Chapter 9

ChIP-on-Chip Analysis of DNA Topoisomerases

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Abstract

Here we describe an adapted ChIP-on-chip protocol for the analysis of DNA topoisomerase chromosomal binding in *Saccharomyces cerevisiae* cells. The ChIP-on-chip technique is based on the immunoprecipitation of crosslinked chromatin (ChIP, chromatin immunoprecipitation), followed by DNA amplification and hybridization to high-density oligonucleotide arrays (Chip). Comparison of the signal intensities of immunoprecipitated and control fractions provides a measurement of the protein–DNA association along entire genomes. ChIP-on-chip analysis of DNA topoisomerase binding to chromosomal DNA opens a window to the understanding of the in vivo contribution of these enzymes to the different DNA transactions taking place concomitantly within the context of the highly organized eukaryotic genome. Chromosomal binding profiles obtained from synchronized cells allow scoring the temporal and spatial restriction of these enzymes at different cell cycle stages. By using this approach, novel aspects of DNA topoisomerase function in chromosome metabolism might be unmasked.

Key words: Top1, Top2, chromatin immunoprecipitation, high-density oligonucleotide arrays, *Saccharomyces cerevisiae*.

1. Introduction

In the last decades, in vitro studies on topologically constrained circular DNA molecules have contributed to the characterization of the ability of DNA topoisomerases to relax torsional stress and to promote catenation/decatenation reactions. Structural studies revealed the fundamental mechanistic aspects through which these enzymes interact with DNA molecules and catalyze the breakage/religation steps that change their linkage status. Nevertheless, the way these topological transactions take place in vivo, within the context of the eukaryotic nucleus is largely unknown.

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Eukaryotic chromosomal DNA exhibits a complex architectural organization. DNA is spatially organized and associated with fixed nuclear structures, such as the nuclear matrix and the chromosome scaffold. The generation and distribution of topological changes, and DNA topoisomerase function, in vivo is presumably subject to the constraints imposed by higher-order chromatin structure and chromosomal architecture. Hence, cells must coordinate the resolution of the topological distortions generated by the different DNA metabolic transactions that occur concomitantly within eukaryotic genomes (e.g., the advancement of replication and transcription machineries). The ChIP-on-chip methodology enables the genomewide analysis of DNA topoisomerase-mediated topological transactions within eukaryotic chromosomes.

The ChIP-on-chip technique exploits the DNA obtained by chromatin immunoprecipitation (ChIP) and PCR amplification as a probe for hybridization to DNA chips. The usage of high-resolution tilling arrays (oligonucleotide DNA chips; i.e., Affymetrix *Saccharomyces cerevisiae* chromosome VI tilling chip array) allows the detection of protein binding to chromosomal DNA at a resolution of 300 bp. Protein–DNA complexes are first crosslinked by formaldehyde treatment (**Fig. 9.1A**). Chromatin is then sheared by sonication to obtain suitable protein-bound DNA fragments, and immunoprecipitation is carried out with specific antibodies. For the ChIP-on-chip analysis in yeast cells, epitope-tagged proteins expressed under their endogenous promoters can be used for immunoprecipitation with highly specific commercial antibodies. Epitopes suitable for ChIP analysis are listed in **Table 9.1**. In this way, two fractions are separated: an IP fraction, enriched in the protein of interest, and a SUP fraction, containing non-immunoprecipitated DNA that is used as a hybridization control. After reversal of the formaldehyde crosslink (**Fig. 9.1B**), samples are treated with proteinase K and RNase. DNA fractions are then evenly amplified by tagged-random primer PCR, DNAse digested, and labeled with biotin. Enriched and non-enriched DNA pools are probed to independent chips and, after staining, washing, and scanning, signal intensities of each locus from IP and SUP-hybridized arrays are compared using an expression analysis protocol and the logarithmic ratio of the loci enrichment between IP and SUP fractions is calculated (**Fig. 9.2**). The statistical significance of the observed differences is based on three criteria (1, 2): (i) the reliability of the signal intensity at each locus, evaluated by the detection of p-value, (ii) the reliability of the IP/SUP detection ratio, judged by the change in p-value, and (iii) when the binding of a protein to a discrete chromosomal site results in the immunoprecipitation of DNA fragments containing that site and neighboring regions, only such clusters containing three or more loci fulfilling the previous two criteria are considered. Integration of the logarithmic expression of the IP/SUP signal ratio and the

