



Carrier ChIP (CChIP) (PROT26)



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Introduction

Chromatin immunoprecipitation (ChIP) arguably represents the most powerful application of antibody technology to epigenetic research. It allows analysis of patterns of histone modification and non-histone protein distribution across genomic regions and underpins large scale epigenetic mapping projects. However, conventional ChIP generally requires at least 10^7 cells, which limits its applicability. To address this, we have developed a new protocol, CChIP, based on the use of carrier chromatin, that allows detailed and reproducible epigenetic analysis of as few as 100 cells. The procedure has been validated with primary mouse embryo material, but should be applicable to cells from various sources, including tissue biopsies and FACS-sorted cell populations. The protocol given here is for analysis of histone modifications in native, unfixed chromatin prepared by micrococcal nuclease digestion. CChIP will undoubtedly be applied to formaldehyde cross-linked chromatin, and thereby used to locate non-histone chromatin proteins, but the generally lower efficiency of precipitation with cross-linked chromatin is likely to increase the numbers of cell required.

Procedure

PART 1: Chromatin preparation from carrier (SL2) and target cells

Before Starting

1. To allow us to monitor the yield of chromatin in later steps, and to ensure accuracy in performing PCR, we routinely label carrier (*Drosophila* SL2) cells overnight with ^3H -thymidine to provide an accurate and sensitive marker for bulk DNA. This is not essential. As an alternative method for determining the amount of DNA in the unbound and bound samples following immunoprecipitation we also use a pico-green assay (Molecular probes) which is also very sensitive;
2. ALL solutions must be ICE COLD so place on ice before harvesting cells. All solutions containing sucrose must be MADE UP FRESH on the day;
3. Remember to add protease inhibitors (whichever your lab uses) to all solutions JUST BEFORE USE. We use 0.1M PMSF and Complete mini protease inhibitors (Roche).

Cells and Harvesting

1. Harvest SL2 cells (see [note 1](#)) by spinning down at $200 \times g$ for 7 minutes and wash x 3 in 40mls of ice cold PBS + 5mM Na butyrate (see [note 2](#)). To achieve a consistent single cell suspension, remember to resuspend the cell pellet first by flicking the base of the centrifuge tube before adding further solutions. Count cells and divide into aliquots of 5×10^7 cells transfer to 1.5ml Eppendorfs;
2. To each aliquot of SL2 cells (5×10^7) add your chosen "target" cells (see [note 3](#));
3. Spin down as above and wash pellet twice in ice cold 1ml [NB buffer](#) + 5mM Na butyrate;
4. Resuspend cell pellet in 2ml [NB buffer](#). Transfer to a 7ml bijou (polyethylene) and add an equal volume of [NB/1% Tween 40](#). Add a small plastic magnetic "flea" and stir gently on ice for 1 hour;
5. Homogenise sample using a hand operated, Dounce all-glass homogeniser with a "tight" pestle. After 10 smooth strokes place the homogeniser on ice until the suspension clears (usually 2-4 minutes). During the cooling/settling period, release of nuclei should be checked by examining a small aliquot under the microscope in a standard counting chamber. Whole cells and nuclei can easily be distinguished at x 40 magnification. Ideally 75-85% of cells should yield intact nuclei and this

- usually requires four cycles of homogenisation/cooling (see [note 4](#));
6. Following homogenisation transfer nuclei to 50ml centrifuge tubes and spin down nuclei at 800 x g for 15 minutes at 4°C;
 7. Resuspend nuclei in 20mls of 5% sucrose/NB and wash once in the same volume (800 x g 15 minutes at 4°C). (Remember to resuspend the pellet first by flicking the centrifuge tube before adding any solutions);
 8. Resuspend nuclei in 5ml [Digestion buffer](#). Check the amount of chromatin by measuring the A₂₆₀ of an aliquot diluted 20-fold in 0.1% SDS, eg. 20µl of Sample + 380µl 0.1% SDS;
 9. An A₂₆₀ reading of 1 (in a cuvette with a 1cm light-path) corresponds to about 50µg/ml of chromatin DNA. So the yield of chromatin (in µg) is given by A₂₆₀ x dilution factor x volume x 50;
 10. Centrifuge sample (800 x g for 10 minutes at 4°C) and resuspend pellet to a chromatin DNA concentration of 0.5mg/ml. Divide into 1ml aliquots in Eppendorf tubes (see [note 5](#));
 11. Digest chromatin with micrococcal nuclease. We use 50U per 250µg chromatin for 5 minutes at 28°C. Stop the digestion by addition of 0.5M EDTA to a final concentration of 5mM and place on ICE for 5 minutes (see [note 6](#));
 12. Centrifuge 12000 x g for 5 minutes, remove supernatant (S1) and keep on ice;
 13. Resuspend and combine pellets (if multiple micrococcal nuclease digestions have been performed) in 1ml final volume of [Lysis buffer](#) (fraction S2). Dialyse S1 and S2 overnight at 4°C against 2 litres of [Lysis buffer](#) (see [note 7](#));
 14. Centrifuge dialysed chromatin at 1800 x g for 10 minutes at 4°C. Remove supernatants and place on ice (fractions S1 and S2);
 15. Resuspend pellet resulting from centrifugation of S2 in 250µl [Lysis buffer](#) (fraction P1);

