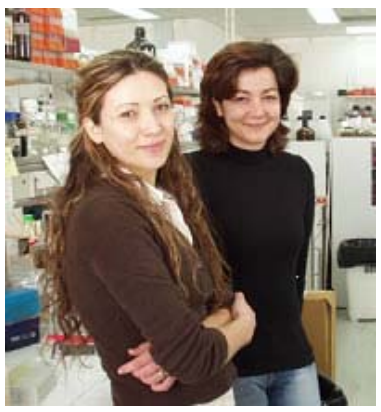




Chromatin Immunoprecipitation (ChIP) Assay (PROT11)



Antigone Kouskouti & Irene Kyrmizi
 Institute of Molecular Biology and Biotechnology
 FORTH
 1527 Vassilika Vouton
 711 10, Heraklion, Crete, Greece

Email feedback to:
akous@imbb.forth.gr

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Introduction

Chromatin Immunoprecipitation (ChIP) assays are used to evaluate the association of proteins with specific DNA regions. The technique involves crosslinking of proteins with DNA, fragmentation and preparation of soluble chromatin followed by immunoprecipitation with an antibody recognizing the protein of interest. The segment of the genome associated with the protein is then identified by PCR amplification of the DNA in the immunoprecipitates. There is a general protocol for ChIP, which is employed by many different labs with minor modifications. Here we describe modified ChIP protocols used successfully in our lab. They include the Standard Protocol, Re-ChIP, Alternative Crosslinking Method and Nucleosome ChIPs.

Procedure

The major modifications of this protocol (Refs. 1-3) compared to those generally found in literature are as follows:

1. After crosslinking of the cells with formaldehyde nuclei are prepared by incubation and dounce homogenization of the cells in [Swelling buffer](#). In our experience, inclusion of this step significantly reduces background.
2. In the widely used ChIP protocols, after crosslinking the cells are lysed in 1% SDS containing buffer. After sonication and before immunoprecipitation SDS is diluted 10 times. In our protocol the cells are lysed in 0.1% SDS-containing buffer and immunoprecipitation is performed in the same solution. The inclusion of at least 0.1% SDS and harsh washing conditions are necessary to reduce background coming from non-specific sticking of chromatin to Protein-G-Sepharose beads. Performing the sonication in a 0.1% SDS-containing buffer compromises shearing efficiency; therefore sonication conditions should be carefully controlled. On the other hand avoiding the use of 1% SDS for lysis, greatly enhanced the ChIP signals obtained with several antibodies (see example in [Figure 1](#) and also see [comment 1](#)).

The step by step protocol is described for cultured cells grown in 150 mm dishes, containing 2-5 x 10⁷ cells per dish.

1. Replace medium with 27 ml DMEM/10% FBS.
2. Add 3 ml formaldehyde (from 10% stock) and mix immediately. Incubate at room temperature for 10 minutes (see [comment 2](#)).
3. Add 3 ml glycine (from 1.375 M stock) and mix immediately.
4. Place the plate on the top of ice and wash 3 times with 20 ml ice-cold PBS/ 0.5 mM PMSF.
5. Scrape cells in 20 ml ice-cold PBS/ 0.5mM PMSF (see [comment 3](#)). Centrifuge at 1000 rpm for 5 minutes in cold centrifuge.
6. Resuspend pellet in at least 10 vol. [Swelling buffer](#). Incubate in ice for 10 minutes. Dounce 10-20 times up-down. (Check nuclei in microscope by mixing an aliquot with equal volume of 0.4% Trypan blue) (see [comment 4](#)).
7. Centrifuge at 2000 rpm for 5 minutes.
8. Resuspend pellet (nuclei) in 5-10 ml [Sonication buffer](#). (See [note 1](#))
9. Sonicate 9 times for 10-20 seconds at 80% setting (VibraCell Sonicator). Keep sample in ice and allow sample to cool in ice for 1 minute between each sonication (fragment size should be 200-1000 nt). (See [comment 5](#) and [note 2](#).)
10. Centrifuge at 14000 rpm for 15 minutes.
11. Take the supernatant and centrifuge again at 14000 rpm for 15 minutes (see [comment 6](#)).
12. Take the supernatant (this contains the crude Soluble Chromatin) and add sonicated DNA (to 1 µg/ml final concentration)