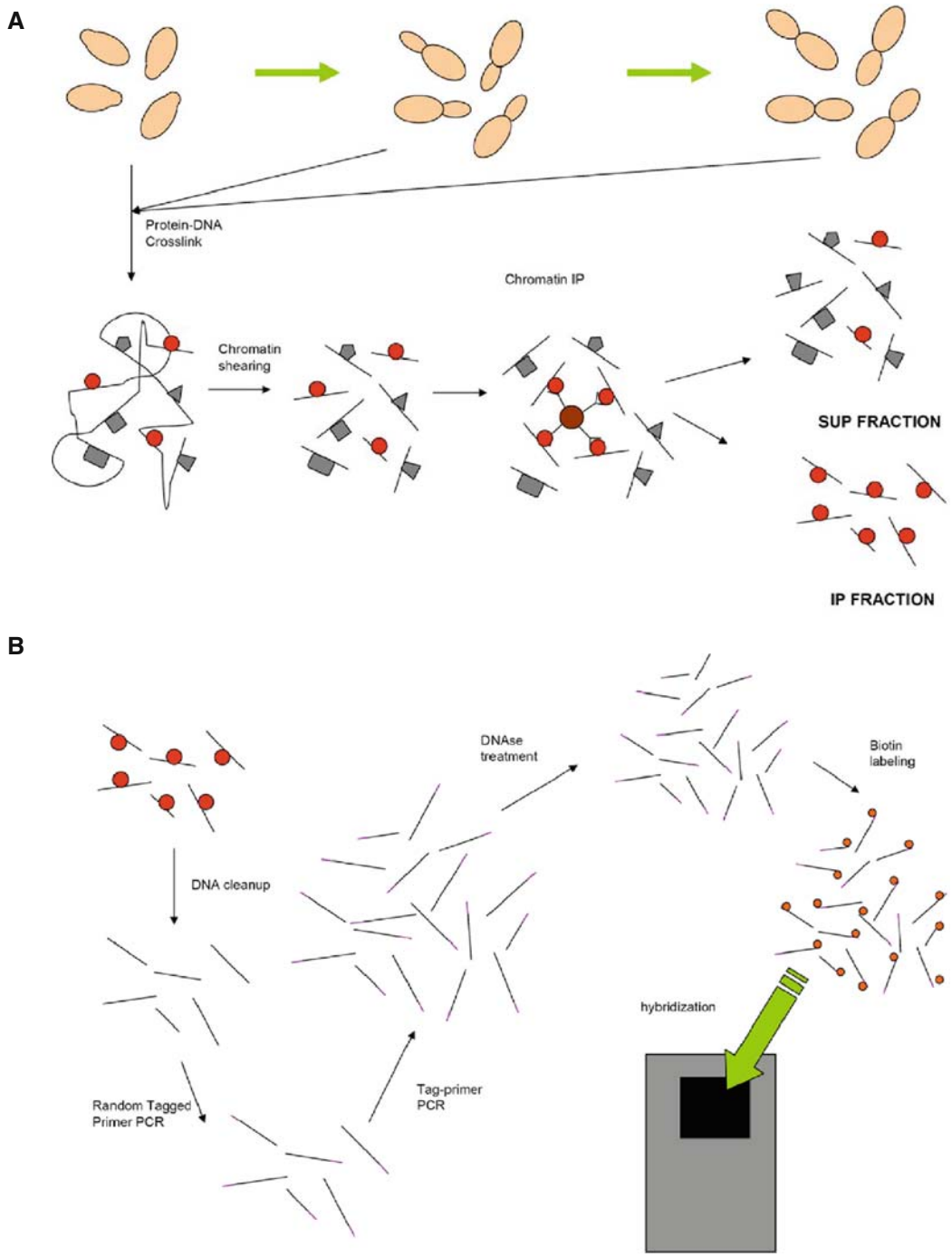

Fig. 9.1. **ChIP-on-chip procedure (I)**. Schematic representation of the Chip-on-chip technique: chromatin crosslink and immunoprecipitation (**A**); purification, amplification, and labeling of the immunoprecipitated and control DNA fractions (**B**) (see text for the details).

Table 9.1
Epitopes available for ChIP-on-chip analysis on budding yeast

TAG	Epitope	Working antibody for ChIP	Antibody_Host
HA	YPYDVPDYA	16B12(BAbCO HA11)	Mouse IgG1
FLAG	DYKDDDDK	M2(SIGMA F3165)	Mouse IgG1
PK	GKPIPNPLLGLD	SV5-Pk1(Serotec MCA1360)	Mouse IgG2a
Myc	EQKLISEEDL	PL14(MBL M047-3)	Mouse IgG1

