



## Chromatin Immunoprecipitation Protocol for Histone Modification & Chromatin Associated Proteins (PROT03)



### Roderick O'Sullivan & Joost Martens

Research Institute of Molecular Pathology (IMP)  
Dr. Bohr-Gasse 7  
A1030 Vienna, Austria

Email feedback to:  
[osullivan@imp.univie.ac.at](mailto:osullivan@imp.univie.ac.at)

**Last reviewed:** 29 Mar 2005 by [Antigone Kouskouti](#) and [Irene Kyrmizi](#), Talianidis Lab, Herakleion. See also their alternative ChIP protocol.

### Introduction

Chromatin Immunoprecipitation (ChIP) experiments are routinely performed in many laboratories around the world to examine the occupancy of proteins or chromatin modifications over particular stretches of the genome. Briefly, chromatin extracts are prepared following fixation with formaldehyde. This bulk chromatin is then sheared into fragments of useful sizes. Aliquots are then incubated with an antibody of choice and immuno-complexed DNA is purified and analyzed, invariably by PCR. However, much discussion remains as to how best these types of experiments are performed, in particular, the method of chromatin lysate preparation, sonication efficiency, antibody concentration and washing of the immuno-complexes. In this protocol, we will describe the method used in our laboratory. This protocol has been successfully used in experiments with Mouse Embryonic Stem (ES) cells, Embryonic Fibroblasts (MEF), Trophoblast Stem (TS) cells, B-lymphocytes and hepatocytes, as well as with U2OS, 293, HeLa, T98G cells from human. Nonetheless, we would urge each investigator to expend much effort in standardizing the method for their specific experimental system.

### Procedure

Typically, when making a lysate we do so in large quantities. We would make a lysate from 7-10 maxi (15cm) dishes, sonicate the bulk chromatin lysate and store the preparation at 4°C. Naturally, the procedure remains the same for smaller scale ChIP experiments.

1. Add 1/10th volume of [crosslinking mix](#) directly to the plate containing growth medium giving a final concentration of 1% and rotate gently for 10 minutes at room temperature.
2. Quench the crosslinking reaction by the addition of 1/10th volume of 1.25M glycine giving 125mM final concentration. Remove the medium from the plate. Then wash the cells twice with 1x PBS.
3. Add 750-1000µl [lysis buffer](#) to each plate, spread on the plate and incubate at 4°C for 3-5 minutes. Harvest the cells by scraping them from the plate and transfer to a falcon tube.

Sonication is now used to disrupt the cell and nuclear membranes and to fragment the cross-linked chromatin fibres. It is crucial to generate the appropriate length of chromatin fragments as it can greatly affect the read-out one obtains at the end of the procedure. Just think, excessive sonication could render a useful chromatin lysate completely useless. A length of ~500bp-1000bp is generally accepted as optimal. Unfortunately, it is very difficult to standardize the number of sonications required from laboratory to laboratory. Therefore, the procedure must be optimized in the individual laboratories and should be carefully performed for each lysate. Also, it is best to keep the chromatin lysate on ice as this will preserve the quality of the chromatin fragments. The model of sonicator used in our laboratory is the Bandelin Sonoplus HD-070, with a MS73 tip.

1. Sonicate for 10 seconds at 50% output, with 2 minutes refractory period between sonications. Ensure to keep the lysate on ice during this time.
2. Take 50µl of lysate and use this to check the efficiency of the sonication. Proteinase K and RNaseA (500µg/ml of each) treat the sample at 37°C for 30 minutes, then transfer to 65°C for a minimum of 3 hours. Purify the DNA either by phenol-chloroform extraction followed by precipitation or by Qiagen purification.
3. Load the sample onto a 1% agarose gel and visualize by Ethidium Bromide staining. Repeat these latter steps until the

desired fragment size is obtained.

- Once the chromatin has been appropriately sonicated, measure the protein concentration of the lysate. We then adjust the concentration to  $\sim 1\mu\text{g}/\mu\text{l}$  by addition of [lysis buffer](#). This lysate can now be stored at  $4^\circ\text{C}$  for a number of months.

