Application of the Chromatin Immunoprecipitation Method to Identify in Vivo Protein-DNA Associations in Fission Yeast

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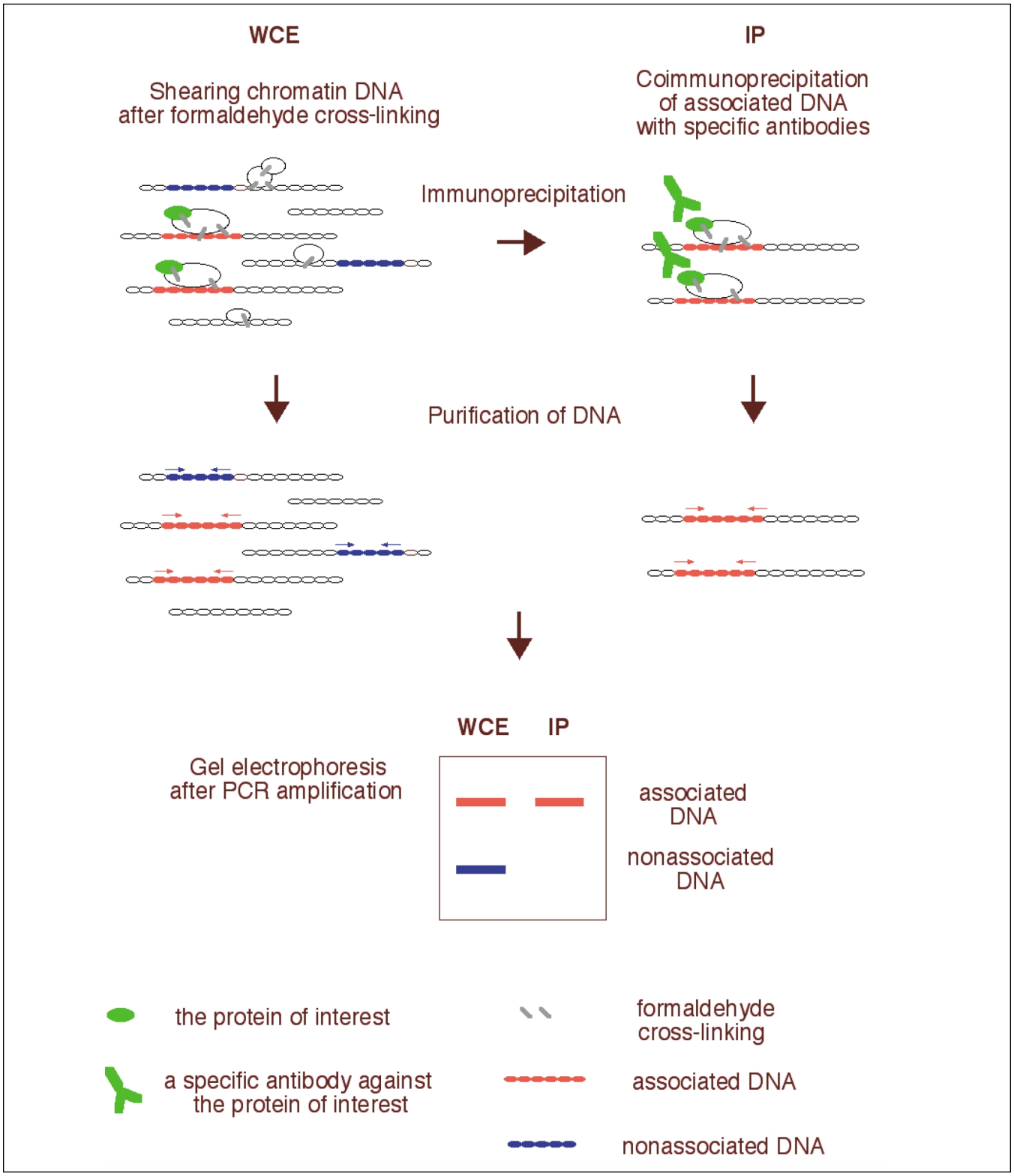

Fig. 1. The principle of the ChIP method used to identify DNA-protein associations in vivo. Living yeast cells are treated with formaldehyde to fix the chromatin structures, then the WCE is prepared and sonicated to shear and solubilize the cross-linked protein-DNA and protein-protein complexes. A selective precipitation of the protein of interest (green) by a specific antibody (green Y) results in the coimmunoprecipitation of the associated DNA (IP). Cross-linking is reversed and the DNA is purified from both the WCE and IP samples. The precipitated DNAs are subjected to PCR amplification followed by gel electrophoresis to identify the associated sequences (red). Nonassociated DNA sequences are shown in blue.