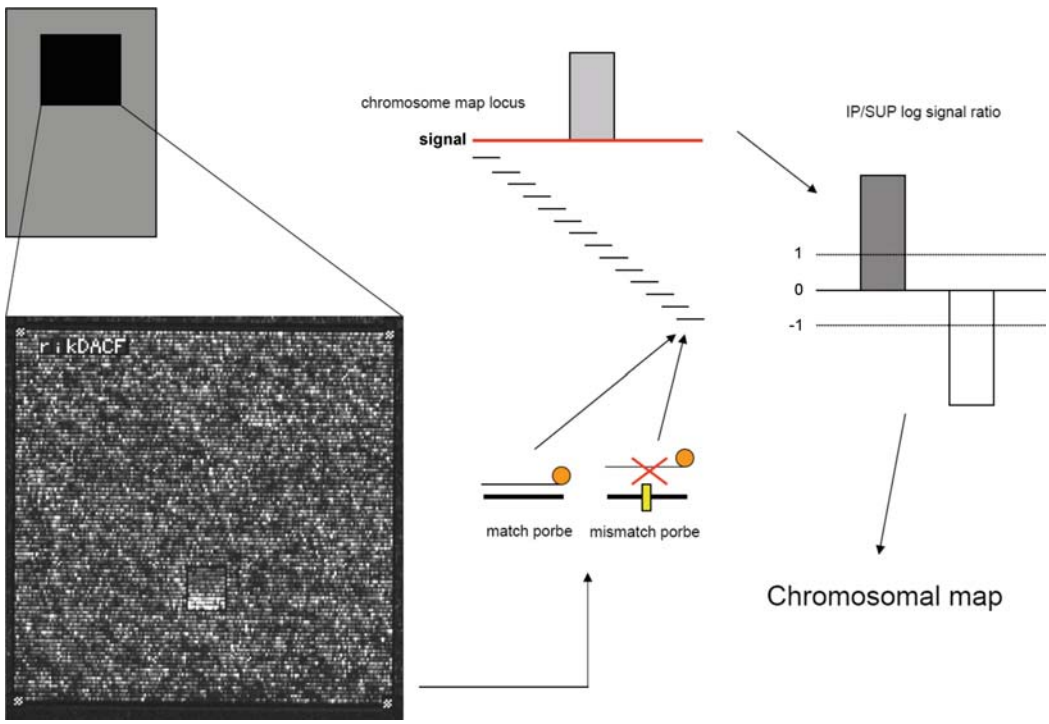

Fig. 9.2. **ChIP-on-chip procedure (II)**. Schematic representation of the statistical algorithm used to calculate the immunoprecipitated versus control signal logarithmic ratio for each chromosomal locus (see text for details).

annotated sequence of *S. cerevisiae* genome (http://www.yeastgenome.org/) allows the construction of maps displaying the binding pattern of the protein of interest along entire chromosomes.

A typical chromosomal binding profile observed for yeast topoisomerase I (Top1) and yeast topoisomerase II (Top2) in S-phase arrested cells is shown in **Fig. 9.3**. Consistent with its role in resolving torsional stress ahead of replication forks and physical association with replisome components, Top1 clusters are detected at regions containing or neighboring active replication

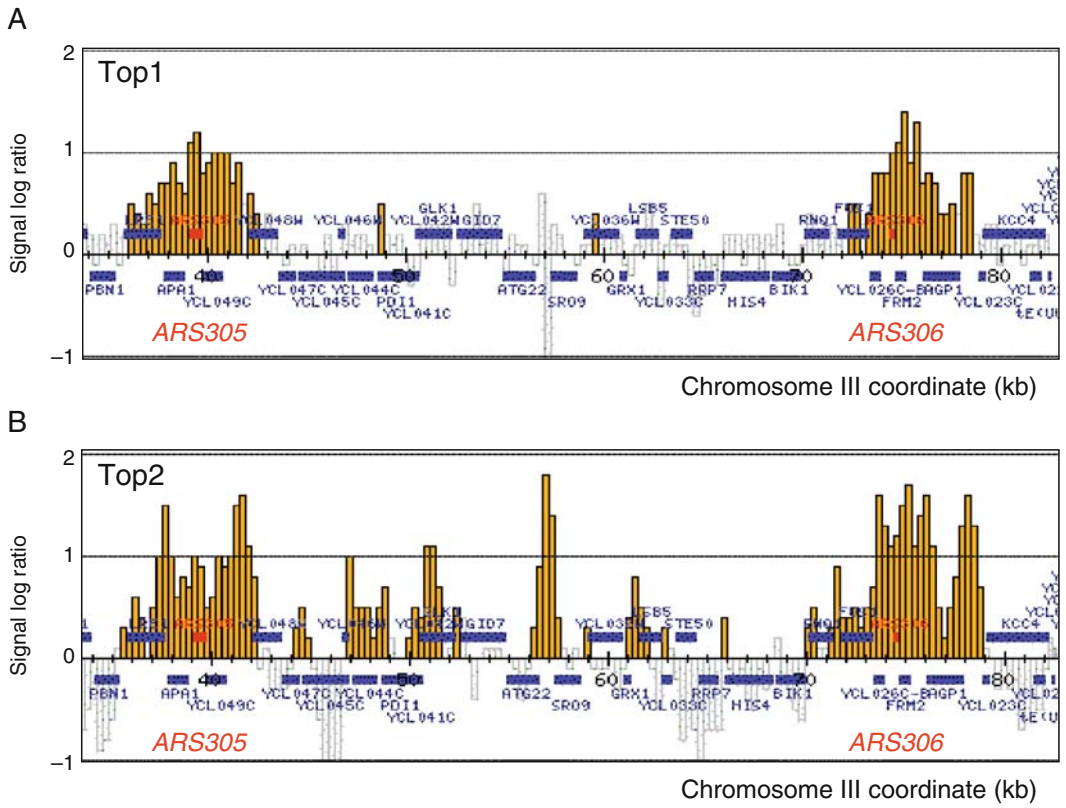

Fig. 9.3. **Chromosomal maps showing Top1 and Top2 distribution in S-phase**. Distribution of Top1 (**A**) and Top2 (**B**) on chromosomal DNA in S-phase arrested cells. The histogram bars in the *Y*-axis show the average signal ratio of loci significantly enriched in the immunoprecipitated fraction along a ~50 kb fragment of chromosome III containing *ARS305* and *ARS306* replication origins in $\log_2$ scale. The *horizontal bars* represent open reading frames (ORFs).

origins (**Fig. 9.3A**). Mutations in the ARS consensus sequence (ACS), that preclude origin firing, abolish the detection of Top1 clusters (4).

