Video Article

A Chromatin Assay for Human Brain Tissue

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Abstract

Chronic neuropsychiatric illnesses such as schizophrenia, bipolar disease and autism are thought to result from a combination of genetic and environmental factors that might result in epigenetic alterations of gene expression and other molecular pathology. Traditionally, however, expression studies in postmortem brain were confined to quantification of mRNA or protein. The limitations encountered in postmortem brain research such as variabilities in autolysis time and tissue integrities are also likely to impact any studies of higher order chromatin structures. However, the nucleosomal organization of genomic DNA including DNA:core histone binding - appears to be largely preserved in representative samples provided by various brain banks. Therefore, it is possible to study the methylation pattern and other covalent modifications of the core histones at defined genomic loci in postmortem brain. Here, we present a simplified native chromatin immunoprecipitation (NChIP) protocol for frozen (never-fixed) human brain specimens. Starting with micrococcal nuclease digestion of brain homogenates, NChIP followed by qPCR can be completed within three days. The methodology presented here should be useful to elucidate epigenetic mechanisms of gene expression in normal and diseased human brain.

Video Link

The video component of this article can be found at https://www.jove.com/video/717/

Protocol

Procedure:

1st Day

1. Homogenize 50-500 mg of frozen post-mortem gray matter tissue with Douncing Buffer.

! CAUTION ! - Human tissue must be handled with care under strict safety conditions. It should be handled at BSL-2 or higher safety standards.

1. Take previously dissected, post-mortem brain from -80°C, dounce them in 5X brain volume of Douncing Buffer, and place in 2.0 mL microcentrifuge tube. Matched sample and control pairs are processed simultaneously.

2. Micrococcal Nuclease (MN) Digestion

1. Add 5U/mL of Micrococcal nuclease to the sample and mix within the solution by pipetting up and down before placing on ice.
   * **CRITICAL STEP -** It is important to do this step quickly since MN has the ability to act at even 4°C.
2. Incubate samples for 7 minutes at 37°C.
3. After the 7 minute incubation, add 0.5M EDTA to a concentration of 10mM to stop the MNase activity.

3. Hypotonisation

1. Place samples into a 15 mL falcon tube. Add 10X the sample volume of 0.2mM EDTA, 1/2000 sample volume of 0.2M benzamidine and 1/1000 sample volume of 0.1M phenylmethanesulphonylfluoride (PMSF). The latter two compounds are used as Protease Inhibitors.
   * **CRITICAL STEP -** It is important to keep the samples on ice during all these steps.
2. Incubate sample for 1 hour, while vortexing it every 10 minutes.
3. At the end of the hour long incubation, add 1/2000 sample volume of 3M DTT, yet another Protease inhibitor.
4. Vortex sample once more and centrifuge at 3175 RCF for 10 minutes at 4°C.
   * **OPTIONAL STEP -** Precleansing with Protein G Agarose.
     1. Take supernatant and put in new 15 mL falcon tube.
     2. Add 500 µL of Protein G Agarose.
     3. Rotate at room temperature for 30 min.

4. Centrifuge at 4000 rpm for 10 min at 4°C.

5. Distribute supernatant so that 500 µL are used as Input control (containing only genomic DNA), and the rest is split into two tubes containing 1600 µL of sample each -- which are for chromatin immunoprecipitation (ChIP) samples.
6. The Input control is placed at -80°C O/N until further use.
7. To the ChIP samples - add 1:10 volume of 10XFSB and 4µg of antibody, then vortex the samples and rotate them at 4°C overnight.
   ! CAUTION ! - Amount and dilution of antibody may require optimization.

2nd Day

! CAUTION ! - Begin 2nd day by washing the Protein G Agarose that will be used to isolate nucleosomal DNA. Since agarose beads are very sensitive, it is necessary to cut off the heads of the tips whenever pipetting any solution containing agarose beads.

1. Probing Protein G Agarose Beads to DNA

1. Add 1.6 mL 1XFSB to 245 µL protein G-agarose (enough for 4 tubes) in a 2 mL microcentrifuge tube (loop).
2. Separate the solution into two 2 mL tubes and refill each up to 1.6 mL with 1X FSB.
3. Rotate at RT for 30 sec and centrifuge at 0.1 RCF for 30 sec.
4. Remove the supernatant using a vacuum.
5. Add 1.6 mL 1X FSB once again. Rotate the samples for 30 sec and then centrifuge at 0.1 RCF for 30 sec.
6. Remove the supernatant once again, and combine both tubes with 1.5 mL 1XFSB.
7. Add 15 µL sonicated Salmon sperm DNA (10mg/mL).
   ! CAUTION ! - This step should, in principle, reduce non-specific binding of the immunoprecipitate to the beads. However, this also could lead to false positives for some of the (human) DNA sequences.
8. Rotate at R.T. for 30 min, then centrifuge at 0.1 RCF for 30 sec.
9. Remove the supernatant. Add 200 µL of 1XFSB.
10. Add 90 µL of agarose beads into each ChIP sample. Rotate at 4°C for 1 hr.
11. Add 1mL of 1XFSB into remaining agarose beads to serve as a negative control and rotate at 4°C for 1 hr.
12. After the hour long incubation, centrifuge the samples and negative control at 0.1 RCF for 30 sec. Discard the supernatant.

2. Washing the Beads

! CAUTION ! - Washing buffers must be kept at 4°C until use, and are to be used only if are less than a month old.