INTRODUCTION

Protein-DNA complexes formed on chromosomes are key platforms for the related signal transduction pathways in the nucleus. A variety of trans-acting proteins directly or indirectly interact with cis-acting chromosomal DNA sequences and form functional complexes that control many nuclear events, such as transcription, replication, and chromosome segregation. The ChIP (chromatin immunoprecipitation) method provides an advantageous tool for investigating these protein-DNA complexes. Whereas other methods, such as the South Western and gel shift assays, analyze direct interactions between protein and DNA in vitro, the ChIP method is suitable for detecting the interactions between proteins of interest and DNAs with known sequences in vivo. The first step of ChIP is fixation of living cells by formaldehyde. As shown in Fig. 1, rapid fixation takes place between proteins and between protein and DNA, leading to the conservation of near-native chromatin structures. Extracts prepared from fixed cells are sonicated to reduce the size of DNA fragments and used for immunoprecipitation.

Coimmunoprecipitated, fragmented chromosomal DNAs are purified and the target sequence is amplified by the polymerase chain reaction (PCR). Note that ChIP can detect the indirect association of proteins with DNA, as the fixation procedure stabilizes large protein complexes that bind DNA. Once the PCR conditions are determined, ChIP permits the analysis of multiple DNA sequences by simply preparing PCR primers for the sequences of interest.

Although the ChIP method is powerful for analyzing chromatin structures, appropriate experimental parameters must be empirically determined for each protein and antibody. ChIP requires primary antibodies suitable for immunoprecipitation. Nevertheless, if standard epitopes [for example, Myc or hemagglutinin (HA)] for tagging can be used, the appropriate monoclonal antibodies are commercially available.

Solomon and Varshavsky were the first to apply the method of formaldehyde cross-linking and immunoprecipitation for the in vivo analysis of chromatin (*1, 2*). Here, we describe the application of ChIP to fission yeast cells. The fission yeast *Schizosaccharomyces*