## Chromatin Immunoprecipitation

The principle variation in this step lies in the antibody concentration. However, as a general rule we find that  $4\mu\text{g}$  of antibody sufficiently immunoprecipitates the corresponding protein: DNA complexes. This has been very successful when using antibodies that detect site specific histone lysine methylation (Peters *et al.* 2003, Martens *et al.* 2005). However, for each different antibody the optimal concentration should be tested and calibrated. The stringency of washing the beads is also critical in order to minimize the effects that background or non-specific binding to the beads can have on the final read-out of the experiment. We carefully wash the beads 4 times in a low salt solution followed by a single wash with a high salt solution. This should minimize the background.

### Day 1

- Bring the lysate to room temperature. Spin down  $420\mu\text{l}$  of lysate for 5 minutes at maximum speed in a bench-top centrifuge. Carefully take off  $400\mu\text{l}$  and dilute to a final volume of 4 ml in a mixture of 9 parts [dilution buffer](#) and 1 part [lysis buffer](#) (in a 15ml Falcon).
- Add  $\sim 4\mu\text{g}$  of antibody per IP and rotate overnight or for a minimum of 6 hours at  $4^\circ\text{C}$ .
- Simultaneously, prepare the protein sepharose beads. Take 0.2g of protein A or  $100\mu\text{l}$  of protein G beads in a final volume of 1 ml of 9:1 [dilution buffer](#) : [lysis buffer](#). Pre-absorb the beads with  $100\mu\text{g}/\text{ml}$  BSA and  $500\mu\text{g}/\text{ml}$  sheared salmon sperm DNA and rotate overnight at  $4^\circ\text{C}$ .

### Day 2-3

- Remove the pre-absorption mix and wash the pre-absorbed beads twice in [dilution buffer](#) and finally resuspend the beads in 9:1 [dilution buffer](#) : [lysis buffer](#).
- Add, of a 1ml stock of beads,  $\sim 100\mu\text{l}$  to each falcon and incubate for 1-2 hours while rotating at  $4^\circ\text{C}$ .
- Gently centrifuge (2000rpm) the falcons and very carefully remove the supernatant. Take up the beads in 1ml of [wash buffer](#) and transfer to a 1.5ml eppendorf tube. Wash beads an additional 3 times in 1 ml [wash buffer](#) and finally once in 1ml [final wash](#) (see [comment 1](#)). Ensure all wash is removed, but do not to disturb the beads. *Do not aspirate the liquid as this can damage the immuno-complexes.* Again, gently spin (2000 rpm) the eppendorfs between washes.
- Elute the immune complexes by addition of  $450\mu\text{l}$  [elution buffer](#) to the beads, add proteinase K and RNase A ( $500\mu\text{g}/\text{ml}$  each) and incubate at  $37^\circ\text{C}$  for 30 minutes. At this time take a  $400\mu\text{l}$  or  $40\mu\text{l}$  aliquot of lysate, bring the volume to  $450\mu\text{l}$  and treat same as with the immunoprecipitated samples. This is the input that will be used in the analysis of the experiment.
- Reverse cross-links by simply placing the eppendorfs at  $65^\circ\text{C}$  overnight or for a minimum of 4 hours (see [comment 2](#)).
- Phenol-Chloroform extract and precipitate the immunoprecipitated DNA.
- Resuspend the DNA in  $50\text{-}100\mu\text{l}$  sterile water. Proceed to PCR reactions.

## Materials & Reagents

<b>crosslinking mix</b>	11% Formaldehyde 100mM NaCl 0.5mM EGTA 50mM HEPES, pH 8.0
<b>lysis buffer</b>	1% SDS 10mM EDTA, pH 8.0 50mM Tris-HCl, pH 8.0, with protease inhibitors
<b>dilution buffer</b>	1% Triton X-100 150mM NaCl 2mM EDTA, pH 8.0 20mM Tris-HCl, pH 8.0, with protease inhibitors
<b>wash buffer</b>	1% Triton X-100 0.1% SDS 150mM NaCl 2mM EDTA, pH 8.0