The Top2 protein is also enriched at regions containing or neighboring early replication origins on chromosomes (**Fig. 9.3B**). Top2 binding is also abolished when inactivating mutations are introduced in the ACS (4), indicating that Top2 acts to sustain DNA replication, most likely contributing to torsional stress removal and/or sister chromatid decatenation at replication forks. Top2 also clusters at specific intergenic regions in a fashion independent of origin activation (4). The Top2 intergenic clusters likely reflect the participation of this enzyme in transcription-dependent topological rearrangements at gene promoters during S-phase.

ChIP-on-chip represents a highly valuable technique for the in vivo analysis of topoisomerase interaction with chromosomal DNA in the nuclear context. In the future, the use of this genomic approach may contribute to the elucidation of the role played by these enzymes in the complex DNA transactions essential for

gene expression, genome duplication, and maintenance of genome integrity. Furthermore, considering that topoisomerases represent the molecular target of several drugs, this approach can also be used to characterize the action of topoisomerase inhibitors.

2. Materials

2.1. Preparation of Magnetic Beads

1. Magnetic beads: Protein A Dynabeads (Dynal, Lake Success).
2. 1.7 ml prelubricated Costar tubes (Corning, NY).
3. Magnetic grid (Dynal MPC–S, Dynal, Lake Success, NY).
4. PBS: 137 mM NaCl, 10 mM phosphate buffer, pH 7.4, 2.7 mM KCl.
5. PBS/BSA: 1X phosphate-buffered saline containing 5 mg/ml bovine serum albumin.
6. Anti-Flag antibody: M2 anti-flag antibody (Sigma).

2.2. Preparation of Chromatin Extracts and Immunoprecipitation

1. *TOP2-FLAG* yeast: *S. cerevisiae* strain expressing *FLAG*-tagged *TOP2* from the endogenous yeast *TOP2* promoter (available upon request).
2. Anti-Flag antibody: Mouse monoclonal antibody that is highly specific for the Flag epitope (*see* **Table 9.1**).
3. Formaldehyde solution: 37% formaldehyde.
4. TBS: 20 mM Tris–HCl, pH 7.5, 150 mM NaCl.
5. Lysis buffer: 50 mM Hepes–KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton-X100, 0.1% Na-deoxycholate.
6. PMSF stock solution: 100 mM phenylmethanesulfonyl fluoride.
7. Antiproteolytic cocktail: Complete protease inhibitor tablets (Roche, Indianapolis, IN).
8. 2-ml O-ring screw-cap tubes.
9. Acid-washed glass beads: 425–600 μm (Sigma).
10. Cell disruptor: Multibeads shocker® (Yasui-kikai, Osaka, Japan) or similar device.
11. Branson Sonifier 2508 (Danbury, CT).
12. 1.7 ml prelubricated Costar tubes.
13. 1.5 ml microcentrifuge tubes.
14. 2X Laemmli buffer: 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris–HCl, pH 6.8.

2.3. Washing the Beads and Crosslink Reversal

1. 1.5 ml microcentrifuge tubes.
2. 2X Laemmli buffer: 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris–HCl, pH 6.8.
3. Magnetic grid (Dynal MPC–S, Dynal, Lake Success, NY).
4. Wash buffer: 10 mM Tris–HCl, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA.
5. Elution buffer: 50 mM Tris–HCl, pH 8, 10 mM EDTA, 1% SDS.
6. TE: 10 mM Tris–HCl, pH 8, 1 mM EDTA.
7. TE/1% SDS: TE with 1% SDS.

2.4. DNA Purification

1. Proteinase K buffer: 895 µl of TE, 30 µl of 10 mg/ml glycogen (Roche), and 75 µl of 50 mg/ml Proteinase K (Roche).
2. 5 M NaCl stock solution.
3. Phenol/chloroform/isoamylalcohol (Sigma).
4. 100% ice-cold ethanol.
5. 70% ice-cold ethanol.
6. RNase A (Sigma): DNAse-free RNase A powder. Store at –20°C.
7. Qiagen PCR purification kit (Qiagen, Valencia, CA).
8. 3 M sodium acetate.
9. 80% ice-cold ethanol.
10. Autoclaved MilliQ water (ddH_2O).

