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Céline Cosseau, Christoph Grunau

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16. Native Chromatin Immunoprecipitation (nChIP)

Céline Cosseau and Christoph Grunau*

Affiliation of both authors:

UMR 5244 CNRS-EPHE-UPVD

Biologie et Ecologie tropicale et méditerranéenne

CBETM, Université de Perpignan

52 Avenue Paul Alduy

66860 Perpignan Cedex, France

Tel 33 (0)4.68.66.21.80

Fax 33 (0)4.68.66.22.81

* corresponding author:

Email: christoph.grunau@univ-perp.fr

Abstract

Native chromatin immunoprecipitation refers to a method that allows for identification and quantification of DNA that is associated with specific chromatin proteins without altering the structure of these proteins. The method has been used with great success in the past and has some advantages over the more widely used cross-linking chromatin immunoprecipitation. We describe here a protocol that was specifically optimized for low cell numbers.

Key Words

native chromatin; histones; immunoprecipitation

1. Introduction

In contrast to cross-linking chromatin immunoprecipitation (xChIP), native ChIP relies on relatively strong interaction between the target proteins and DNA under physiological salt conditions (**1, 2**). Histones are a typical example of such proteins, but nChIP has also successfully been used with transcription factors (**3**). In this protocol we focus on histone isoforms that are typical examples for a chromatin marking system. When histone-style proteins are targeted, we see three advantages of nChIP compared to xChIP: (i) the proteins remain in their native form and there is no danger that crosslinking fixes interactions that do not occur systematically in the cell; (ii) nChIP is 10-100 times more sensitive than xChIP and less starting material is required; and (iii) since enzymatic fragmentation of chromatin is used, no expensive equipment such as a sonicator is necessary. Nevertheless, xChIP and nChIP are complementary, and ideally both approaches should be used in parallel.

2. Material

2.1. *Enzymes and enzyme inhibitors*

1. 100 mM Dithiothreitol (DTT) in distilled water, aliquot to 1 ml (store at -20°C). DTT is a reducing agent that prevents the formation of disulfide bonds in and between proteins.
2. 25 mM Phenylmethanesulfonylfluoride (PMSF) in isopropanol, 10 ml (store at -20°C). PMSF is a serine protease inhibitor.
3. Roche Complete Protease Inhibitor tablets (ref: 11 697 498 001) (store at 4°C)
4. 2.5 M Sodium butyrate in distilled water (Sigma B5887 1g) (store at 4°C). Sodium butyrate is an histone deacetylase (HDAC) inhibitor. Sodium butyrate is not very stable and should not be stored for more than 4 weeks in solution. The product is irritant and the solution has a nauseating odor, wear gloves!

5. 15 U/ μ l Micrococcal nuclease (MNase) (EC 3.1.31.1) (USB 70196Y) in sterile 50% glycerol, aliquot to \sim 10 μ l and store at -20°C. Do not refreeze. MNase digests DNA between nucleosomes (4, 5).
6. 2% NaN₃ in water (store at 4°C) as preservative. Sodium azide is very toxic.

2.2. Antibodies and Sepharose-Protein A

1. aliquot antibodies on arrival to 2-4 μ l and store at -20°C
2. preparation of sepharose-protein A:
 - a. weight 250 mg sepharose-protein A in 15 ml falcon tube
 - b. wash with 10 ml sterile water
 - c. centrifuge 10 min at 4000 rpm
 - d. remove supernatant
 - e. repeat washing step four times
 - f. add sterile water to 5 ml
 - g. add NaN₃ to 0.02% and store at 4°C

250 mg Protein A - sepharose swell to approximately 1 ml gel and bind about 20 mg human IgG.

You will need 50 μ l of the homogeneously mixed sepharose-protein A per ChIP. Protein A has strong affinity to human, mouse and rabbit IgG, for antibodies raised in goat and sheep, sepharose-protein G or protein A/G mixtures should be used. Paramagnetic sepharose particles are available but in our hands, background was on average 20 times higher than in a centrifugation-based separation.

2.3. Other stock solutions

1. 1 M KCl, autoclave
2. 5 M NaCl, filter to prevent formation of crystals later and autoclave

3. 1 M MgCl, autoclave
4. 1 M Tris/Cl pH 7.4 - 7.6, autoclave
5. 0.5 M EDTA, autoclave
6. 1 M CaCl₂, 10 ml, sterile filter or autoclave
7. 20% SDS, sterile filter
8. 20 g/l glycogen solution, aliquot to 100 μ l (store at -20°C)

2.4. Other equipment

1. micro dialysis units (Slide-a-Lyzer 3500 D cut-off, Pierce 69550)
2. if micro dialysis units are not available, prepare dialysis tubes:
 - a. cut tube (e.g. VWR international dialysis tube 0.5 mm) into pieces of 10 - 20 cm length
 - b. boil for 10 min in a large volume of 2% (w/v) sodium bicarbonate and 1 mM EDTA
 - c. rinse the tube thoroughly with distilled water
 - d. boil for 10 min in 1 mM EDTA
 - e. cool and store in this solution at 4°C
 - f. before use, wash tube inside and outside with distilled water
3. centrifuges, stirrers, waterbath or similar, PCR machine, gel electrophoresis

3. Methods

3.1. Step 1: Testing the antibodies

By their very nature, immunoprecipitation methods rely on antibody-antigen interactions.

