CHROMATIN IMMUNOPRECIPITATION (ChIP) FROM CELLULAR PELLET

What is ChIP?

Chromatin immunoprecipitation, or ChIP, is an antibody-based technique used to selectively enrich specific DNA-binding proteins along with their DNA targets. ChIP is used to investigate a particular protein-DNA interaction, several protein-DNA interactions, or interactions across the whole genome or a subset of genes. ChIP utilizes antibodies that selectively recognize and bind proteins, including histones, histone modifications, transcription factors, and cofactors, to provide information about chromatin states and gene transcription. A combination of proteomic analysis and molecular biology techniques used in ChIP allow for the ability to understand gene expression and regulation in cells or tissues of interest.

How does ChIP work?

The principle behind ChIP is relatively straightforward and relies on the use of an antibody to isolate, or precipitate, a certain protein, histone, transcription factor, or cofactor and its bound chromatin from a protein mixture that was extracted from cells or tissues. Hence, the name of the technique: Chromatin Immunoprecipitation. In ChIP-PCR or ChIP-seq, immune-enriched DNA fragments are then able to be identified and quantified using widely available PCR or qPCR reagents and Next Generation Sequencing (NGS) technologies.

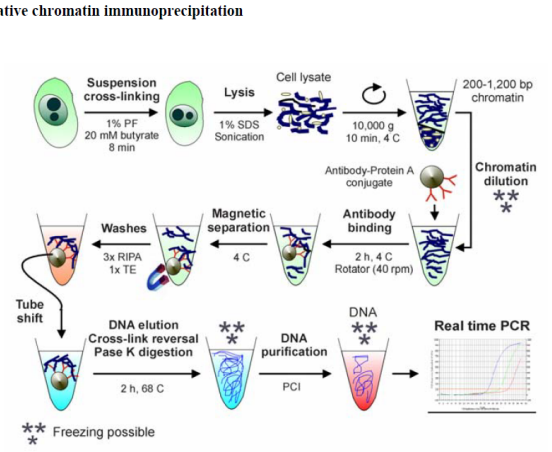


Figure 1. Summary diagram of the Q²ChIP assay. Steps where freezing of the samples is suitable without loss of efficiency are indicated. Reproduced with permission (12).

EQUIPMENT

Fixed Equipment

- Centrifuge
- Rotator
- Bandelin Sonoplus sonicator
- Ice
- Vortex
- Electrophoresis chamber
- Magnetic rack
- Heat block at 68°C
- Pipettes suitable for measuring 10, 200 and 1000 µl, respectively
- Laminar Flow Hood
- DNA Engine Opticon 2 Continuos Fluorescence Detection System (MJ Research)

Consumables

- Polypropylene microcentrifuge tubes
- Filter pipette tips 10, 200, 1000 µl
- Protein A-coated paramagnetic beads (Dynabeads® Protein A)
- Purification columns (DNA Clean & Concentrator®-5, Zymo Research)
- Real Time PCR strips (8 x 0.1 ml)

REAGENTS

- 1X Phosphate Buffered Saline (PBS)
- 1% formaldehyde-PBS
- Lysis buffer (50 mM Tris-HCl, pH 8; 10 mM EDTA; 1% SDS; 1X protease inhibitor cocktail)
- 2M Glycine
- Agarose
- GelRed, 10,000X
- Orange-G, 5X
- 1 kb-size ladder
- RIPA buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 0.5 mM EGTA; 1% Triton X-100; 0.1% SDS; 0.1% Na-deoxycholate; 140 mM NaCl)
- TE buffer (10 mM Tris-HCl, pH 8.0; 10 mM EDTA)
- Antibody: anti-H3K9ac
- DNA Binding Buffer (DNA Clean & Concentrator®-5, Zymo Research)
- DNA Wash Buffer (DNA Clean & Concentrator®-5, Zymo Research)
- SensiFAST SYBR Lo-ROX Mix, 2X
- Hs_ChIP_PDYN forward (100 µM)
- Hs_ChIP_PDYN reverse (100 µM)
- Nuclease-free water

CAUTION!