Chromatin analysis (fractions S1, S2, P)

18. Check A₂₆₀ and ³H-Thymidine dpm of all samples. Calculate distribution of chromatin between the three fractions;
19. Analyse all samples by 1.2% agarose gel electrophoresis (AGE).

DO NOT PLACE ETHIDIUM BROMIDE IN THE AGAROSE OR IN THE ELECTROPHORESIS BUFFER. DUE TO THE PRESENCE OF SDS IN THE SAMPLES THE GEL MUST BE STAINED AFTERWARDS.

20. Preparation of samples for AGE:
 - x µl Chromatin fraction (to give 2µg DNA)
 - y µl ddH₂O (x + y = 25µl)
 - 3 µl 1% SDS (final concentration 0.1%)
 - 2µl Loading buffer
21. Combine S1 and S2 fractions. Check concentration and then perform NChIP.

PART 2: precipitation of chromatin (NChIP)

1. Add 50-200µl affinity-purified antibody (50-100µg immunoglobulin) to 100-200µg unfixed chromatin and add [Incubation buffer](#) (50mM NaCl, 20mM Tris-HCl, pH 7.5, 20mM Na butyrate, 5mM Na₂ EDTA, 0.1mM PMSF) to a final volume of 1ml (see [note 8](#));
2. After overnight incubation (on a very slowly rotating platform) at 4°C, add 200µl pre-swollen [protein A-Sepharose](#) (50% v/v slurry, Pharmacia) and continue the incubation for a further 3 hours at room temperature (see [note 9](#));
3. Centrifuge the antibody-chromatin mixture at 11,600g for 10 minutes. Carefully remove and keep the supernatant on ice. This is the *unbound* fraction and should be depleted in the target protein;
4. Resuspend the [protein A-Sepharose](#) pellet in 1ml [Buffer A](#) containing 50mM NaCl and layer onto 9ml of the same buffer;
5. After centrifugation at 1200 rpm, for 10 minutes at 4°C, remove the supernatant by aspiration and wash the pellet in 10ml [Buffer B](#) containing 100mM NaCl and finally in 10ml of [Buffer C](#) containing 150mM NaCl (see [note 10](#));
6. Elute the bound material from the [protein A-Sepharose](#) by addition of 125µl 1% SDS in [Incubation buffer](#) and incubate for 15 minutes at room temperature with repeated inversion. After centrifugation at 11,600g for 10 minutes remove and **KEEP** the supernatant (**bound 1**) and store on ice;
7. Repeat this step and combine the supernatant with bound 1 to give the final bound fraction. Add an equal volume of [Incubation buffer](#) to the bound fraction to reduce the concentration of SDS to 0.5%;

Isolation of DNA

8. Add one-third volume of phenol:chloroform (1:1) to the input, unbound, and bound fractions;
9. Vortex and centrifuge at 600g for 10 minutes at 4°C to separate the phases;
10. Remove the supernatant and add an equal volume of phenol:chloroform. Repeat the centrifugation;
11. Add an equal volume of chloroform, centrifuge as before, and transfer the supernatant to a 6ml centrifuge tube;
12. Finally precipitate the DNA at -20°C using 1/100th vol of 4M LiCl, 25µl glycogen (2mg/ml) and 2 volumes of ice-cold ethanol (see [note 12](#));

Analysis of DNA following NChIP

13. Check ³H-Thymidine dpm of all samples. Calculate percentage pull-down for each antibody. Compare to the preimmune control (see [note 12](#));
14. Analyse all samples by 1.2% agarose gel electrophoresis (AGE) to check if pull down has worked (see [note 12](#));
15. Perform Species specific PCR on equal amounts of DNA from the unbound and bound samples (see [note 13](#));

Isolation of proteins

16. Precipitate the proteins from the first phenol:chloroform phase by addition of 5µg BSA (carrier), 1/100th vol 10M H₂SO₄, and 12 volumes of acetone;
17. After overnight precipitation at -20°C wash the protein pellets once in acidified acetone (1:6 100 mM H₂SO₄:acetone) and three times in dry acetone (see [note 14](#)).

