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Native Chromatin Immunoprecipitation from Brain Tissue Using Magnetic Beads

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Abstract

We hereby present a protocol for Native Chromatin Immunoprecipitation (NChIP) on brain tissue. Maintaining the chromatin in it's native state, as opposed to cross-linkage by formaldehyde, and using magnetic beads (instead of sepharose beads) facilitates a very sensitive and specific immunoprecipitation (up to 98% enrichment relative to input). Performing qPCR on acetylated H4 precipitated DNA, we found a 12-fold enrichment of the active actin gene compared to the inactive globin gene. Furthermore, very small inter assay variations were found across individual animals. The high sensitivity and specificity of the present protocol circumvents the need for large tissue samples, which is often a limiting factor when working with brain tissue.

Keywords: Epigenetics; Nucleosome; Histone modifications; Native Chromatin Immunoprecipitation; Magnetic beads

Abbreviations: NChIP: Native Chromatin Immunoprecipitation; XChIP: Cross-linked Chromatin Immunoprecipitation; MNase: Micrococcal Nuclease; EB1/2: Extraction buffer ½; qPCR: Quantitative Polymerase Chain Reaction

Introduction

Epigenetics is a field that has grown enormously in the last decade. Numerous biochemical pathways and pathophysiology of many diseases, have been associated with epigenetic changes taking place in cell nucleus [1,2]. Two major epigenetic modifications that influence gene expression directly are DNA methylation and post-translational histone modifications. DNA methylation is a rather stable modification that is used by the cell primarily to delineate repressive chromatin. On the other hand, histone modifications are very dynamic, and are used to regulate gene expression in response to environmental stimuli. Therefore, histone modifications are regarded as a link between the genome and the environment, hence an increasing effort is put into investigating these modifications.

Chromatin immunoprecipitation (ChIP) is a method that allows investigation of association between different proteins and DNA in the chromatin. Two main types of ChIP exist: ChIP based on chemical cross-linking (XChIP) and native ChIP (NChIP) [3]. XChIP protocol is widely used for all tissue types and all proteins that interact with DNA (structural, transcription factors, polymerases etc). These proteins must be cross-linked to the DNA prior to immunoprecipitation, usually by formaldehyde [4]. Such cross-linking decreases yield because it can cause epitope alterations, thereby making the immunoprecipitation inefficient. Furthermore, XChIP uses sonication as a way of chromatin fragmentation, which results in a wide variety of fragment sizes, thereby decreasing the resolution and reproducibility of the assay [5].

NChIP on the other hand, is only applicable to histone proteins. This approach takes advantage of the natural configuration of DNA wrapped tightly around core histones, making it possible to investigate histone modifications and DNA associations in their native form, without chemical cross-linking. NChIP uses micrococcal nuclease (MNase) digestion as a way of preparing the chromatin for immunoprecipitation, a method pioneered by Hebbes [6]. MNase is an endonuclease, unique in its ability to cut double stranded DNA in the linker region between nucleosomes, whereas it only causes single strand breaks in the nucleosome core region, where DNA is tightly wrapped around histones.

The subfield of epigenetics dealing with neurological disorders is developing very fast. Immunoprecipitation from brain tissue is a necessary tool for investigation of multiple histone modifications, but the literature that deals with NChIP from brain tissue is rather scarce. Therefore, we present a complete NChIP protocol for whole brain tissue, which is routinely carried out in our laboratory. This protocol pioneers the use of magnetic beads for immunoprecipitation, as opposed to very popular sepharose beads. We have successfully applied the protocol to both hippocampus and prefrontal cortex of adult rats. Furthermore, several antibodies have been implemented in the procedure, e.g. anti-acetylated histone H4 (anti-H4ac), anti-trimethylated lysine 9 on histone H3 (anti-H3K9me3), anti-trimethylated lysine 4 on histone H3 (H3K4me3), all commercially available and previously proven to be applicable to ChIP (Abcam, Cambridge, UK).

Materials and Methods

Tissue, special materials and conditions

Adult male Sprague-Dawley rats (270-290 g, Charles River Laboratories, Hamburg, Germany) were used in all experiments. To achieve sufficient chromatin yield, between 30 mg and 70 mg prefrontal cortex tissue (or a whole hippocampus) was used. All solutions were ice-cold and all centrifugations were performed at 4°C. Low protein binding tubes were used (Sarstedt, Germany) in order to minimize protein interaction with the surface of the eppendorf tubes.