- and BSA (to 1 mg/ml final concentration). (See [comment 7](#) and [note 3](#).)
13. Preclear the lysate by incubating by constant rotation with Protein-A or G Sepharose (use 40-50 μ l Sepharose per ml lysate) for 2 hours in the cold room. (See [comment 8](#) and [note 4](#).)
 14. Centrifuge samples at 2000 rpm for 5 minutes. Take the supernatant. This is the precleared Soluble Chromatin. (See [note 5](#))
 15. Save a 50 μ l (1/20th of amount used per IP) aliquot at -20°C . (for preparation of INPUT DNA)
 16. Divide the sample into 1 ml aliquots in eppendorf tubes for IP.
 17. Add 5 μ g antibody. Rotate in the cold room for 2 hours. (See [note 6](#).)
 18. Add 40 μ l Protein-A or G Sepharose per IP (equilibrated as above) and incubate overnight by constant rotation in the cold room.
 19. Centrifuge the beads at 6000 rpm for 3 minutes.
 20. Wash 2 times with 1 ml [Sonication buffer](#). (See [note 7](#).)
 21. Wash 2 x with 1 ml [Wash buffer A](#).
 22. Wash 2 x with 1 ml [Wash buffer B](#).
 23. Wash 2 x with 1 ml TE buffer.
 24. Add 200 μ l [Elution buffer](#) to the beads and incubate at 65°C for 10 minutes. Centrifuge at 14000 rpm for 1 minute. Transfer supernatant to a new tube and elute beads again. Combine eluates (400 μ l final volume, adjust with [Elution buffer](#) if necessary) (see [comment 9](#)).
 25. Add 21 μ l NaCl (from 4 M stock). In parallel thaw the input sample (50 μ l) and supplement with 350 μ l [Elution buffer](#). Add 21 μ l NaCl (from 4M stock).
 26. Incubate at 65°C for at least 5 hours. (See [note 8](#))
 27. Add 1 μ l RNase A (from 10 mg/ml, Dnase-free stock) and incubate at 37°C for 1 hour.
 28. Add 4 μ l EDTA (from 0.5 M stock) and 2 μ l Proteinase K (from 10 mg/ml stock).
 29. Incubate at 42°C for 2 hours (see [comment 10](#)).
 30. Extract 2 times with phenol/chlorophorm/isoamylalcohol and once with chlorophorm/isoamylalcohol (see [comment 11](#)).
 31. Add 1 μ l glycogen (from 20 mg/ml stock), 40 μ l Na-acetate (from 3M stock) and 1 ml EtOH (see [comment 12](#)).
 32. Vortex and leave to precipitate -20°C overnight.
 33. Centrifuge at 14000 rpm 30 minutes. Wash 1 x with 80% EtOH. Speedvac (see [comment 13](#)).
 34. Resuspend IP and INPUT samples in 100 μ l 10 mM Tris (pH 7.5). Proceed to PCR analysis.

Re-ChIP assay

Re-ChIP stands for sequential chromatin immunoprecipitations with two antibodies to study the simultaneous presence of two proteins, or different histone modifications in the genome sequence of interest (Refs. 2 and 5) (see [comment 14](#)).

1. After the step 24 of the standard protocol, incubate the beads with equal volume of 10 mM DTT for 30 minutes at 37°C .
2. Centrifuge at 14000 rpm for 1 minute and transfer the supernatant into a new tube.
3. Repeat step 1 and combine the eluates.
4. Dilute the eluted sample 40 times with [Sonication buffer](#), keep 10% of the sample for input and proceed with step 18 of the standard protocol by adding the second antibody.

Alternative method for crosslinking

A general observation in ChIP assays is that the signals obtained for histones and DNA-binding factors are much stronger than those proteins that do not directly contact DNA but recruited via protein-protein interactions. There could be several explanations for this phenomenon. One of them is crosslinking efficiency by formaldehyde. In our experience ChIP signals for such proteins (e.g. CBP and some other cofactors) can be improved 2-4 times by using 3'-dithiobispropionimidate (DTBP from Pierce) in conjunction with formaldehyde. The protocol was adopted from Ref. 4 and is described for cells grown in 150 mm dish.