Formaldehyde is highly toxic. Aqueous solutions of formaldehyde are very corrosive and ingestion can cause severe injury to the upper gastrointestinal tract. Exposure is likely to be by inhalation, skin or eye contact. Contact with skin can result in burns and may result in sensitization. There is evidence linking formaldehyde exposure to nasal cavity cancers. Formaldehyde vapors pose a significant fire hazard. Concentrations in air between 7% and 73% have a potential for explosion, and formaldehyde has a flash point of 64 °C. All procedures should be conducted in an exhausting hood.

1. DNA-PROTEIN CROSS-LINKING

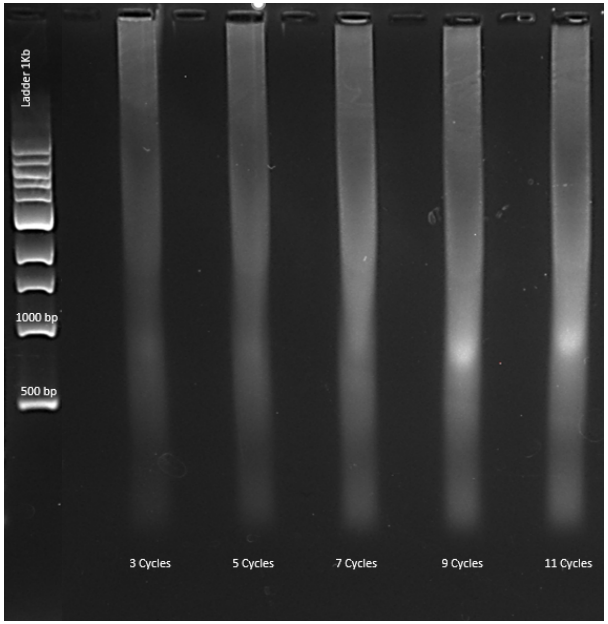
- Resuspend a $1x10^6$ cells-pellet in 500 µL of 1% formaldehyde-PBS, at room temperature.
- Mix by gentle vortexing and put on a rotator for 8 min at room temperature, to allow DNA-protein cross-linking.
- Stop the cross-linking reaction by adding glycine to a final concentration of 125 mM and incubate for 5 min at room temperature.

2. CELL LYSIS AND CHROMATIN FRAGMENTATION

This section describes the fragmentation of chromatin to produce fragments of size suitable for ChIP and PCR analysis of the immunoprecipitated material (200-1,000 base pairs). After chromatin fragmentation, it is necessary to assess fragmentation by agarose gel electrophoresis following DNA purification. This step may be omitted once sonication results are consistent and conditions have been well established for a particular cell type. Carry out all subsequent steps on ice unless otherwise stated.

- Wash the cross-linked cells twice by sedimentation (300 g for 10 min at 4°C) and resuspension in 0.5 ml PBS (for $1x10^6$ cells). Sediment the cells as above, assess pellet volume and discard the supernatant.
- Lyse cells by thorough resuspension of the pellet in 120 µl lysis buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, 1% SDS, broad-range protease inhibitor cocktail). Use room temperature lysis buffer to avoid SDS precipitation.
- Suspend the cells by pipetting and incubate for 5 min on ice.
- Sonicate each sample for 9-11 cycles x 30 sec on ice. Allow a 30 sec pause on ice between each session. We use a Bandelin Sonoplus fitted with a 3-mm probe, at 30% power.
- After sonication, centrifuge the lysate at 12,000 g for 10 min at 4°C. Collect the supernatant into a clean tube placed on ice and take care to avoid aspiration of the pelleted complexes. Do not collect the top lipid layer.
- Analyze chromatin sample by electrophoresis in a 1.2% agarose gel stained with GelRed. It is recommended using a 1000-base pair DNA ladder to evaluate size of the fragments produced.

At this point is possible to freeze fragmentated chromatin at -80 °C or directly proceed with the immunoprecipitation reaction.



Assessment of chromatin fragmentation by sonication. Chromatin was prepared by sonication as described in Section 2. Chromatin was resolved in a 1.2% agarose gel stained with GelRed. 1 kb-size ladder was used.