2.5. DNA Amplification

1. Sequenase Ver2.0 T7 DNA polymerase (USB).
2. Primer A: 5′-GTT TCC CAG TCA CGA TCN NNN NNN NN-3′.
3. Primer B: 5′-GTT TCC CAG TCA CGA TC-3′.
4. 0.1 M DTT stock solution.
5. 3 mM dNTPs stock solution.
6. Microcon centrifugal filter unit (YM30) cartridges (Millipore Corporation, Bedford, MA, USA).
7. KOD-Dash: DNA polymerase with proof-reading and high processivity (TOYOBO, Osaka, Japan).
8. WGA2 GenomePlex Complete Genome Amplification (WGA) Kit (Sigma).

2.6. DNAse Digestion

1. DNAseI: Amplification Grade (Gibco BRL, Grand Island, NY).
2. 10X One-Phor-All-Buffer plus (Pharmacia, Piscataway, NJ).
3. 25 mM $CoCl_2$.

2.7. DNA Labeling

1. Terminal transferase (Roche, Indianapolis, IN).
2. Biotin-11-ddATP (NEN).

2.8. Hybridization

1. rikDACF, chromosome VI tiling array (Affymetrix, CA).
2. GeneChip hybrid oven 320 (Affymetrix, CA).
3. 20X eukaryotic hybridization control (Genechip).
4. Herring sperm DNA: 10 mg/ml dissolved in ddH_2O. Store at –20°C.
5. 20X SSPE: 3 M NaCl, 200 mM sodium phosphate, 20 mM EDTA, pH 7.4.
6. 0.1% Triton-X100.
7. Control OligoB2 (Affymetrix, CA).

2.9. Washing, Staining, and Scanning of the Chips

1. Acetylated BSA (Invitrogen).
2. SAPE: Streptavidin, R-phycoerythrin conjugate, premium grade (Invitrogen).
3. GeneChip fluidics station 450 (Affymetrix, CA).
4. Affymetrix GeneChip Scanner 3000 7G (Affymetrix CA).

3. Methods

Here, we describe a version of the ChIP-on-chip protocol (1) adapted for analysis of the binding pattern of endogenously expressed Flag-epitope-tagged DNA topoisomerases on *S. cerevisiae* chromosome VI (3, 4).

3.1. Preparation of the Magnetic Beads

1. Transfer 60 µl of the magnetic beads to a 1.7-ml prelubricated tube and centrifuge at 2300*g* for 1 min at 4°C.
2. Place the tube in a magnetic grid and aspirate the supernatant with a vacuum pump.
3. Wash beads twice as follows: Resuspend the magnetic beads in 0.5 ml of ice-cold PBS/BSA by removing the tube from the magnetic grid and gently shaking (*see* **Note 1**). Place the tube back in the magnetic grid, and wait until the beads attach to the magnet leaving a clear solution and aspirate the supernatant with a vacuum pump.
4. Resuspend the beads in 60 µl of PBS/BSA and add 20 µg of anti-Flag antibody. Incubate with rotation overnight (*see* **Note 2**).
5. Immediately before use, remove the antibody-containing solution, wash twice with ice-cold PBS/BSA, and resuspend in 60 µl of PBS/BSA. (Magnetic beads, 15 µl, are added to each 0.4 ml lysis buffer aliquot.)

3.2. Preparation of Chromatin Extracts and Immunoprecipitation

1. Collect 100 ml of *TOP2-FLAG* yeast culture at a concentration of 1×10^7 cells/ml and transfer into two 50-ml centrifuge tubes.
2. Add formaldehyde to a 1% final concentration (1.350 ml of a 37% solution to each tube) and incubate at room temperature for 30 min, gently shaking (*see* **Note 3**).
3. Wash the cells three times as follows: Pellet the fixed cells by centrifugation at 1850*g* for 3 min at 4°C and resuspend in 20 ml of ice-cold 1X TBS by vortexing. After the last washing step, discard the supernatant and carefully remove the remaining liquid with a vacuum pump.
4. Resuspend each cell pellet in 0.8 ml of lysis buffer (supplemented with a final concentration of 1 mM PMSF and 1X antiproteolytic cocktail, immediately before use). Transfer 0.4 ml lysis buffer aliquots into 2-ml O-ring screw-cap tubes and add glass beads up to 1 mm below the buffer's meniscus. (You now have four, 2-ml O-ring screw-cap tubes.)
5. Break the cells with a Multibeads Shocker® using the following pattern: 60 min of total time (1 min shaking/1 min pause) at 2500 rpm and 4°C (*see* **Note 4**).
6. Recover the cell lysates as follows: Wipe each of the four O-ring tubes with a tissue paper, puncture the bottom with a hypodermic syringe needle, and put each O-ring tube into a 15 ml centrifuge tube. Centrifuge at 2850*g* for 1 min at 4°C, resuspend the flow-through extracts in the 15 ml tubes, and then transfer to four 1.5 ml microcentrifuge tubes. Repeat the centrifugation step once more and collect the remaining extract.
7. Centrifuge the extracts at 13,400*g* for 1 min at 4°C. Add a 5 µl aliquot of the soluble fraction (SOL) to 5 µl of 2X Laemmli buffer for Western blot analysis of IP efficiency (*see* **Fig. 9.4A**, **Note 5**). Discard the supernatant containing the soluble protein fraction and resuspend the pellets in 0.4 ml of lysis buffer supplemented with PMSF and antiproteolytic cocktail, as described in step 4.
8. Shear the chromatin by applying five sonication cycles of 15 s at 1.5 output (*see* **Note 6**) After each sonication cycle, pellet the chromatin by centrifuging at 2300*g* for 1 min at 4°C (*see* **Note 7**).
9. Centrifuge at $\geq$16,000*g* for 5 min at 4°C and transfer the supernatants to 1.7-ml prelubricated tubes. This is the whole cell extract (WCE).
10. Take a 5 µl aliquot of the WCE and add 5 µl of 2X Laemmli buffer for Western blot analysis (*see* **Fig. 9.4A**).
11. Add the previously washed antibody-bound magnetic beads (15 µl per tube) to the remaining WCE in the 1.7-ml prelubricated tubes. Incubate on a rotating wheel at 4°C overnight (*see* **Note 8**).