1. Wash cells 3x with ice-cold PBS (pH 8.0)
2. Prepare (freshly) 5 mM DTBP in ice cold PBS (pH 8.0) and add to plates sitting on ice. (To cover cells 20-25 ml of this solution is needed per 150 mm plate).
3. Incubate on ice for 30 minutes.
4. Wash cells twice with cold PBS (pH 8.0).
5. Add 20-25 ml ice-cold Quenching buffer (100 mM Tris pH 8.0, 150 mM NaCl) per plate.
6. Incubate on ice for 10 minutes.
7. Take the plates out from ice and wash 3 times with PBS at room temperature.
8. Add 27 ml PBS to each plate + 3 ml formaldehyde (from 10% stock). Mix well and incubate at room temperature for 10 minutes.
9. Add 3 ml Glycine (from 1.375 M stock). Mix well.
10. Wash 3 times with cold PBS/0.5mM PMSF.
11. Continue with step 5 of standard protocol.

Nucleosome ChIP (Ref. 5)

This method allows ChIP analysis at nucleosome resolution by taking advantage of the fact that micrococcal nuclease (MNase) can efficiently digest crosslinked chromatin. It is applicable to study histone modifications and factor occupancy on nucleosomes, but not if the factors are recruited to naked DNA sequence. It should be noted however that, if two neighbouring nucleosomes are bridged via intermediary factors, they are not expected to be resolved (see [Fig. 2](#)). In order to investigate individual nucleosomes, the sonication step of the standard protocol is replaced by micrococcal nuclease digestion of crosslinked chromatin. Complete digestion (i.e. to obtain mononucleosome-sized fragments) requires pure nuclei. While nuclei obtained by the standard protocol step 7 is often fine, we obtain more reproducible results by including the steps below.

1. After step 6 resuspend cells in at least 10 pellet volume of [Sucrose buffer A](#) and perform dounce homogenization (10 up and downs). Check nuclei by trypan blue staining.
2. Layer the nuclear suspension over an equal volume of [Sucrose buffer B](#) and centrifuge for 15 minutes at 3000 rpm. This is performed in Falcon 15 ml tubes, 5 ml nuclear suspension + 5ml [Sucrose buffer B](#).
3. Remove stepwise the supernatant by 1 ml pipette and collect purified nuclei from the bottom of the gradient by resuspension in 1 ml [Buffer NUC](#). Transfer into a clean tube.
4. Wash nuclei with [Buffer NUC](#) once and resuspend them in [Buffer NUC](#), to obtain 0.2 OD260 per 5 µl sample. OD is measured by diluting 5 µl nuclei in 1 ml 1M NaOH.
5. Add CaCl₂ to 3 mM final concentration and immediately add 100 units/ml micrococcal nuclease (MNase, Worthington). (See [note 9](#))
6. Incubate for 5 minutes at 37°C and stop reactions by the addition of an equal volume of 2x [Sonication buffer X](#).
7. Vortex and check lysis in microscope. If not completely lysed perform one short (5 second) sonication. Centrifuge at 14000 rpm for 15 minutes, take the soluble chromatin containing supernatant and proceed to step 14. (See [note 10](#))

Materials & Reagents

Swelling buffer	25 M Hepes, pH 7.8 1.5 mM MgCl ₂ 10 mM KCl 0.1% NP-40 1 mM DTT 0.5 mM PMSF Protease inhibitor cocktail (Roche)
Sonication buffer	50mM Hepes pH 7.9 140 mM NaCl 1 mM EDTA 1% Triton X-100 0.1% Na-deoxycholate 0.1% SDS 0.5 mM PMSF Protease inhibitor cocktail (Roche)
Wash buffer A	50 mM Hepes pH 7.9 500 mM NaCl 1 mM EDTA 1% Triton X-100 0.1% Na-deoxycholate 0.1% SDS 0.5 mM PMSF Protease inhibitor cocktail (Roche)
	This is the same as " Sonication buffer " but contains 500 mM NaCl.
Wash buffer B	20mM Tris, pH 8.0 1mM EDTA 250mM LiCl 0.5% NP-40 0.5% Na-deoxycholate 0.5 mM PMSF Protease inhibitor cocktail (Roche)