3. PREPARATION OF ANTIBODY-BEAD COMPLEXES

- Prepare a working solution of RIPA buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate, 140 mM NaCl).
- Prepare a slurry of Protein A-coated paramagnetic beads (Dynabeads® Protein A): for 20 ChIPs, take out 220 µl of well-suspended bead stock solution, remove the buffer after magnetic capture, remove from the magnet and add 500 µl RIPA buffer. Mix by vortexing and remove the buffer. Repeat the washing procedure once. Resuspend the beads in 210 µl RIPA buffer.
- Aliquot 90 µl RIPA buffer in a 1.5 mL tube, with one tube for each ChIP reaction.
- Add 10 µl of pre-washed Protein A-bead slurry and 2.4 µg of anti-H3K9ac antibody. Incubate at 40 rpm on a rotator for 2 h at 4°C. If necessary, prolong incubation on the rotator until chromatin samples are ready for immunoprecipitation.

NOTE

It is necessary to quantify the chromatin concentration to determine the amount to be used for each ChIP reaction. The recommended chromatin amount per ChIP is ~ 8 µg. HOW CAN THIS BE DONE?

Before proceeding with the immunoprecipitation reaction, it is suggested to take 15 µl of sheared chromatin apart, as input sample, to establish how many microliters are required to reach 8 µg.

The input sample will be indicative for the presence and amount of chromatin used in the ChIP reaction. It is an aliquot taken from the chromatin before washing step (Section 4). The chromatin aliquot is decrosslinked and DNA is isolated. This DNA sample should yield a PCR product with all primer sets used. Besides serving as a positive control, the data derived from the input sample can be used for normalization and to establish the %IP.

4. IMMUNOPRECIPITATION AND WASHES

- Dilute chromatin 10-fold of more in RIPA buffer containing protease inhibitor cocktail, to reduce SDS concentration to ~ 0.1%.
- Briefly spin tubes containing antibody-coated Protein A beads to bring down liquid caught in the lid. Place tubes in an ice-cold magnetic rack, wait for 1 min and discard the supernatant. Remove tubes from magnetic field.
- Transfer 100 µl of diluted chromatin to each tube, and incubate on a rotator at 40 rpm for at least 2 h at 4°C. This step can be carried out overnight if more convenient.
- Remove another 100 µl of diluted chromatin from which DNA will be isolated (see Section 6) and used as input chromatin reference for real-time PCR (see Section 7). Keep this sample on ice until Section 5.
- Wash immune complexes (i.e., the ChIP material) three times in fresh 100 µl RIPA buffer. Always do a brief spin of the tubes to bring down liquid caught in the lid prior to positioning in the magnetic rack. Bring the beads in suspension by gentle manual agitation prior to each wash. Each wash lasts for 4 min on a rotator at 40 rpm at 4°C. Discard the buffer between each wash.
- Wash antibody-bead complexes once in 100 µl of Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA). Incubate for 4 min on a rotator at 40 rpm at 4°C. Do not place tubes in the magnetic rack after washing.
- Transfer the suspension of beads carrying ChIP material into a clean 0.2-ml tube, magnetically capture the beads and remove the TE buffer.

5. DNA ELUTION, CROSS-LINK REVERSAL AND PROTEINASE K DIGESTION

In contrast to procedures reported in conventional ChIP protocols, elution of the DNA from the immunoprecipitated DNA-protein complex, reversal of the DNA-protein cross-link, and digestion of the proteins are combined into a single 2-h step. Also perform cross-link reversal and proteinase K treatment of the input chromatin sample.

- Add 150 µl elution buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM NaCl) containing 1% SDS, and 50 µg/ml proteinase K to the washed ChIP material.
- Put the input chromatin sample at room temperature and add 200 µl elution buffer and proteinase K to a final concentration of 50 µg/ml.
- Incubate samples for 2 h at 68°C at 1,300 rpm, on a Thermomixer.
- Remove from the Thermomixer, briefly spin, capture the beads, collect the supernatant and place it into a clean 1.5-ml tube at room temperature.
- Add 150 µl of elution buffer/SDS/proteinase K to the ChIP material and re-elute for another 5 min at 68°C at 1,300 rpm, on the Thermomixer.
- Remove from the Thermomixer, capture the beads, collect the supernatant and combine it with the first supernatant.

It is possible to move onto the next step, or alternatively, the pooled supernatants can be stored on ice until the next day or frozen at -20°C for up a month.