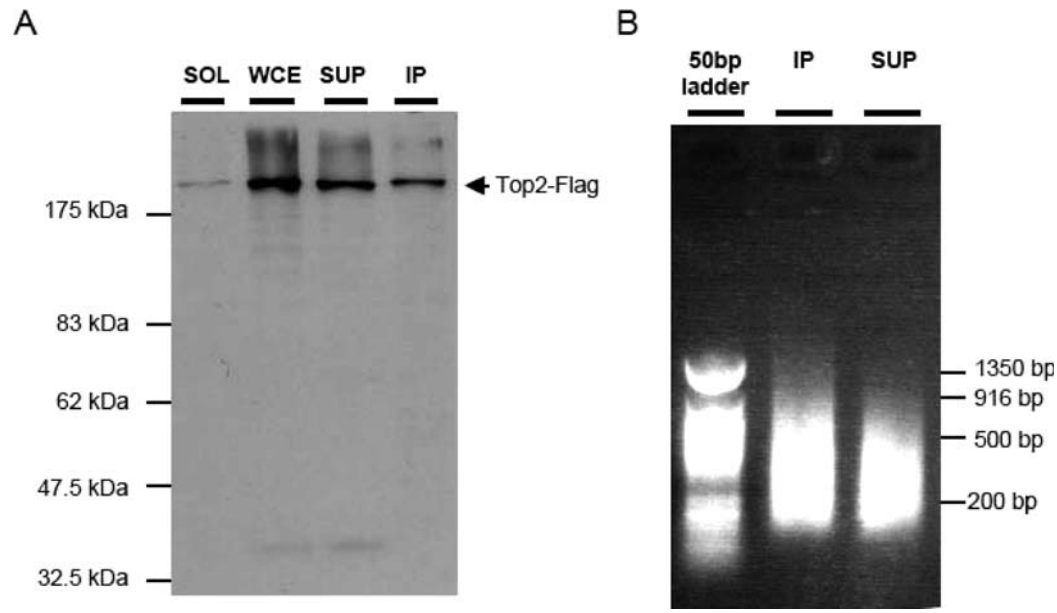

Fig. 9.4. **Immunoprecipitation and amplification controls**. (**A**) Evaluation of the efficiency of chromatin crosslink and immunoprecipitation: Western blot analysis of Top2 abundance in the soluble (SOL), chromatin (WCE), supernatant (SUP), and immunoprecipitated (IP) fractions from a typical ChIP-on-chip experiment. (**B**) Qualitative analysis of DNA amplification: Ethidium bromide-stained agarose gel electrophoresis of an aliquot of the DNA amplified from immunoprecipitated (IP) and control (SUP) fractions. Fragments show a smeared distribution with sizes ranging from 100 to 1000 bp.

3.3. Washing the Beads and Crosslink Reversal

1. Place the four 1.7-ml prelubricated tubes containing the beads and WCE in a magnetic grid. Wait until the beads attach to the magnet leaving a clear supernatant. Transfer 5 µl of the supernatant (the SUP fraction; used in step 7) to a 1.5 ml microcentrifuge tube to be used as the hybridization control and another 5 µl to a tube containing 5 µl of 2X Laemmli buffer for Western blot analysis of IP efficiency (*see* **Fig. 9.4A**).
2. Wash the beads as follows using the magnetic grid as described in **Section 3.1**:
   - 2X with 1 ml of ice-cold lysis buffer (without antiproteolytics).
   - 2X with 1 ml of ice-cold lysis buffer supplemented with 360 mM NaCl.
   - 2X with 1 ml of ice-cold wash buffer.
   - 1X with 1 ml of ice-cold TE.
3. Remove the TE (*see* **Note 9**) and centrifuge the beads at 800*g* for 3 min at 4°C. Place the tubes back in the magnetic grid and remove the remaining liquid with a vacuum pump.
4. Add 40 µl of elution buffer to each tube, resuspend the beads by pipetting up and down, and incubate at 65°C for 10 min (*see* **Note 10**).