Specificity and the strength of this interaction will determine the quality of the ChIP experiment in

terms of specificity and sensitivity. Since histones, the principle targets of nChIP, are highly conserved proteins, most laboratories will prefer to buy antibodies instead of producing their own. Several monoclonal and polyclonal antibodies against histone isoforms are now commercially available. Some of them are “ChIP-certified”, that is to say the manufacturer guarantees success of the immunoprecipitation if the “certified” animal or plant species are used. Nevertheless, antibody specificity and efficiency must always be tested. A simple Western blot gives a good indication of specificity. Already a straightforward protein extraction and separation on a standard SDS-PAGE will provide sufficient information (*see Note 1*). If bands can be detected outside the expected size range of the target proteins, and if the secondary antibody is not the origin of the problem, then antibodies from another source should be tested. Some companies provide antibody samples. While it cannot be excluded that antibodies that show unspecific binding in the Western blot are specific in the IP conditions, such antibodies should be avoided. The non-specific activity is probably due to immunogenic protein carriers used during immunization such as keyhole limpet hemocyanin KLH (**6**). An example is shown in figure 1. Ideally, a single band of the expected size should be visible. In the example in figure 1, antibodies from supplier AB1 should not be used for ChIP since additional bands are detected. Antibodies from supplier AB2 are suitable. However, also absence of any detection does not indicate that the antibody does not work in immunoprecipitation. If the pre-immune serum is available, IgG from this serum must be used as control in all steps of the here described procedure.

3.2. Step 2: Chromatin preparation

The principle of the procedure is shown in figure 2. The procedure follows tightly earlier protocols (**7, 8**) but has improved sensitivity and lower background (we use it routinely with less than 1500 *Schistosoma mansoni* larvae, i.e. roughly 150000 cells (**9**)). The following procedure describes nChIP for 3 samples and one control. One should use 10^6 - 10^7 (at least 10^5) cells for each sample. Cells can be aliquoted and stored at -80°C for up to 6 month or in liquid nitrogen for up to 12 month. However, it is preferable to use fresh biological material.

1. prepare a centrifuge for 10 or 50 ml tubes, up to 8500 rpm and cool it down to 4°C
2. preheat a water bath to exactly 37°C
3. prepare the solutions in tables 1-6 (always freshly)

3.2.1. Cell lysis and purification of nuclei

1. for culture cells or cell suspensions:
 - a. multiply 10^6 - 10^7 cells with the number of antibodies to be used in immunoprecipitation (IP), for the control use the same amount of cells as for one antibody
 - b. for each IP and for the control, transfer the corresponding amount of cells into a 2 ml Eppendorf tube and wash with 1 ml 150 mM NaCl (centrifuge 2500 rpm 10 min 4°C, remove supernatant)
2. for tissue samples: cut into small pieces and wash as above, use 10^6 - 10^7 cells per antibody
3. resuspend pellets completely in 1 ml buffer 1
4. add 1 ml buffer 2 and homogenize for 3 min with Dounce (pestle A) on ice
5. put on ice 7 min
6. fill 8 ml buffer 3 into a 14 or 50 ml corex centrifugation tube, label the tubes appropriately
7. use 2 corex tubes for each sample
8. overlay the 8 ml buffer 3 with 1 ml cell suspension so that the tubes are ready for centrifugation 10 min after buffer 2 has been added to the cells, disturb the interface between buffer 3 and the sample a little bit with the pipette tip
9. if you don't use a swing-out rotor, mark the tubes on the exterior side (to know where to look for the nuclei)
10. centrifuge 8500 rpm, 20 min, 4°C (ideally in a swing-out rotor)

11. remove the supernatant completely and carefully

3.2.2. Chromatin fragmentation with MNase

1. resuspend the two nuclei-containing pellets for each sample in 1 ml MNase digestion buffer
2. aliquot 500 μ l of this suspension into two 1.5 ml Eppendorf tubes
3. add 1 μ l MNase (15 U) and incubate 2-6 min at 37°C (*see Note 2*)
4. to stop the reaction add 20 μ l 0.5 M EDTA to each 500 μ l MNase digest and put the tube on ice
5. centrifuge 13000 g, 10 min, 4°C
6. transfer the supernatant to a new tube (S1) and keep the pellet (P1) on ice
7. quantify chromatin in S1 by measuring OD at 260 nm against MNase buffer (In general we find about 50 μ g/ml DNA in the undiluted S1, OD_{260/280} values can be bad because there is a lot of protein in the solution. DNA quantification is therefore not precise but sufficient for reproducibility.)
8. store S1 at -20°C
9. humidify Slide-a-Lyzer with 50 μ l dialysis buffer
10. resuspend the pellet P1 in 100 μ l dialysis buffer and dialyze overnight at 4°C against 50 ml dialysis buffer with gentle stirring. This dialysis step is important for liberation of chromatin fragments from the nuclear debris. For some cell types this step can be skipped, if no DNA can be detected in the pellet. In general, the dialysis step is however necessary (figures 3 and 4).
11. the next day, prepare a 2% 0.5x TBE agarose gel with 20 μ l slots
12. thaw yesterdays supernatant S1, transfer dialyzed sample to Eppendorf tubes and...