Elution buffer	50mM Tris, pH 8.0 1mM EDTA 1% SDS 50mM NaHCO ₃
Sucrose buffer A	0.32mM sucrose 15mM Hepes pH 7.9 60mM KCL 2mM EDTA 0.5mM EGTA 0.5% BSA 0.5mM spermidine 0.15mM spermine 0.5mM DT
Sucrose buffer B	0.32mM sucrose 15mM Hepes pH 7.9 60mM KCL 2mM EDTA 0.5mM EGTA 0.5mM spermidine 0.15mM spermine 0.5mM DTT This is the same as Sucrose buffer A without BSA + 30 % sucrose.
Buffer NUC	15mM Hepes pH 7.5 60mM KCL 15mM NaCl 0.34mM sucrose 0.15mM mercaptoethanol 0.15mM spermine 0.5mM spermidine
2x Sonication buffer X	For Nucleosome ChIP 90mM Hepes pH 7.9 220mM NaCl 10mM EDTA 2% Triton X 100 0.2% Na-deoxycholate 0.2% SDS 0.5mM PMSF Protease inhibitor cocktail (Roche)

Authors Notes

1. The volume depends on the number of nuclei. Use a volume to obtain approximately $2-3 \times 10^6$ nuclei per ml (see [comment 15](#)).
2. This step has to be optimized for each cell type and instrument. Pilot experiments using different settings and times should be performed and after decrosslinking and phenol extractions the size of the DNA is evaluated by agarose gel electrophoresis (see [comment 16](#)).
3. Sonicated salmon sperm DNA can also be used in place of λ DNA.
4. Before the preclearing step the columns should be washed 3x with [Sonication buffer](#) and then preincubated for at least 4 hours with [Sonication buffer](#) containing 1 μ g/ml sonicated λ DNA + 1 mg/ml BSA. We get much nicer results (less background), if preclearing is done twice with new Protein-G Sepharose (see [comment 17](#)).

5. At this point the samples can be frozen to -80°C (see [comment 18](#)).
6. The concentration of the antibody should be empirically determined (see [comment 19](#)).
7. Each wash includes 10 minute constant rotation of the tubes in the cold room.
8. This is the de-crosslinking step, which can also be done overnight (see [comment 20](#)).
9. In most cases this amount of MNase is sufficient to obtain mononucleosome-sized fragments, but may need to be optimized for the particular experiment.
10. At the end of the procedure the purified DNA fragments are around 146 bp in size. In order to design primers for PCR analysis the positions of the nucleosomes should be determined. In our experience low resolution mapping by indirect end-labeling of partially digested chromatin with MNase is sufficient.

Reviewer Comments

Reviewed by: [Sergei Denisov](#) and [Arjen Brinkmann](#), Stunnenberg lab, Amsterdam