5. Centrifuge the tubes for 1 min at $\geq$16,000*g* at RT.
6. Place the tubes back in the magnetic grid (this is the IP fraction) and transfer 5 µl to a tube containing 5 µl of 2X Laemmli buffer. [For Western blot analysis of the IP efficiency, boil the samples at 95°C for 30 min prior to separation on an SDS-PAGE gel, then following blotting, probe the membrane with α-flag antibody at a dilution of 1:30,000 (*see* **Fig. 9.4A**, **Note 11**).]
7. Transfer the remaining IP fractions (about 35–40 µl per tube) to four new 1.5-ml microcentrifuge tubes containing 4 volumes (140–160 µl) of TE/1% SDS.
8. Add 95 µl of TE/1% SDS to the SUP fraction (collected in step 1).
9. Incubate overnight at 65°C to reverse the crosslink (*see* **Note 12**).

3.4. DNA Purification

1. Consolidate the SUP and IP fractions by pulse-spinning and, if necessary, add TE to each of the IP samples to reach a volume of 200 µl.
2. Add 100 µl of proteinase K buffer to the IP samples and 44.75 µl of proteinase K buffer to the SUP sample.
3. Mix, without vortexing, and incubate at 37°C for 2 h.
4. Add 12 µl or 6 µl of a 5 M NaCl stock to the IP or SUP samples, respectively.
5. Extract twice by adding an equal volume of phenol/chloroform/isoamylalcohol and centrifuging at 13,400*g* for 5 min at RT.
6. Add 2 volumes of cold 100% ethanol, vortex, and incubate at –20°C for at least 20 min (*see* **Note 13**).
7. Centrifuge at $\geq$13,400*g* for 10 min at 4°C.
8. Discard the supernatants and wash with 1 ml of cold 80% ethanol.
9. Let the pellets dry (*see* **Note 14**), resuspend each pellet in 30 µl of TE containing 10 µg of RNase A. Incubate for 1 h at 37°C. (The RNase A powder should be added to the TE immediately before use and boiled for 10 min.)
10. Pool the 30 µl IP samples together to obtain two 60 µl samples and purify the IP and SUP DNA samples using a PCR purification kit following the manufacturer's instructions. Elute the DNA from each sample with 50 µl of EB buffer.
11. Pool the two 50 µl IP samples together.

12. Precipitate the IP DNA sample by adding 5 µl of 3 M sodium acetate, 2 µl of glycogen (10 mg/ml), and 2.5 volumes of cold 100% ethanol. Precipitate the SUP DNA sample by adding 2.5 µl of 3 M sodium acetate, 1 µl of glycogen (10 mg/ml), and 2.5 volumes of cold 100% ethanol.
13. Incubate at –20°C for at least 20 min.
14. Centrifuge at ≥13,400*g* for 10 min at 4°C.
15. Discard the supernatants and wash with 0.5 ml of cold 70% ethanol.
16. Resuspend the pellets in 7 µl of TE or 10 µl of ddH_2O (for amplification using method I or method II, respectively).

3.5. DNA Amplification

Amplification of the IP and SUP DNA is required to obtain a sufficient amount (≥5 µg) of DNA to be labeled and used as hybridization probes. We describe here two methods for random PCR amplification that give comparable outcomes.

3.5.1. Method I

1. Prepare Round A setup as follows:

DNA	7 µl
5X sequenase buffer	2 µl
Primer A (40 mM)	1 µl

2. Prepare reaction mix (for each reaction):

5X sequenase buffer	1 µl
dNTPs (3 mM each)	1.5 µl
DTT (0.1 M)	0.75 µl
BSA (0.5 mg/ml)	1.5 µl
Sequenase	0.3 µl

3. Incubate Round A setup in a thermocycler as follows: 94°C for 2 min, 10°C for 5 min, hold. During the hold time add 5.05 µl of reaction mix to each sample.
4. Ramp to 37°C over 8 min. Hold at 37°C for 8 min, at 94°C for 2 min, and then at 10°C for 5 min. During this time, add 1.2 µl of diluted sequenase (diluted 1:4 in 5X sequenase dilution buffer).
5. Ramp to 37°C over 8 min.
6. Concentrate the DNA using a Microcon centrifugal filter unit (YM30) according to the manufacturer's instructions, and adjust the sample volume to 40 µl with ddH_2O.
7. Prepare Round B reaction as follows:

DNA template	40 µl
KOD-Dash	2 µl
10X KOD-Dash PCR buffer	10 µl

dNTPs (2 mM each)	10 µl
Primer B (100 µM)	1 µl
ddH_2O	37 µl

8. Perform the following amplification cycle (*see* **Note 15**): 94°C for 5 min (94°C for 20 s, 40°C for 30 s, 50°C for 30 s, 74°C for 3 min) × 32 cycles, 74°C for 7 min.

3.5.2. Method II

1. Use the WGA2 GenomePlex Complete Genome Amplification (WGA) Kit. Follow the manufacturer's instructions from the *Library Preparation* step onward.
2. Check the amplified DNA by running an aliquot (2.5 µl for Method I, 1.9 µl for Method II) of the reaction on a 1.2% agarose gel. A smear ranging from 100 to 1000 bp should be observed (*see* **Fig. 9.4B**).
3. Concentrate the DNA using a Microcon centrifugal filter unit (YM30) according to the manufacturer's instructions.
4. Measure the DNA concentration by spectrometry at A260.

