protein co-localizes with PCNA in S-phase cells, suggesting that Timeless is associated with replication forks [5]. Fork association of the protein can also be confirmed by examining physical association of the protein with a known replication fork component by immunoprecipitation. Indeed, PCNA was co-precipitated with Timeless using anti-Timeless antibodies [6]. However, these methods do not demonstrate the dynamics of protein movement along the chromosome during DNA replication. Accordingly, chromatin immunoprecipitation (ChIP) has been widely used to investigate direct association of replication factors with specific chromosome loci. Because cells are directly treated with formaldehyde to cross-link proteins onto DNA in a ChIP experiment, *in vivo* protein–chromatin association is preserved.

In this method, we describe a simple ChIP method to investigate the association of Timeless with a well-characterized replication origin at the *MYC* gene (c-Myc) region in human cells [5]. This method utilizes Chelex 100 resin, allowing for efficient ChIP experiments whereby a high reproducibility can be achieved [7]. We use HeLa cells synchronized at the beginning of S phase. Cells are treated with formaldehyde and released into the cell cycle.

DNA fragments precipitated with the anti-Timeless antibody are analyzed by quantitative PCR to monitor cell cycle-dependent association of Timeless with the MYC origin. The method described in this chapter is a typical ChIP assay that can be easily modified for detecting other chromatin-binding factors, such as replication and DNA damage response proteins.

3.1 Preparation and Synchronization of HeLa Cells by a Double-Thymidine Block and Release

1. Plate 4×10^6 cells in a 15-cm culture dish containing 20 mL growth medium (*see Note 4*). Multiple dishes are required depending on a time course schedule. For example, six culture dishes are required for 0-, 1-, 2-, 3-, 6-, and 9-h time points after the release from cell cycle arrest. Grow the cells overnight (16–24 h) at 37 °C to allow the cells to attach the dish surface.
2. Add 400 μ L of 100 mM thymidine to the culture dish (final concentration is 2 mM), mix gently, and incubate the cells for 16–19 h.
3. Aspirate the medium, add 10 mL of 1 $\times$ PBS, and gently shake the dish to wash the cells. Repeat this washing step once more with 10 mL of 1 $\times$ PBS and once with 10 mL of fresh growth medium.
4. Aspirate the medium, add 20 mL of fresh growth medium to the dish, and grow the cells at 37 °C for 9 h.
5. Add 400 μ L of 100 mM thymidine to the culture dish (final concentration is 2 mM), mix gently, and incubate the cells for 16 h.
6. Wash the cells as described in **step 3**.
7. Aspirate the medium, add 20 mL of fresh growth medium to the dish, and grow the cells for desired length of time (*see Note 5*) until cell fixation described in Subheading **3.2**.

3.2 Cross-linking Cells

1. To fix the cells (1×10^7 cells), add 0.8 mL of 37 % formaldehyde (final concentration is 1.42 %) directly to the culture dish, mix the medium by gently shaking the dish, and incubate the cells for 15 min at room temperature.
2. To quench formaldehyde, add 1.1 mL of 2.5 M glycine (final concentration is 125 mM) directly to the dish, mix the medium by gently shaking the dish, and incubate the cells for 5 min at room temperature.
3. Aspirate the medium, add 10 mL of 1 $\times$ PBS, and gently shake the dish to wash the cells. Repeat this washing step once more.
4. Aspirate the buffer, add 1 mL of 1 $\times$ PBS, scrape the cells off the dish by a cell scraper, and transfer cell suspension to a 15-mL conical tube.
5. Centrifuge the cells at $800 \times g$ for 5 min at 4 °C.
6. Aspirate the buffer and immediately freeze the cell pellet at –80 °C (*see Note 6*).