Materials & Reagents

NB buffer	[Make up 500ml store at 4°C] 15mM Tris-HCl (pH 7.4) 60mM KCl 15mM NaCl 5mM MgCl ₂ 0.1mM EGTA 0.5mM 2 mercaptoethanol 0.1mM PMSF
NB/1% Tween 40	[10ml] Ensure that the Tween 40 is completely dissolved. We normally warm under the tap and keep at room temperature. If kept on ice for prolonged periods the Tween 40 comes out of solution as a white precipitate.
25% SucroseNB	[100 ml] Dissolve 25g sucrose in NB buffer and make final volume up to 100ml.
Digestion buffer	[100 ml] 0.32M Sucrose 50mM Tris-HCl (pH 7.4) 4mM MgCl ₂ 1mM CaCl ₂ 0.1mM PMSF
Lysis buffer	[2 litres] 2mM Tris-HCl (pH 7.4) 0.2mM EDTA 5mM Na butyrate 0.2mM PMSF 0.4M Glycine Plus additional protease inhibitors (Complete mini, Roche, or your equivalent).
Incubation buffer	50mM NaCl 20mM Tris-HCL (pH 7.5) 20mM Na butyrate 5mM Na ₂ EDTA 0.1mM PMSF
Buffer A	50mM Tris-HCL (pH 7.5) 10mM EDTA 50mM NaCl 5mM Na butyrate

Buffer B	50mM Tris-HCL (pH 7.5) 10mM EDTA 100mM NaCl 5mM Na butyrate
Buffer C	50mM Tris-HCL (pH 7.5) 10mM EDTA 150mM NaCl 5mM Na butyrate
protein A-Sepharose	Pre-swell protein A-Sepharose overnight in Buffer A at 4°C. After centrifugation (2000rpm 10 minutes MSE Chilspin) resuspend pellet in an equal volume (50% w/v) of Buffer A .

Authors Notes

1. *Drosophila*

SL2 cells are grown at 26°C in Schneider's medium (Gibco) supplemented with 8% foetal calf serum (Gibco) and antibiotics (50 units per ml Penicillin, 50µg per ml Streptomycin). We usually grow these cells in standard 75cm² tissue culture flasks laid flat and with sealed caps (gassing is unnecessary). The cells grow in suspension as loose clumps and are usually split 1 to 4 every two weeks.

Pellets of SL2 cells (ideally 5x10⁷

cells) can be safely stored at -80°C for two months or more prior to use in CChIP. But if you use frozen SL2 cells, then use only one round of homogenisation (i.e. 10 strokes) to release the nuclei (step 4 below). Check the nuclei: cell ratio and if necessary give 5 more strokes. The frozen nuclei are more fragile than fresh ones and more easily lysed.