13. centrifuge both at 13000 g, 10 min, 4°C
14. Transfer the supernatants to new tubes and repeat the centrifugation and transfer steps 2 times, discard the pellets (P2). These triple centrifugations are important! They reduce the unspecific background! If you still observe a pellet after the third centrifugation, repeat the centrifugation step.
15. The final supernatants are fractions S1 (non-dialyzed) and S2 (dialyzed).
16. use 50 µl of S1 and S2 for phenol/chloroform extraction, centrifuge and load 20 µl of supernatant on a 2% 0.5x TBE agarose gel (100V, 25 min) to verify the presence of mono- to pentanucleosomes.

3.2.3. Incubation with the antibody

When the antibodies have passed the initial Western blot test, the right antibody-to-chromatin ratio must be determined. For a given amount of chromatin, the antibody must be in excess, and the amount of immunoprecipitated DNA must not depend on the amount of antibody used. This titration procedure can be done with the below outlined procedure, using a constant quantity of chromatin and increasing amounts of antibodies. From a certain quantity of antibody on, the amount of immunoprecipitated DNA should remain constant (figure 5). For further experiments, an antibody concentration above this threshold must be used. If no threshold can be reached, the antibody is not suitable for IP since the amount of precipitated DNA will be a function of the used antibody amount. The advantage is that even if antibody concentrations are unknown, the suitable amount (in µl) can easily be determined. The procedure is costly since relatively large amounts of antibodies are consumed and much biological material is required, but it assures that in the following steps reproducible results can be achieved. Failure to determine the correct antibody to chromatin ratio would lead to many difficulties in subsequent experiments.

1. Prepare a dilution series of your chromatin in MNase buffer starting with 20 - 40 μg chromatin DNA for histone ChIP and use 2-20 μg antibody (if the concentration is not known use 2 - 20 μl antibody).
2. Add appropriate amounts of stock solutions to generate the antibody incubation buffer (150 mM NaCl, 20 mM sodium butyrate, 5 mM EDTA, 0.2 mM PMSF, 20 mM Tris/Cl pH 7.5) by taking into consideration the amount of S1 and S2 and their respective buffers.
3. Dilute S1 and S2 in 1ml final volume of antibody incubation buffer.
4. Add the appropriate amount of antibody (typically 4-8 μg). Add an equal volume to the control tube without antibody.
5. Incubate overnight at 4°C on a slowly rotating wheel.

3.3. Immunoprecipitation and DNA extraction

3.3.1. Immunoprecipitation

1. prepare 50 μl of sepharose-protein A for each tube
2. wash the beads to remove NaN_3 : short spin, remove supernatant and replace with equal volume of sterile water
3. add 50 μl of sepharose-protein A to each tube, including the control tube
4. incubate at least 4 h at 4°C on a rotating wheel
5. prepare washing buffers (10 ml / tube) and cool down to 4°C (table 7)
6. centrifuge chromatin-antibody-beads mixture 10 min 4°C 11600 g
7. keep the supernatant in a 2 ml tube. This is the unbound fraction UB.
8. resuspend the pellet in approximately 1 ml washing buffer A and transfer into a 15 ml Falcon tube containing 9 ml washing buffer A

9. mix for 10 min on a rotating wheel at 4°C
10. centrifuge 10 min 4000 rpm 4°C and pour off supernatant
11. add 10 ml washing buffer B, mix for 10 min on a rotating wheel at 4°C and centrifuge 10 min 4000 rpm 4°C
12. pour off supernatant
13. add 10 ml washing buffer C, mix for 10 min on a rotating wheel at 4°C and centrifuge 10 min 4000 rpm 4°C
14. pour off supernatant
15. centrifuge 10 min 4000 rpm 4°C
16. remove remaining supernatant completely (centrifuge if necessary)
17. resuspend pellet in 500 µl elution buffer (table 8) at room temperature
18. transfer to a 1.5 ml tube and incubate 15 min at room temperature on a rotating wheel
19. centrifuge 10 min, 11600 g, 18-20°C
20. transfer supernatant to a 1.5 ml tube. This is the bound fraction B.

To make sure, that the correct protein was immunoprecipitated, it is advisable to do a Western blot for fraction B. This is often not trivial since Protein A will leach into the SDS gel, and bands corresponding to the heavy and light chains of the antibody will be detected. Using beads coated with recombinant Protein G can in some cases provide a solution.

3.3.2. DNA extraction

1. use a standard phenol/chloroform extraction of DNA (*10*) in fractions B and UB
2. add 1 µl of a 20 g/l glycogen stock solution

3. add NaCl to 250 mM (26 μ l and 52 μ l for B and UB, respectively) and add 1 volume isopropanol
4. put overnight at -20°C or 30 min at -80°C
5. precipitate by centrifugation and wash with 70% ethanol
6. dry the pellet and resuspend in 40 μ l 10 mM Tris/Cl
7. for single-copy loci use 2.5 μ l of this DNA for PCR in 10 μ l reactions (quantitative real-time PCR) or continue with massive sequencing (see chapter 22). In qPCR, single locus DNA should deliver Ct values of around 25 when starting with 150000 cells.