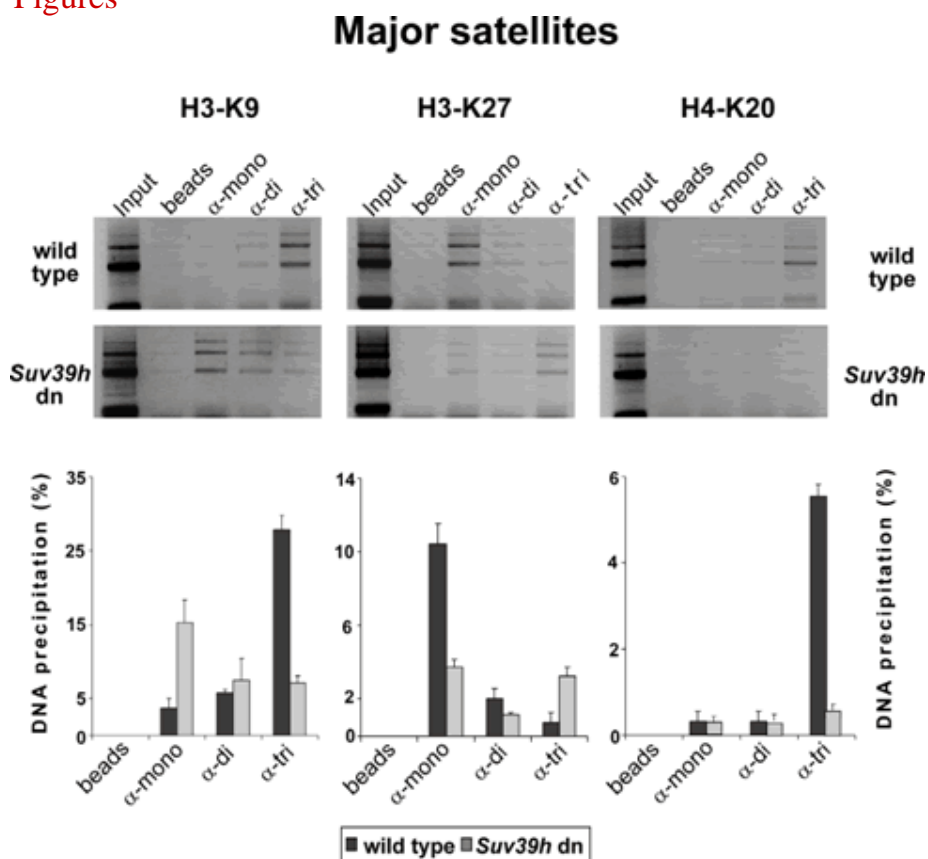
	20mM Tris-HCl, pH 8.0, with protease inhibitors
<b>final wash</b>	1% Triton X-100 0.1% SDS 500mM NaCl 2mM EDTA, pH 8.0 20mM Tris-HCl, pH 8.0, with protease inhibitors
<b>elution buffer</b>	1% SDS 100 mM NaHCO <sub>3</sub>

## Reviewer Comments

Reviewed by: [Antigone Kouskouti](#) and [Irene Kyrmizi](#), Talianidis Lab, Herakleion. See also their alternative ChIP protocol.

1. Inclusion of a further wash step with the following buffer (B) may be beneficial.  
**Washing 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).
2. The presence of 200mM NaCl is important for efficient reversal of the crosslinks during heating in our hands.

## Figures



**Figure 1.**

An example of a typical result obtained with the state specific histone methylation antibodies that have been developed in our laboratory. We show the abundance of H3-K9, H3-K27 and H4-K20 mono-, di- and tri-methylation at major satellite chromatin in wild-type and Suv39h dn ES cells. The immunoprecipitated DNA was amplified both by standard PCR (gel pictures) and by real-time PCR (bar diagrams).

## References

1. Peters AH, Kubicek S, Mechtler K, O'Sullivan RJ, Derijck AA, Perez-Burgos L, Kohlmaier A, Opravil S, Tachibana M, Shinkai Y, Martens JH, Jenuwein T. (2003) Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol Cell*. **12**(6):1577-89
2. Martens JH, O'Sullivan RJ, Braunschweig U, Opravil S, Radolf M, Steinlein P, Jenuwein T (2005) The profile of

repeat-associated histone lysine methylation states in the mouse epigenome. *EMBO J.* **24(4)**:800-12



---

© 2004-2009 Epigenome Network of Excellence  
Printed from: <http://www.epigenome-noe.net/researchtools/protocol.php>