Isolation of nuclei

Solutions (all chemicals were purchased from Sigma, USA, unless otherwise stated):

Extraction Buffer 1 (EB1): 0.4 M sucrose, 10 mM Tris-HCl pH 7.9, 5 mM sodium butyrate (freshly added), 5 mM β-mercaptoethanol

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(freshly added), protease inhibitor cocktail (Roche, Basel, Switzerland) (freshly added).

Extraction Buffer 2 (EB2): 0.25 M sucrose, 10 mM Tris-HCl pH 7.9, 5 mM sodium butyrate (freshly added), 5 mM β-mercaptoethanol (freshly added), protease inhibitor cocktail (Roche, Basel, Switzerland) (freshly added), 1% Triton X-100, 10 mM $MgCl_2$.

Digestion Buffer: 0.32 M sucrose, 50 mM Tris-HCl pH 7.5, 5 mM Na+Butyrate, protease inhibitors cocktail (Roche, Basel, Switzerland) (freshly added), 4 mM $MgCl_2$, 1 mM $CaCl_2$.

Procedure

1. The frozen tissue is placed in 10 mL EB1 and disrupted 3x 10 seconds by T10 Ultra-Turrax homogenizer (IKA, Staufen, Germany) using 8 mm pistil and by increasing speed each time. The time and speed of the tissue homogenizer should be optimized to the applied tissue. We used 10 seconds on each of three consecutively increasing speed levels (starting from 3 on the homogenizer's scale) to be appropriate for both tissue types. After homogenization the sample must be spun down immediately for 20 min at 3000 g.

2. Discard the supernatant and re-suspend the pellet in 1 mL EB2. Centrifuge the samples for 10 min at 12,000 g.

3. Discard the supernatant and re-suspend the pellet in 1 mL Digestion Buffer.

Quality control of the isolated nuclei

1. To ensure the presence of intact nuclei, DAPI staining should be performed at this stage.

2. Mix nuclei with DAPI solution (1 mg/mL) in a ratio 1:19 and incubate 5 min at RT.

3. Centrifuge the sample at 12,000 g for 5 min and re-suspend the pellet in 100 µL MilliQ water.

4. Analyze the samples by a fluorescent microscope with an appropriate filter set (DAPI excitation= 364 nm, emission=454 nm).

5. The nuclei isolated from the rat brain can be seen as individual ovals (Figure 1a) or in groups of several (Figure 1b), but all nuclei should have a nice round (oval) shape and look intact.

Micrococcal nuclease digestion

Before the MNase digestion, the amount of chromatin present in the sample should be assessed by spectrophotometry in the presence of 0.1% SDS. Subsequently, dilute the chromatin to a concentration of 0.5 mg/mL with Digestion Buffer (see above).

Solutions:

Resuspension Buffer: 10 mM Tris-HCl pH 7.9-8, 1 mM EDTA pH 8, 5 mM sodium butyrate (freshly added).

Procedure

1. 50 U of MNase (Thermofischer Scientific, USA) per 0.5 mg chromatin should digest the sample in 4 min, at 37°C. It is optimal to use a thermomixer set to 600 rpm to avoid sedimentation of the nuclei.

2. Stop the digestion by adding EDTA to a final concentration of 5 mM and place on ice.

3. In order to avoid over- or under digestion of the chromatin, a time-course optimization should be performed on the desired tissue, followed by gel electrophoresis on 1.2% agarose in the presence of 0.1% SDS. Ethidium Bromide staining should be performed after the electrophoresis, as SDS binds to ethidium bromide.

4. Centrifuge the sample for 5min at 11,600 g and transfer the supernatant to a new tube – this is the first supernatant fraction (S1).

5. Resuspend the pellet in 0.5 mL Resuspension Buffer and transfer the solution to a dialysis tube (10 kDa pore width). Dialyse the sample in 4 L Resuspension Buffer overnight at 4°C on magnetic stirrer.

6. Transfer the sample to an eppendorf tube and centrifuge for 10 min at 2000 g. Retain the supernatant – this is the second supernatant fraction (S2).