3.4. Step 5: Quantification of precipitated DNA

The amount of genomic region of interest (RoI) must be determined in the unbound and bound fractions of the control without antibody, and in the bound fraction with antibody (B). The unbound control fraction corresponds to the input (I), and the bound control fraction is the background (BG), i.e. DNA that sticks to the beads (figure 2). If no DNA can be detected in fraction B, the antibody unbound fraction UB must be analyzed, in order to exclude that DNA was entirely degraded. Essentially, there are two possibilities to determine the quantity of RoI, either by comparing to an internal standard (reference locus method), or by determining the proportion of immunoprecipitated DNA compared to the input (% input recovery method).

3.4.1. Relative quantification (reference locus method)

In this case, in fraction B, I and BG a reference RoI and target RoI are quantified by quantitative PCR. The relative enrichment is calculated according to the following simplified formula:

$$\text{enrichment factor} = 2^{-\Delta\Delta Ct}$$

$$\text{where } \Delta\Delta Ct = (Ct_{\text{target}} - Ct_{\text{reference}})_{\text{B}} - (Ct_{\text{target}} - Ct_{\text{reference}})_{\text{I}}$$

In this case it is assumed that the amplification efficiency of the PCR is close to 2 and identical for the two qPCR (for a detailed description of the underlying assumptions of the method and possible pitfalls see **(II)**). It could also be envisaged to use an efficiency-corrected comparative quantification or multiple reference genes.

By using a standard curve, the amount for each locus can be determined based on a dilution series of DNA of known concentration. The PCR efficiencies can be different. In this case:

$$\text{enrichment factor} = [\text{ng target(B)}/\text{ng reference(B)}] / [\text{ng target(I)}/\text{ng reference(I)}]$$

The standard curve method should be preferred if sufficient qPCR reactions can be performed simultaneously. The advantage of this quantification method is that target to reference DNA ratio in the same tube is measured. Even if DNA is lost during the purification process, the relative enrichment remains the same. Despite the relatively complex procedure, enrichment factors are very reproducible; in our hands standard errors are around 10%. The reference locus can for instance be in the body of a housekeeping gene. The enrichment factor described how much more chromatin modifications are associated with the target locus compared to the reference. A principal caveat of this quantification method is that it assumes that chromatin structure in the reference locus does not change. This is probably true for regions in the body of housekeeping genes, but it cannot be excluded, that under particular conditions, these regions change their chromatin status in parallel with the target locus. In this case, the relative quantification method would not be appropriate.

3.4.2. Absolute quantification (% input recovery method)

A solution could be to quantify directly the amount of precipitated target RoI (bound) and to compare it to the amount of target RoI in the input I (unbound fraction of control). In general, this ratio is expressed in % input recovery **(I2)**:

$$\% \text{ input recovery} = 100 * \text{PCR-efficiency}^{(\text{Ct-input} - \text{Ct-bound})}$$

The problem with this method is that DNA in two different tubes is compared (B and UB-C). If more DNA is lost during the preparation process in one tube, this will induce errors that cannot be

detected. The method is sensitive to pipetting errors, requires careful standardization and, naturally, several technical and biological duplicates must be performed. Even then, day-to-day variations can be high (figure 6). Input recovery of BG should be $\leq 0.1\%$.

In conclusion, for a successful nChIP, it is indispensable to test the specificity of the antibodies and to determine the optimal amount by titration, prior to the experiment. For titration, a suitable target region in the genome must be known. For histone isoforms this will be relatively straightforward, for other chromatin proteins with restricted target regions, this might be more difficult.

Nevertheless, and for obvious reason, this positive control region is necessary. In the experiment itself, input recovery in the background must be low ($\leq 0.1\%$). In our hands, quantification via the reference locus method gives better reproducibility. The % input recovery method has relatively high day-to-day variations and, apart from the comparison to the background, it should only be applied if a reference locus cannot be used.

4. Notes

1. Protein extraction and Western Blot: For high-resolution analysis of histones and histone isoforms, classical acid extraction and Triton-Acetic Acid-Urea (TAU) gels should be used (**13**). For antibody testing we recommend the faster following method: Roughly 3×10^5 cells are suspended in denaturation buffer (0.2 % bromophenol, 10% sucrose, 3% SDS, and 0.2 M DTT, 62.5 mM Tris/Cl, pH 6.8) treated by sonication (e.g. 60% intensity of Vibra cell sonicator 75185, 6 times 15 sec, with cooling intervals on ice) and boiled 10 min at 95°C. Proteins are separated by conventional 15% SDS-PAGE and transferred for 1 hour to nitrocellulose membranes (e.g. Amersham RPN203D) by the semi-dry method. The membranes are blocked overnight at 4°C in blocking buffer (150 mM NaCl, 0.05% v/v Tween 20, 5% w/v fat-free dry milk, 20 mM Tris/Cl, pH 7.5) and incubated with the desired antibody for 2 hours in blocking buffer. The membranes are washed with washing buffer (150 mM NaCl, 0.05% v/v Tween 20, 20 mM Tris/Cl, pH 7.5) and incubated with a

peroxidase-coupled secondary antibody for 30 min in blocking buffer. Bands are revealed by chemical luminescence (e.g. ECL Pierce) and direct exposure to x-ray film (Amersham EmNo.27304). For stripping, incubate the membrane at 50°C for 1 hour in stripping solution (2% SDS, 0.8% beta-mercaptoethanol, 62 mM Tris/Cl pH 6.8) in a tightly closed 50 ml tube and wash with distilled water several times until the smell of beta-mercaptoethanol has completely disappeared. A membrane can be used 3-4 times.