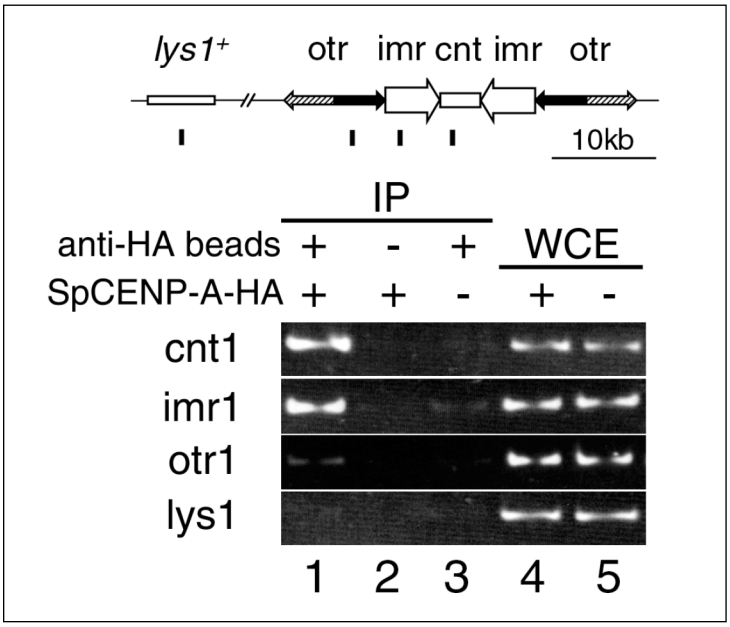

Fig. 2. An example of results from the ChIP assay. SpCENP-A is an inner centromere-specific histone H3 variant. SpCENP-A tagged with a triple HA epitope was expressed in wild-type cells and immunoprecipitated for ChIP analysis with an antibody against HA that was conjugated to beads. Coimmunoprecipitated DNAs were amplified by PCR with four different primers (their location is shown as vertical lines in a schematic drawing of centromere 1). Approximately the same amount of PCR product was obtained from WCEs of cells with and without SpCENP-A-HA (lanes 4 and 5). IPs of SpCENP-A-HA yielded the PCR products of *cnt* and *imr* but not of *otr* or *lys1* (lane 1), indicating that SpCENP-A is bound to the inner centromeres. Lanes 2 and 3 are the control lanes using beads alone or the extract without SpCENP-A-HA, respectively. Quantification of the band intensity after background titration revealed that the amounts of the PCR products of *otr* and *lys1* were less than 5% of those of *cnt* and *imr*, almost equal to those of the control lanes.

pombe is an excellent model organism for studying control of the eukaryotic cell cycle and chromosome segregation (*3*, *4*) for several reasons. Many of its mitotic features are conserved in mammals: Chromosomes are condensed and the spindle apparatus forms only during mitosis; after metaphase is established, anaphase A occurs. Different stages in mitosis and meiosis can be resolved by application of the FISH (fluorescence in situ hybridization) method (*5*, *6*) or the live charge-coupled device microscopic technique using a variety of cytological markers (such as centromeres, spindle pole bodies, and microtubules) tagged with green fluorescent protein (GFP) (*7*, *8*). Many mutants defective in mitotic or meiotic chromosome behavior have been isolated and characterized (*3*, *9*). These cytological and genetical merits for analyzing chromosome dynamics in *S. pombe* are reinforced by application of the ChIP method. The protocol presented below is based on those described for budding yeast [(*10*); see (*11*) for a protocol with useful commentary]. The protocol described here has been used in this laboratory to identify a number of chromosomal DNA regions that interact with kinetochore proteins or sister chromatid cohesion molecules (*12*, *13*) (Fig. 2). Other experiments that used the ChIP method are described in Partridge *et al*. (*14*) and Ogawa *et al*. (*15*).

MATERIALS

dNTPs (2.5 mM)

Ethanol

Gel-loading pipette tips (Gel-Saver, USA Scientific)

Glass beads (~0.5 mm diameter)

Glycogen

Needles (26-G)

Phenol/chloroform/isoamyl alcohol, 25:24:1 (v/v/v)

Primary antibody against the protein of interest

Protease inhibitors [phenylmethylsulfonyl fluoride (PMSF) and Trasylol (aprotinin; Bayer Leverkusen)]

Protein assay (Bio-Rad Laboratories)

Protein G-Sepharose or equivalent (protein A or secondary antibody coupled to agarose beads)

Sodium acetate (3 M, pH 5.2)

Taq polymerase (AmpliTaq DNA polymerase or Taq DNA Gold polymerase, Perkin-Elmer)

Yeast culture

EQUIPMENT

PCR machine: PTC-200 Peltier Thermal Cycler (MJ Research)

Sonicator: Sonifier 450 (Branson)

Power vortex: We use a reformed vortex machine with powerful vortexing capability.

Note: If a high-power vortex is not available, a regular vortex in a cold room may be used with larger tubes (10 ml or more.) DIsruption of yeast does not occur efficiently in 1.5-ml tubes if a regular vortex machine is used.

RECIPES

Recipe 1: EMM2 (Synthetic Yeast Growth Medium)

20× EMM2 salt stock	50 ml
50× Salts stock	20 ml
100× Minerals stock	10 ml
1000× Vitamins stock	1 ml
Glucose	20 g

Distilled water to 1000 ml

Autoclave and store at room temperature.

20× EMM2 salt stock

Potassium hydrogen phthalate	60 g
Na_2HPO_4	44 g
NH_4Cl	100 g
Distilled water to 1000 ml	
Autoclave and store at 4°C.	

50× Salts stock

$MgCl_2•6H_2O$	52.5 g
$CaCl_2•2H_2O$	0.735 g
KCl	50 g
Na_2SO_4	2 g
Distilled water to 1000 ml	
Autoclave and store at 4°C.	