3.3 Preparation of Cell Extract

1. Thaw cell pellets on ice, suspend the cells in 1 mL of ice-cold ChIP lysis buffer, and transfer the cell suspension to a 1.5-mL microcentrifuge tube.
2. Incubate the cell suspension on ice for 10 min.
3. Place the sample tube in a special float (*see Note 1*) that fits into a 100-mL glass beaker filled with ice water.
4. Sonicate the sample using a microtip: output 5, ON time of 1 s, OFF time of 5 s. The total ON time for each step is 15 s (15 cycles of 1 s ON, 5 s OFF).
5. Place the sample tube on a separate icebox, and perform **step 4** for all other samples (*see Note 7*).
6. Repeat **steps 4** and **5** for five times (*see Note 8*).
7. Centrifuge samples at the maximum speed in a microfuge at 4 °C.
8. Transfer the supernatant (cell extract) to a new 1.5-mL microcentrifuge tube and keep on ice.
9. Measure protein concentration using Bio-Rad Protein Assay Dye Reagent Concentrate.
10. Adjust protein concentration by diluting samples by ice-cold ChIP lysis buffer to the protein concentration of the lowest concentrated sample.
11. Transfer 10 µL of each extract to a new tube to use for the input control (*see Subheading 3.4*).
12. Perform immunoprecipitation with the rest of the cell extracts.

3.4 Immuno-precipitation

1. Prior to immunoprecipitation, prewash protein A-Sepharose beads (protein A beads) as follows: Use 50 µL (50 % slurry, 25 µL bead volume) of protein A beads for each sample. Transfer the required amount of beads to a 1.5-mL microcentrifuge tube, centrifuge briefly at 7,000 rpm in a microfuge, aspirate supernatant, and resuspend beads in 1 mL of ice-cold ChIP lysis buffer, in order to wash beads. Repeat this washing procedure two more times, and resuspend beads in ice-cold ChIP lysis buffer to obtain 50 % slurry of protein A beads. Keep on ice until needed (*see Note 9*).
2. Separate the cell extract obtained in Subheading 3.3 into two separate microcentrifuge tubes (450 µL each).
3. Add 7 µg of the anti-Timeless antibody to one tube and 7 µg of rabbit IgG to the other tube. Rotate samples overnight at 4 °C.
4. Add 50 µL of the pre-washed protein A beads to the samples. Rotate samples for 2 h at 4 °C.
5. To wash protein A beads, centrifuge briefly at 7,000 rpm in a microfuge, aspirate supernatant, and add 1 mL of ice-cold ChIP lysis buffer. Repeat this washing procedure five times, and aspirate supernatant (*see Note 10*).

3.5 DNA Extraction

1. Add 100 μL of Chelex 100 resin (10 % slurry in sterile H_2O) to the washed beads (*see* Subheading 3.4, **step 5**) and the input samples (*see* Subheading 3.3, **step 11**), mix well by vortex, and boil the samples for 10 min, in order to reverse cross-link and to extract DNA.
2. Cool samples to room temperature, add 2 μL of 10 mg/mL proteinase K, and incubate the samples at 55 $^{\circ}\text{C}$ for 30 min. Mix samples occasionally.
3. Boil the samples for 10 min to inactivate proteinase K.
4. Centrifuge the samples at the maximum speed in a microfuge at 4 $^{\circ}\text{C}$ for 1 min.
5. Carefully transfer 80 μL of supernatant (extracted DNA) to a new microcentrifuge tube (*see* **Note 11**).
6. Add 120 μL of dd H_2O to the beads, mix by vortexing.
7. Centrifuge the samples at maximum speed in a microfuge at 4 $^{\circ}\text{C}$ for 1 min.
8. Carefully transfer 120 μL of supernatant (extracted DNA) to the 80 μL previously collected (total 200 μL). Store the extracted DNA at -20°C until needed for PCR (*see* **Note 11**).

3.6 PCR Using SYBR Green-Based Real-Time PCR Analysis

DNA immunoprecipitated by this protocol can be amplified and quantified using various PCR-based quantification methods. Below, we describe a simple quantification using the SYBR green-based real-time PCR. In our assay, we use primers that are designed to amplify the MYC replication origin region. This method can be applied to other chromosomal loci, in order to measure the quantity of DNA precipitated with antibodies. An example of this analysis is shown in our previous studies [5].