2. Optimization of MNase digestion: The digestion time must be determined experimentally and can be different for each cell type and each MNase preparation. To determine the optimal digestion time, transfer every minute 100 µl of the reaction mix into Eppendorf tubes containing 10 µl 0.5 M EDTA on ice. Mix 50 µl of each fraction with 50 µl phenol-chloroform, vortex, centrifuge and apply 20 µl of the upper phase on the 2% 0.5x TBE agarose gel. Separate the fragments (100V, 25-35 min) and observe after ethidiumbromide or other DNA staining. Optimal digestion is achieved when DNA fragments corresponding to 1 - 5 nucleosomes are visible. For CHIP followed by massive sequencing (CHIP-Seq), mononucleosomes are preferred, however, these mononucleosomes should not be produced by prolonged digestion with Mnasel since this will introduce a bias in heterochromatin to euchromatin ratio. Other methods such as sucrose gradient centrifugation should be used (7). In our hands, typical incubation times for 10^6 cells and 37°C are 2-6 minutes. An example is shown in figure 4A. In this case, liver tissue of a Syrian hamster (*Mesocricetus auratus*) was used. After 8 min, virtually all chromatin is digested to mononucleosomes. Figure 4B illustrates the distribution of DNA fragments in the different fractions of the chromatin preparation. Fraction S2 is enriched in larger fragments. It should be noted that Mnasel incubation time influences also on the relative amount of DNA in the different fractions. This is shown in table 9. In this example, after 4 min Mnasel treatment, the distribution of different loci in the hamster genome is relatively homogenous. In contrast, after incubation for 8 min with Mnasel, distribution is uneven. Since the majority of the chromatin in S1 and

S2 is digested to mononucleosomes of around 150 bp, this might also reflect problems in PCR amplification for larger primer distances.

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table 1: 2x base buffer	
1x: 60 mM KCl, 15 mM NaCl, 5 mM MgCl ₂ , 0.1 mM EDTA, 15 mM Tris/Cl, pH 7.5	
6 ml	1 M KCl
1.5 ml	1 M Tris/Cl
0.3 ml	5 M NaCl
0.5 ml	1 M MgCl ₂
20 μ l	500 mM EDTA
to 50 ml with autoclaved distilled water, put on ice	
2 Roche protease inhibitor tablets	

table 2: buffer 1	
0.3 M sucrose, 5 mM CH ₃ CH ₂ CH ₂ COONa, 0.1 mM PMSF, 0.5 mM DTT, 60 mM KCl, 15 mM NaCl, 5 mM MgCl ₂ , 0.1 mM EDTA, 15 mM Tris/Cl, pH 7.5	
2.58 g	sucrose
12.5 ml	2x base buffer
50 μ l	2.5 M sodium butyrate
100 μ l	25 mM PMSF
125 μ l	100 mM DTT
to 25 ml with autoclaved distilled water, put on ice	

table 3: buffer 2	
0.3 M sucrose, 5 mM CH ₃ CH ₂ CH ₂ COONa, 0.1 mM PMSF, 0.5 mM DTT, 0.8% (v/v) NP40, 60 mM KCl, 15 mM NaCl, 5 mM MgCl ₂ , 0.1 mM EDTA, 15 mM Tris/Cl, pH 7.5	
10 ml	buffer 1
put on 37°C to allow Nonidet P-40 (NP40) to be pipetted into the buffer	
80 μ l	NP40 or replacement product (Sigma-Aldrich has replaced Nonidet P-40 with a nonionic, non-denaturing substitute detergent (Fluka 74385). The principle is that the detergent will destroy the cytoplasmic membrane but not the nuclear membrane.

table 3: buffer 2	
	Centrifugation separates the intact nuclei by precipitation.)
put on 37°C to fully dissolve NP40, mix well but do not vortex and put on ice	

table 4: buffer 3 for 3 samples and 1 control	
1.2 M sucrose, 5 mM CH ₃ CH ₂ CH ₂ COONa, 0.1 mM PMSF, 0.5 mM DTT, 60 mM KCl, 15 mM NaCl, 5 mM MgCl ₂ , 0.1 mM EDTA, 15 mM Tris/Cl, pH 7.5	
20.55 g	sucrose
25 ml	2x base buffer
100 µl	2.5 M sodium butyrate
200 µl	25 mM PMSF
250 µl	100 mM DTT
to 50 ml with autoclaved distilled water, put on ice	