7. Visualization of the two supernatant fractions by 1.2% agarose gel electrophoresis should be performed to assess the degree of digestion (Figure 2). The measurement of DNA quantity should be performed using spectrophotometer. Typical yield from hippocampus was approx. (2.5 mg/mL) and from PFC approx. (0.5 mg/mL).

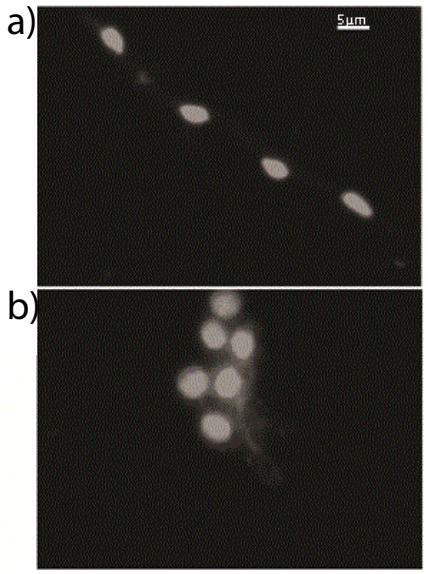

Figure 1: DAPI staining of nuclei isolated from rat hippocampus a) spread nuclei b) grouped nuclei.

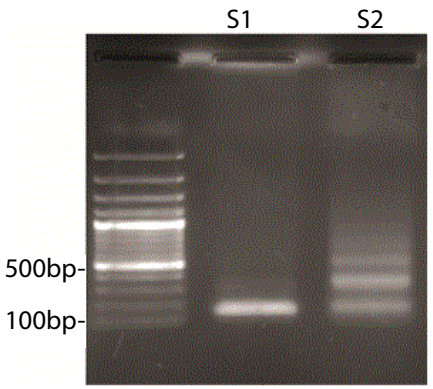

Figure 2: Gel electrophoresis of S1 and S2 chromatin fractions. S1 fraction is dominated by mononucleosomes. DNA associated with up to 4 nucleosomes is visible in the S2 fraction. DNA ladder loaded in the first lane- GeneRuler 100 bp Plus DNA ladder (Thermofisher Scientific, USA).

Immunoprecipitation

For immunoprecipitation S1 and S2 should be pooled. In our laboratory the anti-H3K4me3 antibody raised in rabbit (Millipore, cat# 06-866) is routinely used for NChIP, and therefore rabbit serum is used as mock-control. We and others used the same antibody in X-ChIP (Abcam), though NChIP gives a much better yield as also reported elsewhere [5], see below. We suggest using freshly prepared chromatin in this step, as freezing the chromatin in -20 degrees lowers immunoprecipitation efficiency dramatically. Other groups reported that snap freezing in liquid nitrogen could be applied at this step (Gozani O. group, personal communication).

Solutions:

Dilution Buffer: 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 7.9-8, 167 mM NaCl.

Equilibration Buffer: 6 µg sonicated λ DNA, 3 mg BSA in Dilution Buffer (see above).

Low Salt Wash Buffer: 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 7.9-8.

High Salt Wash Buffer: 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 7.9-8.

LiCl Wash Buffer: 0.25 M LiCl, 1% Igepal (NP-40), 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 7.9-8.

TE-buffer: 10 mM Tris-HCl pH 7.5, 1 mM EDTA.

Elution Buffer: 1% SDS, 0.1 M NaHCO3 (freshly added).

Procedure

1. Mix 0.1 mL sample with 0.4 mL Dilution Buffer, then add 6 µL serum (mock) or antibody (sample). Incubate overnight at 4°C on a rotatory shaker (upside down rotation) at 20 rpm.

2. Depending on the chromatin yield from different tissue preparations, the amount of sample can be lowered at this step if a high mock qPCR signal is present.

3. Prepare the Dynabeads (Invitrogen, USA) (30 µl per sample) by washing them twice in 0.5 mL Dilution Buffer. Afterwards, incubate the beads in 0.5 mL Equilibration Buffer overnight as in step 1 to block the beads preventing subsequent unspecific binding of the chromatin.

4. Wash the beads three times in 0.5 mL Dilution Buffer. After the final wash, resuspend the beads in the same amount of Dilution Buffer as initially taken from the stock (i.e. 30 µL per sample) and transfer it to the samples. Incubate for 3 h at 4°C on the rotatory shaker at 20 rpm.