3.6. DNAse Digestion (see Note 16)

1. Prepare DNAse reaction (for 13 samples):

DNAse I (1 U/µl)	2 µl
10X One-Phor-All-Buffer plus	2 µl
25 mM $CoCl_2$	1.2 µl
ddH_2O	14.8 µl

2. Prepare the following reaction mix:

DNAse I setup	1.5 µl
10X One-Phor-All-Buffer plus	4.85 µl
25 mM $CoCl_2$	2.9 µl
DNA (5–10 µg) + ddH_2O (IP/SUP) samples	40.75 µl

3. Incubate at 37°C for 30 sec and then transfer to 95°C for 15 min.

3.7. DNA Labeling

1. Transfer the DNA into new 1.5-ml microcentrifuge tubes.
2. Add 5 µl of TdT reaction buffer, 1 µl terminal transferase (25 U/µl), and 1 µl biotin-N11-ddATP (1 nM/µl).
3. Mix and incubate at 37°C for 1 h.

3.8. Hybridization

Hybridization, chip-staining, washing and scanning, as well as discrimination analysis, are performed as described by the manufacturer's instructions (Affymetrix), as follows:

1. Prehybridize the chips with 200 µl of prehybridization solution. Incubate in an Affymetrix oven at 42°C for 10–20 min.

2. Prepare a mix with the DNA samples in 1.5 ml tubes:

DNA (5–10 μg)	55 μl
3 nM control OligoB2	3.3 μl
Herring sperm DNA (10 mg/ml)	2 μl
20X eukaryotic hybridization control	10 μl
20X SSPE	60 μl
0.1% Triton-X100	10 μl
ddH2O	64.7 μl

3. Boil the mix for 10 min and transfer immediately to ice.
4. Spin the samples at 14,000*g* for 2–3 min in a microcentrifuge.
5. Remove the prehybridization solution and spot the boiled DNA probes onto the microarray chips. Hybridize with 150 μl of probe for 16 h at 42°C in a hybridization oven at 60 rpm.

3.9. Washing, Staining, and Scanning of Chips

1. Remove the hybridization solution.
2. Add 200 μl of washing buffer (wash A) to the chips.
3. Prepare 600 μl of staining buffer as follows:

2X stain buffer	300 μl
ddH_2O	270 μl
Acetylated BSA (50 mg/ml)	24 μl
SAPE (1 mg/ml)	6 μl

4. Insert the chips in a Fluidics Station and run the washing and staining protocol (Midi_euk1 ver2) provided by Affymetrix.
5. Scan the chips.
6. Compare the signal intensities of each locus on the DNA chips hybridized with IP and SUP fractions using GCOS expression analysis.
7. Discrimination analysis can be carried out using the statistical algorithms developed by Affymetrix (extensive information can be downloaded from the manufacturer's web site: http://www.affymetrix.com). Comparison analysis of the IP/SUP fractions is employed to generate DNA topoisomerase binding profiles (1, 4).

4. Notes

1. During the washes, excessive shaking is not desirable as it might revert the magnetic bead–antibody crosslinking.

2. Incubation time can be shortened, but bead–antibody crosslinking times under 2 h are not recommended.
3. Crosslink efficiency can be extended by gently shaking the extracts overnight at 4°C, though immunoprecipitation efficiency might decrease.
4. Breakage time can be extended if broken cells are not over 90% of the total. Cell breakage can be assessed by analyzing a small aliquot of the lysate by phase contrast microscopy. Broken cells appear phase-dark while intact cells appear bright.
5. Western blot detection of this fraction indicates the proportion of protein that is not associated with chromatin after the formaldehyde crosslink.
6. During the sonication step, it is important to avoid sample overheating that can result in crosslink reversal.
7. The size of the sheared chromatin can be assessed by running the extract on a 1.2% TAE agarose gel. A smear of DNA fragments ranging from approximately 100–1000 base pairs should be observed. If not, additional sonication cycles can be performed.
8. The duration of the chromatin immunoprecipitation can be reduced down to 5 h, but overnight incubation is recommended to obtain a better efficiency.
9. After the last wash, TE should be removed with a micropipette to avoid bead aspiration.
10. Flick the tubes three times to resuspend the beads during the incubation time.
11. A good IP efficiency is essential to obtain a significant enrichment ratio. A distinct band should be observed for the IP fraction by Western blotting.
12. Crosslink reversal can be performed in an oven to avoid condensation of the sample.
13. The duration of the precipitation steps at –20°C can be extended without a decrease in the DNA yield.
14. A heated vacuum-centrifuge can be used at this step to completely remove ethanol from the sample.
15. The number of cycles of Round B PCR can be raised to increase the DNA yield. It is not advisable to perform more than 40 cycles as background DNA can be amplified.
16. Short DNAse incubation is performed to reduce the size of the amplified DNA and increase its suitability for the hybridization on the array.

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