100× Minerals stock

Boric acid	50 mg
$MnSO_4$	40 mg
$ZnSO_4•7H_2O$	40 mg
$FeCl_2•6H_2O$	20 mg
Molybdic acid	4 mg
KCl	10 mg
$CuSO_4•5H_2O$	4 mg
Citric acid	100 mg
Distilled water to 1000 ml	
Autoclave and store at 4°C.	

1000× Vitamins stock

Pantothenic acid calcium salt	1 g
Nicotinic acid	10 g
Meso-inositol	10 g
D-Biotin	10 mg
Distilled water to 1000 ml	
Autoclave and store at −20°C.	

Recipe 2: YPD (rich yeast growth medium)

Polypeptone	20 g
Yeast extract	10 g
Glucose	20 g

Distilled water to 1000 ml

Autoclave and store at room temperature.

Recipe 3: 11% Formaldehyde Solution

Formaldehyde	11% (v/v)
NaCl	100 mM
EDTA-Na (pH 8.0)	1.0 mM
EGTA-Na	0.5 mM
Tris-Cl (pH 8.0)	50 mM

Note: Formaldehyde vapor is toxic. Prepare in a chemical hood immediately before use.

Recipe 4: Buffer I

Hepes/KOH (pH 7.5)	50 mM
NaCl	140 mM
EDTA (pH 7.5)	1 mM
Triton X-100	1% (v/v)
Sodium deoxycholate	0.1% (w/v)

Sterilize by filtration and store at room temperature.

Recipe 5: Buffer II

Hepes/KOH (pH 7.5)	50 mM
NaCl	500 mM
EDTA (pH 7.5)	1 mM
Triton X-100	1% (v/v)
Sodium deoxycholate	0.1% (w/v)

Sterilize by filtration and store at room temperature.

Recipe 6: Buffer III

Tris-Cl (pH 8.0)	10 mM
LiCl	250 mM
EDTA (pH 7.5)	1 mM
Nonidet P-40	0.5% (v/v)
Sodium deoxycholate	0.5% (w/v)

Sterilize by filtration and store at room temperature.

Recipe 7: 1 mg/ml Proteinase K Solution

Proteinase K	1 mg/ml
Tris-Cl (pH 8.0)	50 mM
$CaCl_2$	1 mM

Store at –20°C.

Recipe 8: 1 mg/ml RNase A Stock Solution

Dissolve 1 μg/mlmg RNase A in 900 μl of 10 mM sodium acetate (pH 5.2).

Heat the enzyme solution to 100°C, 15 min and cool it down to room temperature.

Add 100 μl of 1 M tris-Cl (pH 7.6) to the solution.

Store at –20°C.

Recipe 9: 10% SDS

Prepare a 10% (w/v) sodium dodecyl sulfate solution.

Recipe 10: TE Buffer

Tris-Cl (pH 7.6)	10 mM
EDTA (pH 7.5)	1 mM

Recipe 11: PCR Reaction Cocktail

10× PCR buffer	2.0 μl
2.5 mM dNTP	1.6 μl
10 pmol/μl primers	1.0 μl each
Taq DNA polymerase	0.25 μl
(or 0.1 μl of Taq DNA Gold polymerase)	
Distilled water	4.15 μl

Note: The use of Taq DNA Gold polymerase (Perkin-Elmer) results in reduced background. The volume of distilled water should be increased to 4.9 μl if Taq DNA Gold polymerase is used to maintain a final volume of 10 μl for the reaction cocktail.

INSTRUCTIONS

Formaldehyde Cross-Linking in Vivo

1. Culture the yeast cells in 50 to 100 ml of synthetic or rich medium (Recipes 1 and 2) to 2×10^6 to 10×10^6 cells/ml.
2. Add 1/10 volume of 11% formaldehyde solution (Recipe 3) to the culture (final concentration, 1%) and incubate the culture for 10 min at 26°C with gentle shaking.
3. Chill the cell culture in ice water bath for 50 min with occasional shaking.
4. Pellet the cells by centrifugation at 1000*g* at 4°C for 5 min. Discard the supernatant.
5. Wash the cell pellets with 1 ml of ice-cold buffer I (Recipe 4).
6. Wash the cell pellets three times with ice-cold buffer I (Recipe 4), centrifuging as in step 4 between each wash.
7. Freeze the cell pellets in liquid nitrogen and store at –80°C until ready to use.