5. Collect the beads on a magnet. From the mock-control, transfer the buffer to another tube – this is the input fraction containing all the chromatin. Buffer from antibody samples can be discarded.

6. Wash the beads in 1 mL of the following stringent buffers for 10 min each at 4°C and 30 rpm on the rotatory shaker:

7. Low Salt Wash Buffer

8. High Salt Wash Buffer

9. LiCl Wash Buffer

10. Three times in TE Buffer

11. To elute the immune complexes, incubate the beads in 0.25 mL Elution Buffer for 15min at 65°C and 1000 rpm in a thermomixer. Repeat this step and combine the two eluates afterwards, obtaining 500 µL final eluate.

12. Mix 0.1 mL input fraction (step 4) with 0.4 mL Elution Buffer and incubate continuously (total of 30 min.) alongside the samples as in step 6.

13. To remove histone proteins for more efficient DNA purification, add 10 µL EDTA (0.5M), 20 µL Tris-HCl (1M pH 6.5-6.8) and 20 µg Proteinase K (Thermofisher Scientific, USA) to all the samples. Incubate for 3h at 45°C and 600 rpm in a thermomixer.

DNA analysis by qPCR

After Proteinase K treatment the samples contain DNA fragments ready for further processing. We have used several DNA purification kits, e.g. MinElute PCR Purification (Qiagen, Germany) or ChIP DNA Clean & Concentrator (Zymo Research, USA), with satisfactory yield for many qPCR investigations.

Materials: Maxima SYBR Green qPCR master mix (2x), ROX solution provided (Thermofisher Scientific, USA)

Primers: actin forward: GAGGCCGGTGAGTGAGCGAC, reverse: GTTGCGCCGCCGGGTTTTAT; globin forward: GTGTGAGGTCTAGAAGCTTGGAGATGA, reverse: TGACCAATAGTCTCGGAGTCCTGGGGA

Results

We have run all reactions initially in triplicates, and after confirming the reproducibility we continued running them in duplicates. In order to check the specificity of the NChIP on the rat brain tissue, we chose an active gene (actin (Actb), AC_000080.1) associated with acetylation on histone H4, and a gene which is not expressed in the brain (gamma globin (Hbe1), NC_005100.3), thus not associated with acetylated histone H4 to the same degree. The results indicate a very high degree of histone H4 acetylation on the actin gene promoter as compared to the globin gene (Figure 3). We have calculated 12-fold enrichment in the histone H4 acetylation of the actin gene compared to globin gene using the following equation: fold enrichment= 2^(Ct input actin-Ct H4Ac actin)/2^(Ct input globin-Ct H4Ac globin), Ct input actin=31.16, Ct H4Ac actin=32.09, Ct input globin= 32.71, Ct H4Ac globin=37.32.

Comparing the relative enrichment of the pull-down between XChIP and NChIP methodologies, using the same antibodies, reveal both high specificity and sensitivity of NChIP superior to XChIP. Abcam as an antibody manufacturer uses XChIP with sepharose beads to validate their products as ChIP-grade. However, less enrichment relative to input is achieved by their approach, compared to the present protocol. For the activating H3K4me3 modification, their best enrichment is ~27% of input, whereas we precipitate 40-88% of input with this antibody (Figure 4a) (Abcam) [7]. An even clearer picture is true for the repressive modification H3K9me2. Using this antibody, Abcam precipitates ~4.5% of input, whereas we enrich by 25-98% of input (Abcam) [8]. Furthermore, Figure 4 clearly demonstrates the specificity of the NChIP protocol, since active and inactive genes are more/less associated with active and repressive modifications, respectively. The minute SEM-values of Figure 4 validates the reproducibility of the NChIP protocol, as these results stem from 6 different animals.

Concluding Remarks

We hereby present a protocol for native chromatin immunoprecipitation from rat brain tissue. We used 30-70 mg of prefrontal cortex or hippocampus, which is far less than reported elsewhere [5]. We believe that the use of magnetic beads,

which is described for the first time in this paper, increases the immunoprecipitation efficiency, therefore the amount of tissue used in the experiment can be lowered. This is an important issue, as the amount of tissue is often a limiting factor. The final result indicates a 12-fold increased association of H4-Ac with the active gene compared to an inactive gene, thereby confirming high specificity of the protocol.

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