- We have added Na butyrate to prevent deacetylation during the isolation procedure and this is present in all our solutions, along with protease inhibitors.
- This will usually be between 100 and 10,000 cells. The minimum number of cells on which we have successfully performed CChIP is 50-100, BUT at such low cell numbers, the number of PCR reactions that can be performed on Bound and Unbound fractions is inevitably small. Detection of target cell DNA requires a very high efficiency of immunoprecipitation and minimal experimental losses.
- It is important that steps 4 and 5 are carried out promptly and that cell lysis and release of nuclei is efficient. The presence of large numbers (>20%) of intact cells has a detrimental effect on the quality of the chromatin after micrococcal nuclease digestion.
- We have found that a more reproducible digestion is achieved if the micrococcal nuclease step is performed in 1ml aliquots rather than larger volumes.
- This step MUST be carefully timed and controlled. Over-digestion will lead to sub-nucleosomal particles and possible degradation of target sequences. Under-digestion will lead to a reduced overall yield of chromatin and possible under-representation of chromatin from more condensed regions of the genome. Note that the NChIP procedure has consistently given preferential precipitation of oligonucleosomes in comparison to mononucleosomes. For efficient precipitation, a chromatin preparation consisting predominantly of oligonucleosomes (centered around 3-5mers) is ideal.
- We have found that it is not necessary to dialyse S1 at this point. However, if S1 is not dialysed, be sure to add glycine to S1 at a final concentration of 0.4M to make it equivalent to the S2 fraction. If this is not carried out then S1, S2 and P fractions will electrophorese differently on agarose gels.
- If after combining S1 and S2 the sample is too dilute to achieve the concentration required for ChIP (ideally 200mg/ml) then concentrate the sample using a Centricon concentrator.
- We have found that the use of affinity-purified antibodies reduces the amount of non-specific binding. The optimum amount of antibody added is dependent on its titer and on the amount of target protein present in the chromatin and must be determined for each antiserum. Commercially available antiserum has been employed successfully in XChIP using very small amounts (5-50µl) and subsequent analysis by PCR. We always use affinity purified antisera for ChIP and either whole sera or 50% saturated ammonium sulphate cuts for Western blotting.
- [protein A-Sepharose](#)
is available commercially as a freeze-dried powder and should be preswollen in 50mM Tris-HCl, 5mM Na EDTA, 50mM NaCl. The concentration of the NaCl can be increased depending on the affinity of the antibody for its target protein. Increasing the NaCl concentration reduces the amount of non-specific binding, but may also reduce the binding affinity of the antibody for the target protein. Preliminary experiments changing the NaCl concentration should be employed to determine the optimum conditions for the antibody used.
- The concentration of the NaCl can be increased to reduce non-specific binding. We have found that large volume washes, carried out in 15-ml siliconized centrifuge tubes, greatly reduce non-specific binding.
- We routinely add glycogen (5µg) as a carrier to maximize the precipitation of DNA from the bound fraction. Ethanol used in the precipitation should be molecular biology grade (e.g., Analar-BDH) and stored at -20°C to ensure rapid precipitation. The volume you re-suspend your pellet in following immunoprecipitation is VERY IMPORTANT in CChIP. Remember that both the Unbound and Bound samples will have low amounts of 'target' DNA in them. If you have spiked with a very low number of cells (100 say) then remember that you will have only 200 molecules of any particular DNA fragment. With an overall yield of 40%, then you will have 80 molecules in the Bound and Unbound fractions combined. Decide how

many fragments do you need per PCR reaction and resuspend accordingly. The total amount of DNA in each fraction is determined by the amount of [³H] thymidine present (using scintillation counting) and one must assume that the recovery for the 'carrier' and target will be equivalent. Remember to keep the first phenol:chloroform phase for subsequent protein isolation. We routinely analyze the DNA samples by electrophoresis on 1.2% agarose gels followed by staining with ethidium bromide.

13. In order to detect your 'target' DNA **species-specific** PCR must be performed. All 'target' primers should be checked for cross reactivity against 'carrier DNA', both by BLAST search and by visualisation following test PCR. We have found that PCR primer sets that should be mouse-specific according to the BLAST search, can still cross react with *Drosophila* when tested by radioactive PCR, normally giving a higher molecular weight product. We have chosen to use radioactive PCR followed by analysis on DNA PAGE, drying down of gels and exposure to phosphorimager screens for our routine analyses. Initial analyses establish the linear range for each primer set. This has proved a reliable and quantitative method that confirms both that the assayed product is of the right size and absence of cross-reactivity with the 'carrier' DNA. Alternatively, analysis of samples could be performed by Real Time Quantitative-PCR, though we would recommend use of Taqman probes for the added specificity they provide.
14. We routinely analyze the proteins by electrophoresis on SDS-polyacrylamide gels. If proteins are to be analyzed on acid/urea/Triton (AUT) gels, the acetone pellet should be resuspended in 500µl double-distilled H₂O and centrifuged through microconcentrators (Amicon) for 30 minutes at 11,600 x g to reduce the volume. This dilution:concentration step is repeated (to reduce residual SDS) and 2vol of AUT loading buffer (8M urea, 5% 2-mercaptoethanol, 1M glacial acetic acid, plus a few drops of tracking dye (pyronineY)) is added to the final concentrated sample. Western blotting and immunostaining are carried out using standard methodology.

References

1. O'Neill, L.P. and Turner, B.M. (2003) Immunoprecipitation of native chromatin: NChIP, *Methods*, **31(1)**: 76-82.
2. O'Neill, L.P., VerMilyea, M.D. and Turner, B.M. (2006) Epigenetic characterization of the early embryo with a chromatin immunoprecipitation protocol applicable to small cell populations, *Nature Genetics*, **38(7)**: 835-841.