Preparation of Whole-Cell Extracts

1. Resuspend the cell pellets in 500 µl of ice-cold buffer I (Recipe 4) containing protease inhibitors (1 mM PMSF and 1/100 volume of Trasylol).
2. Add 1 volume of glass beads to the suspension.
3. Vortex the cells vigorously for 30 s and chill on ice for at least 1 min to cool down.
4. Repeat step 3 two or three times until more than 80% of the cells are lysed.

   Note: Check for cell lysis by taking a small aliquot to observe the cells with a microscope.

5. Invert the tubes and puncture bottom with a 26-G needle to selectively filter the glass beads by centrifugation.
6. Insert the tube into a fresh tube of the same size and then centrifuge the cells at 1000*g* for 3 min at 4°C. Discard the upper tube containing glass beads.
7. Resuspend the pellets by pipetting, and transfer the suspension to a fresh 1.5-ml microcentrifuge tube kept on ice.
8. Sonicate the suspension for 30 s, four times with a Sonifier 450 sonicator (Branson; output 3.0, duty cycle 30%) on ice. There should be at least a 1-min interval on ice between each pulse in order to cool the sample. This sonication step shears the chromatin DNA to sizes ranging between 0.5 and 1.0 kb.

   Note: Foaming of the solution during sonication results in unequal shearing of DNA samples. To prevent foaming, keep the tip end of a sonicator near the bottom of the sample tubes. Placing the tip end near the surface induces foaming. The sizes of the sheared DNA can be confirmed by 2% agarose gel electrophoresis stained with ethidium bromide.

9. Centrifuge the suspension at 14,000 rpm for 15 min at 4°C and then transfer the supernatant to a fresh 1.5-ml microcentrifuge tube on ice.
10. Measure the protein concentration of each supernatant with the Bio-Rad protein assay kit according to the manufacturer's instructions.
11. Adjust the protein concentration of each supernatant to 20 µg/µl by diluting with ice-cold buffer I (Recipe 34). The supernatants are referred to as whole-cell extracts (WCE).

Immunoprecipitation of the Protein of Interest

1. Add 10 µl of protein G-Sepharose beads to 200 µl of the WCE and incubate on a rotating wheel for 1 hour at 4°C.
2. Pellet the beads by centrifuging at 12,000 rpm for 20 s at 4°C and transfer the supernatant to a fresh 1.5-ml microcentrifuge tube on ice. This step removes proteins, which interact nonspecifically with the beads, from the WCE.
3. Add the primary antibody against the protein of interest to the supernatant and incubate on a rotating wheel for 1 to 2 hours at 4°C.

   Note: Appropriate antibody concentrations should be predetermined by performing preliminary experiments to maximize the level of specific coimmunoprecipitated DNA and to minimize the precipitation of nonspecifically bound DNA.

4. Add 10 µl of protein G-Sepharose beads to the mixture and incubate on a rotating wheel for 2 hours at 4°C.
5. Pellet the beads by centrifuging at 5000 rpm for 20 s at 4°C and discard the supernatant.
6. Add 1 ml of ice-cold buffer I (Recipe 4) to the glass beads and incubate on a rotating wheel for at least 10 min at 4°C.
7. Pellet the beads by centrifuging at 5000 rpm for 20 s at 4°C and carefully remove all of the supernatant using a 200-µl micropipettor with a gel-loading pipette tip (Gel-Saver, USA Scientific).

   Note: Special care should be taken not to remove any beads from the sample when discarding the supernatant during this and subsequent washing steps. A gel-loading pipette tip with an opening much smaller than the diameter of the glass beads prevents the removal of any beads.

8. Wash the beads with 1 ml of ice-cold buffer I (Recipe 4). Pellet the beads by centrifuging as in step 7 and carefully remove the supernatant as in step 7.
9. Wash the beads with 1 ml of ice-cold buffer II (Recipe 5) twice. Pellet the beads between each wash by centrifuging as in step 7 and carefully remove the supernatant as in step 7.
10. Wash the beads with 1 ml of ice-cold buffer III (Recipe 6) twice. Pellet the beads between each wash by centrifuging as in step 7 and carefully remove the supernatant as in step 7.