1. In our hands preparing chromatin using lysis buffer containing 1% SDS, followed by dialysis against the same buffer with lower SDS concentration clearly increases the recovery for some antibodies. 1% SDS appears to improve sonication efficiency.
2. Cross-linking time influences the ChIP-efficiency. For instance, for ChIPping histone modifications 10' cross-linking is perfect, while for ChIPping transcription factors longer cross-linking times can be employed (up to 30'). We normally incubate 30 minutes.
3. We do not use toxic PMSF or any other protease inhibitors at this step.
4. We wash cross-linked cells first with 30 ml cold PBS and then with buffer similar to the [Swelling buffer](#).
5. We sonicate 12 times x10 seconds with intervals 30 seconds, hold tube on ice-ethanol (-16°C). We found very important that sample stays cold during sonication.
6. We centrifuge 5 minutes once.
7. When performing ChIP-on-chip, such competitor DNA should be omitted because of the random amplification step involved. We try to avoid contamination with any non-relevant DNA.
8. We use Protein A/G agarose beads from Santa Cruz.
9. We elute once with 400 μl of [Elution buffer](#), 30 minutes rotation at room temperature.
10. We do not perform steps 27-29.
11. We extract once each step.
12. We add 10 μg of glycogen.
13. We wash with 70% ethanol, then carefully remove it and leave tubes open for few minutes so no ethanol left. Speedvac with such a tiny pellet can be not desirable.
14. We perform elution with "[Elution buffer](#)" (contains 1% SDS). We found it crucial to immobilize antibodies on prot A/G beads to avoid leakage of antibody from first ChIP to the second ChIP. We incubate a fraction of eluate from the first ChIP with empty beads as an antibody leakage control.
15. We dilute cells in broad ranges: 106 cells per 30-300 μl . Branson250 sonicator works efficiently and almost independently of cell concentration.
16. Typically, short sonication results in high recoveries (%ChIP/input) but low resolution, while longer sonication times result in lower recovery but higher resolution. Gel image can be not sufficient and conclusive. ChIP experiment will provide better clues about specific signal-to-background ratio.
17. We found preclearing step is not necessary. We skip it because it makes no differences as for single gene ChIP as for our ChIP-on-chip experiments.
18. The use of fresh chromatin clearly increases the recovery, although cross-linked chromatin can be stored at -80°C . Storage longer than 1-2 months is not recommended. Chromatin prepared in lysis buffer containing 1% SDS can be stored at 4°C for 1-2 days until use.
19. Optimal ratio can be $\sim 2-4$ μg of antibody against transcriptional factors per 1-2 million cells. Different ratio was found for histone Abs.
20. We de-crosslink 4 hours at 65°C , incubation o'n can be a problem.

Figures

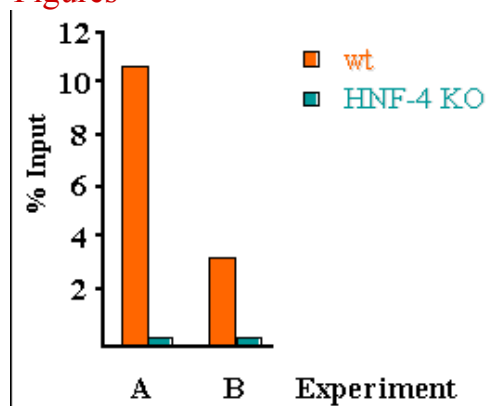
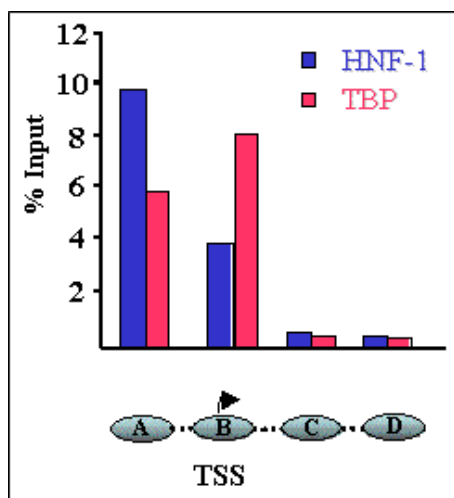


Figure 1.

An example of results obtained with an HNF-4 antibody using the standard ChIP protocol in wild type and HNF-4KO hepatocytes. In Experiment A the cells were lysed and sonicated with 0.1% SDS-containing [Sonication buffer](#), while in Experiment B the cells were lysed and sonicated in 1% SDS-containing buffer and diluted 10 times before IP. The bars represent normalized real-time PCR values on target promoter (HNF-1) expressed as % of input. Note the 3-fold increased specific ChIP signal in Experiment A.

**Figure 2.**

An example of results obtained with HNF-1 and TBP antibodies using the Nucleosome ChIP protocol. The bars represent normalized real-time PCR values on the nucleosomes of the HNF-4 gene. Primer sets amplifying the DNA spanning nucleosome (B) located at the transcription start site (TSS) were used and those amplifying the DNA spanning nucleosomes located upstream (A) and downstream (C and D) of TSS. The resolution is demonstrated by the background signals obtained in downstream nucleosomes. A significant signal with TBP antibody is also detected at the nucleosome A (which spans the HNF-1 binding site) and with the HNF-1 antibody in nucleosome B. This can be explained by taking into consideration that a large preinitiation complex is formed in this area and due to efficient crosslinking through intermediary factors antibodies recognizing TBP or HNF-1 may eventually bring down both nucleosomes.

References

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