1. Add 1 mL of each washing solution to the beads and rotate for 3 min at RT.
2. Centrifuge at 0.1 RCF for 30 sec.
3. Discard supernatant using vacuum.

* Each washing solution

-- Low salt washing buffer

-- High salt washing buffer

-- Lithium chloride solution - **only rotate at RT for 1 minute!**

-- TE buffer (10 mM Tris, 1 mM EDTA pH=8)

3. Elution

*** CRITICAL STEP** - Make fresh Elution Buffer for every experiment on the day it is to be used.

1. Add 250 µL of freshly prepared elution buffer to each sample.
2. Rotate for 15 min at RT, then centrifuge at 0.4 RCF for 1 min.
3. Save the supernatant in a 2.0 mL microcentrifuge tube (Loop).
4. Add 250 µL of elution buffer to each sample and vortex by hand for a few seconds. Then, vortex samples for 15 minutes on a mulitvortexer.
5. Centrifuge at 16 RCF rpm for 4 minutes and save supernatant in same 2.0 mL tube (Loop).

4. Digest Protein

1. Add 10 µl 0.5M EDTA, 25 µl 0.8M Tris-HCl, pH 6.5, 10 mg/ml Proteinase K (1/200 sample) to each ChIP sample.
2. To each Input control, add lysis buffer for proteinase K digestion (1/10 of sample buffer) and 10mg/ml Proteinase K (1/200 of sample buffer).
3. Incubate Input and ChIP samples in 52°C for at least 3 hrs.

5. Phenol/Chloroform extraction

! CAUTION ! - Experiment requiring phenol/chloroform should be performed under the hood. Use nitrile gloves when handling phenol/chloroform.

1. After the 3 hour incubation, we add the 500 µl phenol-chloroform to each sample.
2. Vortex all samples for several seconds, then centrifuge at 13 RCF rpm for 5 min.
3. At this point, two phases will be present and we are interested in the content of the top phase. Take out the top phase and put it into a 2 mL microcentrifuge tube.

4. Add a mixture of 2µL of Glycogen and 50µL of 3M sodium acetate to each sample along with 1.375 mL of 100% Ethanol.
5. Vortex all samples vigorously. Place them in -80°C overnight.

3rd Day

1. Remove samples form -80°C and place them on ice for them to thaw.
2. Centrifuge at 15 RCF for 10 minutes at 4°C.
3. Carefully remove supernatant without disturbing the pellet at the bottom of the tube.
4. Add 1 mL of cold 75% Ethanol to each sample, then invert them 4-6 times.
5. Centrifuge at 18 RCF for 5 min at 4°C.
6. Remove supernatant once more and allow the pellets to air dry.
7. Dissolve dried pellets in 50 µL of 4mM Tris-HCl, pH8 and store at -80°C until further use.

Discussion

The protocol outlined here is particularly useful for investigators interested in histone and/or DNA methylation signatures of human brain, because these chromatin markings may be less prone to postmortem artifacts as compared to other types of modifications, including (histone) acetylation and phosphorylation[1, 2]. The postmortem brain is amenable to the study of mono-nucleosomal preparations; the DNA remains largely attached to the core histones, at least in specimens with representative autolysis intervals (the time between death and freezing/ storing of the tissue) typically in the range of several hours up to 1.5 days[1]. However, mono-nucleosomal preparations could be sensitive to changes in nucleosomal positioning and densities, particularly around sequences surrounding transcription start sites of genes[3]. Therefore, control experiments with modification-independent anti-histone antibodies should be included. Alternatively, it may be possible to isolate poly-nucleosomal fractions from human brain extracts, using shorter incubation times for the micrococcal nuclease digest in conjunction with additional purification steps (incl. ultracentrifugation). Finally, a recent study on genome-wide transcription factor binding in postmortem brain simply sheared chromatin via sonication[4]. Notably, preparation of chromatin by fixation prior to sonication or enzyme-based digestion may not be ideal from the viewpoint of postmortem studies, because breakdown and/or artificial reconfiguration of higher order chromatin structures after death are potential confounds difficult to control for. Hence, ChIP assays on postmortem brain are likely to be useful for a limited number of molecules, including nucleosomal core histones and other proteins tightly attached to the genomic DNA. Even with this caveat taken into account, the approaches outlined in this presentation are likely to provide novel insights into chromatin-associated mechanisms governing neuronal and glial functions in the normal and diseased human brain.

We have used this technique successfully primarily for measuring of histone methylation and histone occupancies at specific promoters using gene by gene qPCR[1, 5, 6]. As stated previously the human postmortem brain seems to be amenable to the study of mono-nucleosomal preparations because the DNA remains largely attached to the core histones. Typically, when starting with 75 mg of human child or adult cerebral cortex (grey matter) one can expect using the protocol presented above- a yield of 20-30 ng/µl in a total volume of 50 µl for the Input and 10-15 ng/µl in a total volume of 50 µl for ChIP, at least when modification specific anti-histone antibodies are used. The so called ChIP to Input ratio is the unit of measure. Specificity of the reaction is monitored by melting curve analysis, gel electrophoresis, and sequencing. In addition, negative controls (i) lacking the specific antibodies or (ii) containing (non-specific) immunoglobulin should be processed by qPCR in parallel to the ChIP and Input samples and should not result in specific product. Be aware that when salmon sperm is used as a blocking agent, control samples may result in a smear when run on a gel.

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