11. Wash the beads with 1 ml of ice-cold TE (Recipe 10). Pellet the beads between each wash by centrifuging as in step 7 and carefully remove the supernatant as in step 7.
12. Add 100 µl of TE containing 10 µg/ml RNase A (Recipe 8 for stock RNase A solution) to the beads WCE (designated the IP sample). Incubate for 15 min at 37°C. (This is the experimental sample.)
13. Add 100 µl of TE containing 10 µg/ml RNase A to 20 µl of the (designated the WCE sample) from step 10 in Preparation of WCE. Incubate the mixture for 15 min at 37°C. (This is the control sample.)

Purification of the Coimmunoprecipitated DNA

1. Add 2.5 µl of 10% SDS (Recipe 9) and 2.5 µl of 1 mg/ml Proteinase K solution (Recipe 67) to the IP or WCE samples and incubate at 37°C for at least 8 hours.
2. Incubate the samples at 65°C for at least 6 hours to reverse the protein-DNA cross-linking.
3. Add 10 µl of 3 M sodium acetate (pH 5.2) and 100 µl of phenol/chloroform/isoamyl alcohol to the samples.
4. Vortex well.
5. Centrifuge the samples at 14,000 rpm for 5 min at room temperature.
6. Transfer the upper aqueous phase containing the DNA to a 1.5-ml microcentrifuge tube.

   Note: For the WCE sample, a second phenol/chloroform/isoamyl alcohol extraction may be required. The same amount of the upper phase should be taken from each sample as carefully as possible.
7. Add 20 µg of glycogen and 250 µl of 100% ethanol to the DNA-containing upper phase, mix well, and incubate for 30 min at –20°C.
8. Precipitate the DNA by centrifuging for 15 min at 14,000 rpm at room temperature.
9. Wash the pellets with 1 ml of 70% ethanol, centrifuge the resulting suspension for 5 min at 14,000 rpm, discard the supernatant, and dry the DNA pellet.
10. Resuspend the pellets from the IP sample or the WCE sample in 50 µl of TE buffer (Recipe 10) and store at –20°C (hereafter referred to as the IP DNA solution and the WCE DNA solution, respectively).

PCR Amplification of the Coimmunoprecipitated DNA

1. Place 10 µl of the template DNA solution (either IP DNA solution or WCE DNA solution) in each PCR tube on ice.
2. Add 10 µl of the ice-cold PCR reaction cocktail (Recipe 11) and mix it by gentle pipetting (See Notes and Remarks for preliminary experiments to determine the appropriate concentrations of the template DNAs).

   Note: Taq polymerase tends to become inactivated by rough handling. To avoid inactivation, prepare 10 µl of the template DNA solution first and then add 10 µl of the PCR reaction cocktail to the tube on ice. Taq polymerase should be mixed with the reaction cocktail immediately before use by gentle pipetting.
3. Perform PCR reaction using the following parameters.

1 cycle:	94°C	3 min
26 cycles:	94°C	1 min
	52°C	1 min
	72°C	1 min
1 cycle:	72°C	5 min

   Note: The use of Taq DNA Gold polymerase has reduced our background; however, a preheating cycle (1 cycle at 95°C for 9 min) must be performed before the first cycle above.
4. Separate the PCR products by 2.5% agarose gel electrophoresis and visualize the DNA with 1 µg/ml ethidium bromide.

TROUBLESHOOTING

Lack of Reproducibility

Foaming during the sonication step in preparation of the WCE can result in unequal levels of chromatin DNA shearing among different samples. Ideally, the uniformity of size among the sheared samples (or among the experiments) should be confirmed by 2% agarose gel electrophoresis stained with ethidium bromide.

Another source of error can occur during the washes of the glass beads in the immunoprecipitation part of the procedure. To maximize sample uniformity, take care not to remove any beads from the sample when discarding the supernatant in the washing step of immunoprecipitates.