6. DNA PURIFICATION

The DNA Clean & Concentrator®-5 (DCC®-5) provides a hassle-free method for the rapid purification and concentration of high-quality DNA from PCR, endonuclease digestions, cell lysates, and other impure DNA preparations. It can also be used for post-RT cDNA clean-up and purification of sequencing-ready DNA from M13 phage. Simply add the specially formulated DNA Binding Buffer to your sample and transfer the mixture to the supplied Zymo-Spin™ Column. There is no need for organic denaturants or chloroform. Instead, the product features Fast-Spin column technology to yield DNA that is free of salts and contaminants in just 2 minutes. The purified DNA is ideal for DNA ligation, sequencing, labeling, PCR, microarray, transfection, transformation, and restriction digestion procedures**.**

Buffer Preparation

- *Before starting*: Add 24 ml 100% ethanol (26 ml 95% ethanol) to the 6 ml DNA Wash Buffer concentrate. Add 96 ml 100% ethanol (104 ml 95% ethanol) to the 24 ml DNA Wash Buffer concentrate.

Sample Processing

- In a 1.5 ml microcentrifuge tube, add 2-7 volumes of DNA Binding Buffer to each volume of DNA sample (see table below). Mix briefly by vortexing.

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Application	DNA Binding Buffer : Sample	Example
Plasmid, genomic DNA (>2 kb)	2 : 1	200 µl : 100 µl
PCR product, DNA fragment	5 : 1	500 µl : 100 µl
ssDNA[1] (*e.g.* cDNA, M13 phage)	7 : 1	700 µl : 100 µl

For efficient recovery of genomic or large DNA (> 20 kb to > 200 kb), use the **Genomic DNA Clean & Concentrator® (Cat. Nos. D4010, D4011)**.

- Transfer mixture to a provided Zymo-Spin™ Column in a Collection Tube.
- Centrifuge (≥ *12,000 g*) for 30 seconds. Discard the flow-through.
- Add 200 µl DNA Wash Buffer to the column. Centrifuge for 30 seconds. Repeat the wash step.
- Add 6 µl DNA Elution Buffer or 20 µl of nuclease-free water directly to the column matrix and incubate at room temperature for one minute. Transfer the column to a 1.5 ml microcentrifuge tube and centrifuge for 30 seconds to elute the DNA.

Ultra-pure DNA is now ready for use or can be stored at -20°C.

7. REAL TIME PCR ANALYSIS AND EXPRESSION OF CHIP RESULTS

The immunoprecipitated DNA is analyzed by real time PCR using your laboratory's standard operating procedure, starting from 1 µl template DNA per 10 µl reaction. Starting quantities of DNA in the ChIP samples and the input sample are determined based on the ΔΔCt method (Livak) and converted to relative expression ratio $2^{(-\Delta\Delta Ct)}$ for statistical analysis.

Real Time PCR mix preparation

Reaction mix composition:

Reagent	Final concentration	Volume
SensiFAST SYBR Lo-ROX Mix, 2X	1X	
Primers mix F+R, 10 µM	1 µM	
Nuclease-free water	-	
DNA	-	1 µl
Reaction volume	-	**10 µl**

NOTE

Both forward and reverse stock primers concentrations are 100 µM. It is recommended to dilute each of them in a single F+R primers mix in a volume of 100 µl of nuclease-free water.

Real Time PCR, thermocycler set up and run

Dispense 10 µl of the reaction mix into each well of Real Time PCR strips and load the strips inside the DNA Engine Opticon 2 Continuos Fluorescence Detection System instrument.
Run the experiment with the following settings:

1. Incubate at 95.0 °C for 00:05:00 (enzyme activation)
2. Incubate at 95.0 °C for 00:00:10 (denaturation step)
3. Incubate at 55.0 °C for 00:00:15 (annealing step)
4. Incubate at 72.0 °C for 00:00:20 (extension step)
5. Plate read
6. Go to line 2 for 49 more times
7. Incubate at 72.0 °C for 00:05:00 (final extension)
8. Melting Curve from 65.0 °C to 95.0 °C, read every 0.5 °C, hold 00:00:02
9. Incubate at 4.0 °C forever

END