table 5: MNase digestion buffers	
0.3 M sucrose, 5 mM CH ₃ CH ₂ CH ₂ COONa, 0.2 mM PMSF, 4 mM MgCl ₂ , 1 mM CaCl ₂ , 50 mM Tris/Cl, pH 7.5	
1.1 g	sucrose
0.5 ml	1M Tris/Cl
80 µl	25 mM PMSF
40 µl	1 M MgCl ₂
20 µl	2.5 M sodium butyrate
10 µl	1 M CaCl ₂ (essential for the enzyme)
to 10 ml with autoclaved distilled water, put at 37°C	

table 6: Dialysis buffers	
5 mM CH ₃ CH ₂ CH ₂ COONa, 0.2 mM PMSF, 0.2 mM EDTA, 1 mM Tris/Cl, pH 7.5	
10-20 ml	autoclaved distilled water
400 μ l	25 mM PMSF
100 μ l	2.5 M sodium butyrate
50 μ l	1M Tris/Cl
20 μ l	500 mM EDTA
to 50 ml with autoclaved distilled water, put on ice	

table 7: Washing buffers A,B and C (example 100 ml)	
5 mM CH ₃ CH ₂ CH ₂ COONa, 10 mM EDTA, 75 mM (A) / 125 mM (B) / 175 mM (C) NaCl, 50 mM Tris/Cl, pH 7.5	
1.5 ml (A)	5 M NaCl
or 2.5 ml (B)	
or 3.5 ml (C)	
200 μ l	2.5 M sodium butyrate
5 ml	1M Tris/Cl
2 ml	500 mM EDTA
to 100 ml with autoclaved distilled water, put on ice	

table 8: Elution buffer	
1% SDS, 50 mM NaCl, 5 mM EDTA, 20 mM sodium butyrate, 0.1 mM PMSF, 20 mM Tris/Cl pH 7.5	
500 μ l	20% SDS
200 μ l	1M Tris/Cl
100 μ l	5 M NaCl
80 μ l	2.5 M sodium butyrate
40 μ l	25 mM PMSF

table 8: Elution buffer

to 10 ml with autoclaved distilled water

Table 9: % DNA in the different fractions after 4 and 8 min Mnase I digest at 37°C

Locus	4 min Mnase I				8 min Mnase I				Quantification method
	S1	S2	S1+S2	P2	S1	S2	S1+S2	P2	
total DNA	49	18	67	33	37	15	52	48	nanodrop
P53 (Genbank U08134)	74	15	89	11	13	69	82	18	qPCR (176 bp)
5S (Genbank J00063)	66	8	74	25	11	4	15	85	qPCR (101 bp)
Heterochromatic repeat(Genbank AB185090)	79	6	85	15	10	3	13	86	qPCR (151 bp)

Figure 1: Luminescence-revealed Western blots of *S.mansoni* protein extracts, separated on 15% SDS-PAGE and incubated with antibodies against histone H3 (left) and histone H3 trimethylated at lysine 9 (middle). In each lane, the same amount of protein was applied. Antibodies from two different suppliers (AB1 and AB2) were used and revealed with a peroxidase coupled secondary antibody. On the right, only the secondary antibody was used as control.

Figure 2: Schematic representation of the nChIP procedure.

Figure 3: PCR products separated on agarose-gels used for the detection of 28S rDNA genes in different fractions during the nChIP process. Stained with ethidiumbromide. Image inverted. On the left (first lane) PCR on DNA extracted from the S1 pellet before dialysis. Clearly, much chromatin is still present. In contrast, no DNA remains on the beads after DNA extraction that follows immunoprecipitation (second lane). C+ is genomic DNA, c- PCR without template (negative control).

Figure 4: (A) Optimization of the M_naseI digestion process. DNA was extracted from the S1 fraction and fragments were separated on a 2% agarose gel (50V, 90 min). (B) Comparison of DNA extracted from fractions S1, S2, P1 and P2. Dialysis of P1 gives soluble fraction S2 and pellet P2. P2 is discarded because it contains cell debris that would interfere with the subsequent centrifugation steps. S2 fraction of 8 min digest was barely visible and the photo was software improved.

Figure 5: Example for the results of a titration experiment. In this case, antibody saturation was reached at around 10 μ g antibody. The antibody had been sold as “ChIP-grade” and supplier recommended amount was 2 μ g.

Figure 6: Frequency distribution of % input recovery for anti-acetyl H3K9 in the *S.mansoni* alpha-tubulin promotor region in 38 experiments. The graph illustrates the relatively high day-to-day variations with the quantification method.

The % input recovery corresponds to the proportion of DNA in a specific locus that is precipitated (fraction B), compared to the unbound fraction in the tube without antibody (input I). Differences in these fractions do not only reflect differences in binding vs. non-binding to the beads but can be introduced through experimental errors (e.g. pipetting errors, loss of precipitated material). In half of the shown experiments, input recovery in this locus was around 20%, but in other experiments it was higher. In one case it exceeded 80%, probably due to a loss of material during the purification of the input DNA that served as reference.