1. Mix the following PCR reagents for each reaction (*see* **Note 12**):
 - (a) 3 μL of template DNA (input control or ChIPed DNA).
 - (b) 5 μL of iQ SYBR Green Supermix.
 - (c) 0.3 μL of Forward Primer (10 μM).
 - (d) 0.3 μL of Reverse Primer (10 μM).
 - (e) 1.4 μL of H_2O .
2. Perform PCR using the following cycles (*see* **Note 13**):
 - (a) Denaturation of DNA and hot-start activation of iTaq DNA polymerase: 95 $^{\circ}\text{C}$, 2 min.
 - (b) Denaturation: 95 $^{\circ}\text{C}$, 10 s.
 - (c) Annealing: 55–60 $^{\circ}$, 30 s.
 - (d) Extension: 72 $^{\circ}\text{C}$, 30 s (perform SYBR Green detection here).
 - (e) Go to (b)–(d), repeat for 39 additional cycles.

- (f) To degrade the polymerase enzyme prior to melt curve analysis: 72°, 7 min.
- (g) (Optional) Melting curve: 55–95 °C, (SYBR Green signal detection ever 0.5 °C) (*see* **Note 14**).
3. Obtain cycle threshold (Ct) values for each sample, IgG control, and input (*see* **Notes 15** and **16**).
4. After the Ct values have been ascertained, determine the relative enrichment of your protein of interest at a locus using the following equations:
 - (a) Protein occupancy = $2^{2.2 \% \text{ Input Ct} - \text{Timeless ChIP Ct}}$.
 - (b) Background correction = $2^{2.2 \% \text{ Input Ct} - \text{IgG ChIP Ct}}$.
 - (c) Background corrected occupancy = Protein occupancy – Background correction.
 - (d) The input sample must be corrected for the lower volume compared to the sample, in this case 2.2 % (10 µL of input/450 µL for the IP). Thus, Final Occupancy = (Background Corrected Occupancy)/45.
 - (e) To convert this number to the percentage of immunoprecipitated DNA from the total sample, multiple the Final Occupancy by 100 to obtain a percentage value.

4 Notes

1. For a floating rack, we use a thin Styrofoam plate cut into a circle to fit into a beaker. One hole is cut into the middle of the rack to hold 1.5-mL microfuge tubes above the ice water.
2. In this protocol, we use the anti-Timeless antibodies described in our previous studies [5, 8]. However, anti-Timeless antibodies are readily available from commercial sources. Also, antibodies specific to proteins of interest can be used to monitor their chromatin association.
3. Primers should be designed to amplify chromosome regions of interest. For SYBR-green based real-time PCR, the ideal amplicon size is between 80 and 200 bp, and amplicons over 300 bp are not recommended. To amplify the *MYC* (c-Myc) origin region, we use MYC11-F (5'-TAT CTA CAC TAA CAT CCC ACG CTC TG-3') and MYC11-R (5'-CAT CCT TGT CCT GTG AGT ATA AAT CAT CG-3') primers as described [5, 9]. Other defined origins we have used include *HBB* (β-globin), *LB2* (lamin B2) and *MCM4* (Mcm4) loci [9–11]. As a non-origin control, we use GACT-F (5'-GCT GTT CCA GGC TCT GTT CC-3') and GACT-R (5'-ATG CTC ACA CGC CAC AAC ATG C-3') primers to amplify the *ACTG1* (γ-actin) locus [11].