Sample variation can also be introduced during the phenol/chloroform extractions. The same volume of the aqueous phase containing the DNA should be removed from each sample during extraction. The IP solutions should be matched to each other and the WCE solutions should be matched to each other, but the IP and WCE solutions do not have to be identical.

Lack of a PCR Product in the Whole-Cell Extracts Samples

Taq polymerase tends to become inactivated by rough handling that results in foaming of the sample. To avoid inactivation, keep the template DNA solution and the PCR reaction cocktail on ice and mix the Taq polymerase with the reaction cocktail immediately before use by gentle pipetting.

NOTES AND REMARKS

An Example of ChIP Assay Results

Fig. 2 shows an example of data obtained using the ChIP assay. This experiment demonstrates that the fission yeast histone H3-like protein, SpCENP-A, is associated with inner centromere DNA (*imr* and *cnt*), but not with outer repetitive DNA (*otr*) or pericentric *lys1* locus (*13*). WCE DNA samples (lanes 4 and 5) were amplified to determine the relative amounts of chromatin DNA in different samples and to determine the efficiency of different pairs of the primers in the PCR reaction. The amount of amplified IP DNA (lane 1) must be normalized to that of the corresponding WCE DNA (lane 4). The relative enrichment of the amplified IP DNA for *imr* and *cnt* relative to those for *otr* and *lys1* indicates that SpCENP-A predominantly localizes in *imr* and *cnt* regions. Two controls (lanes 2 and 3) were performed to demonstrate the specificity of the signals of IP DNA observed in lane 1. A mock immunoprecipitation by nonconjugated beads (lane 2) and immunoprecipitation with extracts prepared from a nontagged, isogenic strain of yeast (lane 3) yielded no detectable signals.

Temperature Variability

One source of variability to consider is that the results of an experiment can vary with temperature used to grow and fix the yeast. Under the conditions described above, there is a general tendency for the PCR signals derived from cells fixed at a high temperature (33° or 37°C) to be stronger than those fixed at a low temperature (20° or 26°C). This tendency may reflect a temperature dependency on the efficiency of formaldehyde cross-linking (*11*). When a temperature shift experiment is carried out, this effect should be taken into consideration in order to correctly interpret the results.

Resolution

The resolution of ChIP analysis depends on the degree of fragmentation of the chromatin DNA by sonication. For example, if the majority of the sizes of the fragmented DNA range between 500 and 1000 bp, a considerable amount of adjacent nonassociated DNAs located within 1 kb of the actual protein binding site will be coimmunoprecipitated. A shorter shear size of the chromatin DNA will be required for better resolution. However, care should also be taken because repeated sonication to achieve smaller DNA fragments might cause denaturation of the proteins in the extract.

PCR Conditions

The parameters of the number of PCR cycles and the amount of the template DNAs (IP and WCE) must be determined experimentally for each protein of interest and its antibody. The conditions described in this protocol provide an example of the parameters optimized for the ChIP analysis of one kinetochore protein, Mis6, tagged with the triple HA sequence using monoclonal antibody 12CA5, which recognizes the HA epitope (*12*). It is important to select PCR conditions under which amplification is within a linear range. To determine the optimal conditions, we recommend performance of a preliminary PCR amplification with threefold serially diluted template DNAs (1/50, 1/150, 1/450, and 1/1350 dilution for the WCE DNA solution, and 1/2, 1/6, 1/18, and 1/54 dilution for the IP DNA solution).

Quantification

To accurately quantify the results, it is necessary to design a set of PCR primers such that the PCR products are much shorter than the average size of the fragmented DNA. Otherwise, a significant fraction of the coimmunoprecipitated DNA cannot bind to both primers, resulting in a reduction in the PCR signal. We recommend primers that yield products approximately 200 and 300 bp in length. Another method used to obtain quantitative PCR results is multiplex PCR using several sets of primers in the same reaction tube (*15*). This method allows the quantification of the relative amounts of PCR products from different sequences at the same time. Another factor that affects quantification is the binding properties of the proteins of interest. If the DNA has multiple binding sites for the protein (for example, histone binds to DNA approximately every 200 bp), then a significant decrease in the bound protein may not produce a correlative decrease in the PCR signal, because binding of the protein to a single site may be sufficient to coimmunoprecipitate the DNA.

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