H3

tri-meth. H3K9

ABI

AB2

ABI

AB2

sec.AB

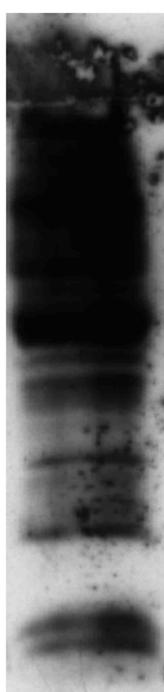
110k

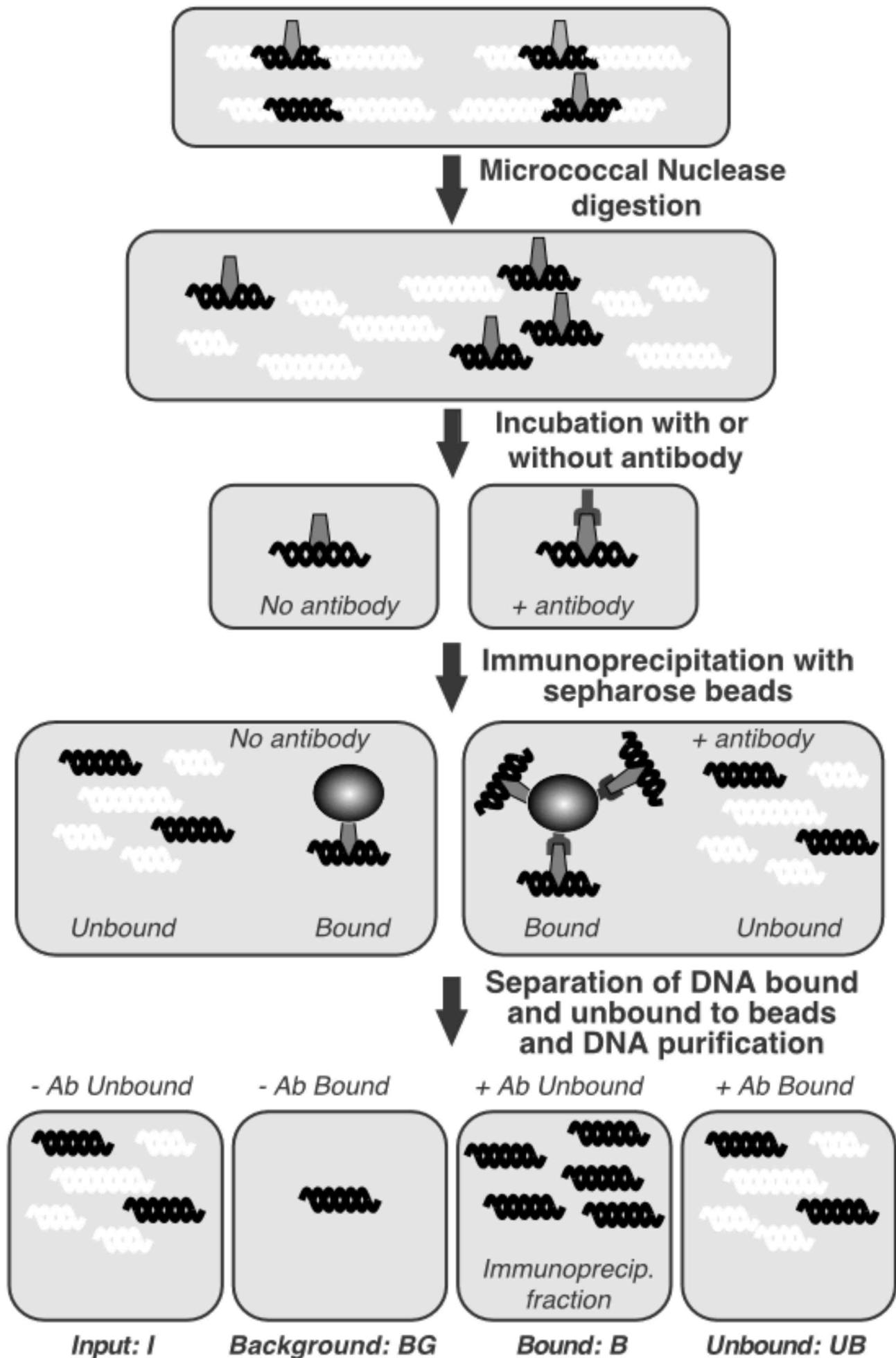
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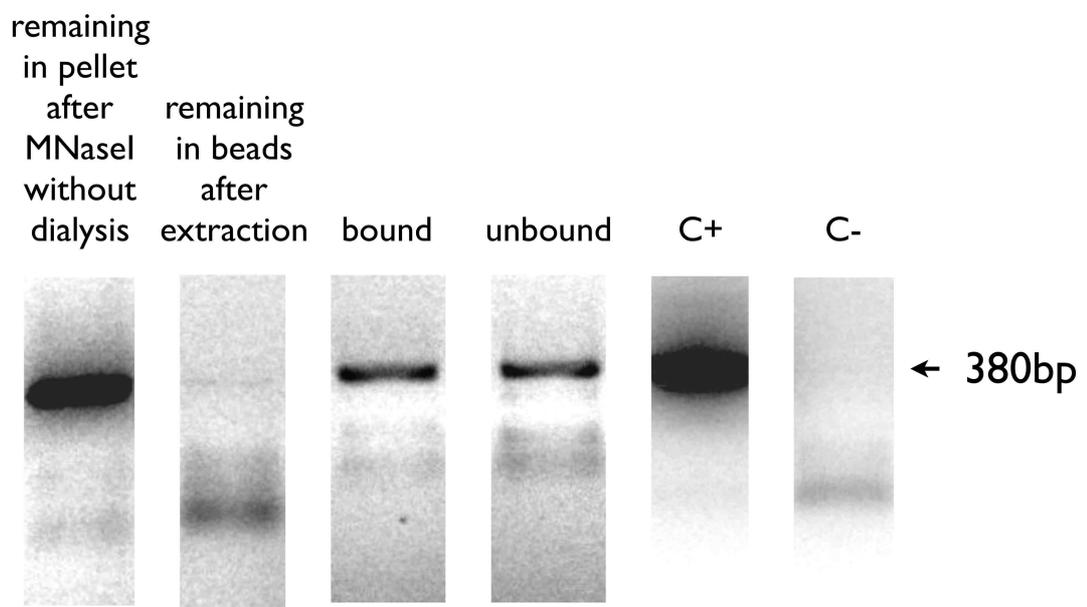
47k