4. HeLa cells should be plated at approximately 25 % confluency considering that cells will be incubated further. This prevents cells from becoming confluent at the time of cell collection. Plating cell numbers need to be adjusted for each cell type.
5. We often take time points at 0, 1, 2, 3, 6, and 9 h after the release.
6. Use liquid nitrogen or dry ice–ethanol to snap-freeze cell pellets.
7. Continuous sonication of the same sample causes sample heating. To prevent this problem, sonication is performed at intervals.
8. This condition allows us to shear DNA into 500- to 700-bp fragments using our equipment. However, the sonication conditions should be optimized in individual laboratories by extracting and monitoring cross-linked DNA after sonication.
9. Prepare 20–30 % more pre-washed beads than needed.
10. Use caution not to aspirate any beads, as these contain the immunoprecipitated DNA.
11. At this point, a small volume of liquid will remain over the beads. This is normal, if Chelex 100 resin is transferred over to the new tube (for the final DNA sample), it will interfere with downstream amplification steps.
12. For consistent results, generate a master mix of all components minus the template DNA. As described in **Note 3**, to amplify the *MYC* origin, use MYC11-F and MYC11-R primers. As a control, use GACT-F and GACT-R primers to amplify the γ -actin gene region. Prepare excess master mix to ensure equal volumes in all PCR reactions.
13. Annealing temperature needs to be adjusted depending on the melting temperatures of the primers. For PCR conditions, see iQ SYBR Green Supermix supplier's instructions.
14. By running a melting curve analysis, one can determine the melting temperature of the PCR fragment generated during the PCR reaction. The melting temperature can be determined by the inflection point of the melting curve. It is important that only one inflection point is detected during melting curve analysis, indicating that only PCR product has been generated.
15. The method depends upon the software used and user preferences. For an accurate measurement of DNA precipitated by antibodies, it is vital to use a consistent method for Ct determination between the sample, IgG control, and input.
16. The efficiency of the PCR primer set can be determined experimentally using a standard curve. A variety of efficiency calculators are freely available online to determine the efficiency of the primer set. Using a value of 2 for the efficiency assumes a primer amplification efficiency of 100 %.

Acknowledgements

This work was supported in part by NIH grants (AG035480 to A.R.L. and GM0776043 to E.N.). We would like to thank Chiaki Noguchi for technical support and troubleshooting during protocol development.

References

1. Masai H, Matsumoto S, You Z, Yoshizawa-Sugata N, Oda M (2010) Eukaryotic chromosome DNA replication: where, when, and how? *Annu Rev Biochem* 79:89–130
2. Leman AR, Noguchi E (2013) The replication fork: understanding the eukaryotic replication machinery and the challenges to genome duplication. *Genes* 4(1):1–32
3. Hustedt N, Gasser SM, Shimada K (2013) Replication checkpoint: tuning and coordination of replication forks in S phase. *Genes* 4(3):388–434
4. Moldovan GL, Pfander B, Jentsch S (2007) PCNA, the maestro of the replication fork. *Cell* 129(4):665–679
5. Leman AR, Noguchi C, Lee CY, Noguchi E (2010) Human timeless and tipin stabilize replication forks and facilitate sister-chromatid cohesion. *J Cell Sci* 123(Pt 5):660–670
6. Yang XH, Shiotani B, Classon M, Zou L (2008) Chk1 and Claspin potentiate PCNA ubiquitination. *Genes Dev* 22(9):1147–1152
7. Nelson JD, Denisenko O, Sova P, Bomsztyk K (2006) Fast chromatin immunoprecipitation assay. *Nucleic Acids Res* 34(1):e2
8. Leman AR, Dheekollu J, Deng Z, Lee SW, Das MM, Lieberman PM, Noguchi E (2012) Timeless preserves telomere length by promoting efficient DNA replication through human telomeres. *Cell Cycle* 11(12):2337–2347
9. Sibani S, Price GB, Zannis-Hadjopoulos M (2005) Decreased origin usage and initiation of DNA replication in haploinsufficient HCT116 Ku80+/- cells. *J Cell Sci* 118(Pt 15):3247–3261
10. Schaarschmidt D, Ladenburger EM, Keller C, Knippers R (2002) Human Mcm proteins at a replication origin during the G1 to S phase transition. *Nucleic Acids Res* 30(19):4176–4185
11. Tan BC, Chien CT, Hirose S, Lee SC (2006) Functional cooperation between FACT and MCM helicase facilitates initiation of chromatin DNA replication. *EMBO J* 25(17):3975–3985