32k

25k





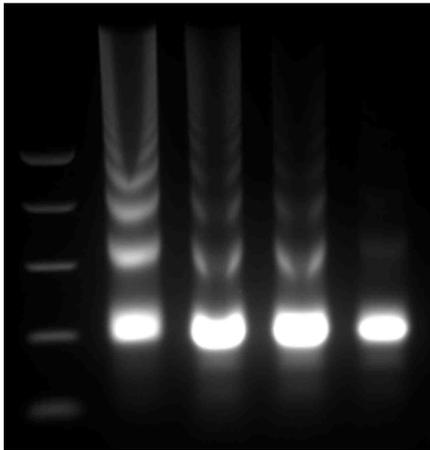


(A)

SI

min Mnasel at 37°C

2 4 6 8



(B)

4 min Mnasel

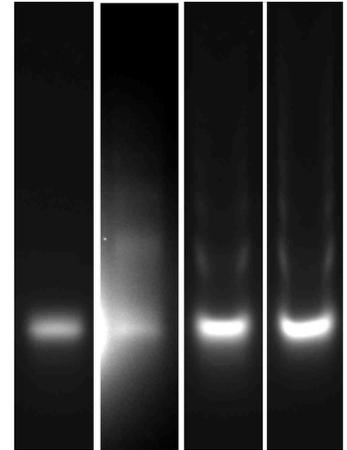
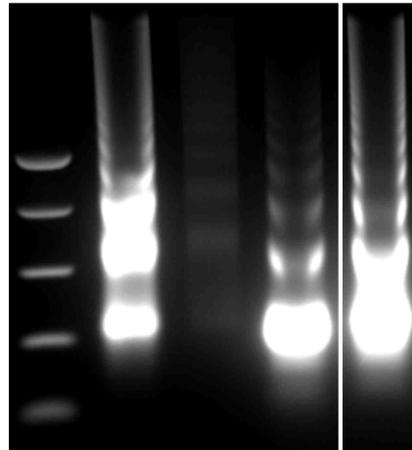
8 min Mnasel

SI S2 P2 PI

SI S2 P2 PI

bp

500
400
300
200
100



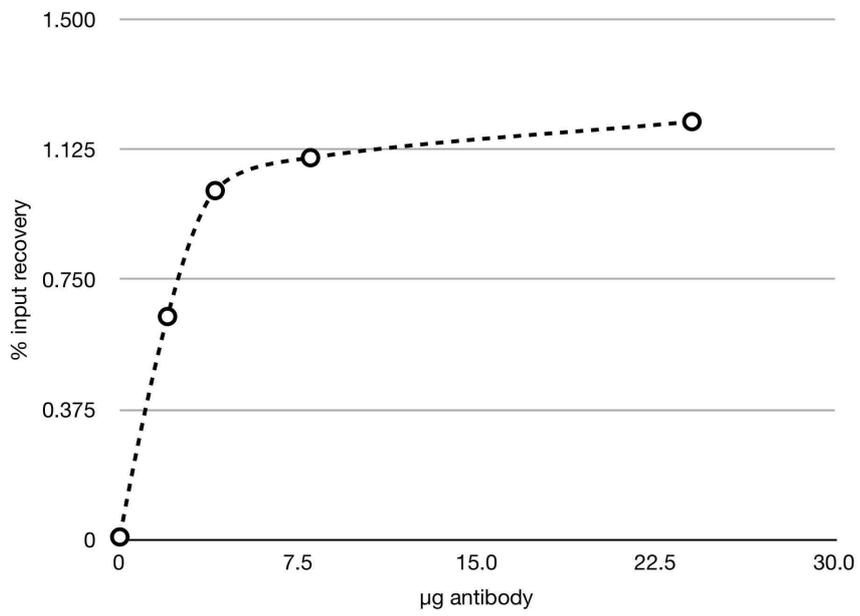
ChIP

discarded

ChIP

discarded

titration of anti trimethylated-H3K4 antibody in the alpha-tubulin locus



**frequency distribution of % input recovery in different experiments
using the same antibodies and qPCR target regions in each experiment**

