Introduction

The discovery of 5-methylcytosine oxidative products led to the development of a large number of techniques to allow the detection of cytosine modifications and to map them genome-wide. Here is described an antibody-based method to detect 5-hydroxymethylcytosine (5-hmC) in mammalian cells. This protocol consists of an extraction of genomic DNA followed by a three-day immunoprecipitation procedure. 5-hmC containing immunoprecipitated DNA fragments can be further analysed by qPCR or submitted to high throughput sequencing. It is important to note that this approach allows the mapping of 5-hmC only at low resolution and, as a consequence, other techniques must be used for single nucleotide resolution.
Procedure

Extraction of genomic DNA

Genomic DNA from mammalian cultured cells is prepared using the DNeasy® Blood & Tissue kit (Qiagen) according to the manufacturer’s instructions with the following modifications:

1. Volumes (step 1c: 400 µL ice cold PBS, 60 µl proteinase K from kit; step 2: 600 µL buffer AL; step3: 400 µL ethanol; see Note 1).
2. Step 2, after incubation at 56°C: short spin, add 800 µg of RNase A (8 µL of a stock at 100 mg/ml) and incubate 30 min at 37°C under mix.
3. Step 6: supplementary centrifugation for 1 min at 20,000 g followed by incubation of columns for between 3 to 5 min at 65°C to ensure ethanol evaporation.
4. Step 8: second elution with 100 µL of Buffer AE.

Day 1: DNA fragmentation and incubation with antibody

DNA is first sonicated to obtain fragments ranging from 200 to 500 bp length and then denatured to make the 5-hmC available for immunoprecipitation.

1. Put 20 µg of DNA in 300 µl TE buffer in a 1.5 ml microfuge.
2. Sonicate DNA in a cold room with a Bioruptor sonication system (Diagenode): twice 7 min (30 sec on/30 sec off), then twice 14 min (30 sec on/30 sec off). Short spin between each sonication.
3. Take an aliquot of 20 µl for input sample and keep at -20°C.
4. The remaining 280 µl of sample are used for immunoprecipitation: add 120 µl of TE for a final volume of 400 µl.
5. Denature DNA at 95°C for 10 min, then cool on ice. Short spin.
6. Add 100 µl of IP buffer 5X.
7. Add 5-hydroxymethylcytosine antibody (see Note 2).
8. Incubate the mix overnight at 4°C on a rotating platform.

Day 2: Precipitation of the DNA-antibody complexes with beads, washes and proteinase K digestion

Next day, Protein-A Sepharose beads are saturated to avoid background (see Note 3) and added to the sample in order to precipitate the DNA-antibody complexes. Immunoprecipitated complexes are then washed followed by a digestion with proteinase K to facilitate further DNA extraction.

1. Annotate a new series of tubes corresponding to each sample and put 50 µL of sepharose beads (50% slurry) per tube. Add 10 volumes of PBS/BSA 0.1%.
2. Incubate 10 min on a rotating platform at 4°C.
3. Centrifuge 2 min at 800 g, 4°C, discard supernatant and add 10 vol of PBS/BSA 0.1%.
4. Incubate 5 min on a rotating platform at 4°C.
5. Centrifuge 2min at 800 g, 4°C and discard supernatant.

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6. Add the 500 µl of DNA/ Antibody mix on the 25 µl pellet of beads.
7. Incubate 2 h on a rotating platform at 4°C
8. Wash beads 3 times with IP buffer 1X:
   a. Centrifuge mix DNA/ Antibody/ beads 2 min at 800 g, 4°C. Discard supernatant.
   b. Add 1 ml IP buffer 1X. Incubate on a rotating platform 5 min at 4°C.
   c. Centrifuge 2 min at 800 g, 4°C. Throw away supernatant.
   d. Repeat steps b and c twice again (3 washings in all)
9. Resuspend beads in 250 µl of Digestion buffer
10. Add 70 µg of proteinase K.
11. Incubate overnight in a thermomixer at 55°C, 1400 rpm.

**Day 3: Extraction of immunoprecipitated DNA**

After a phenol/chloroform extraction, immunoprecipitated DNA is purified by ethanol precipitation.

1. Add 250 µl phenol (TE, pH 8) and 250 µl of isoamylc alcohol/chloroform to each sample. Vortex for 30 sec.
2. Centrifuge 5 min at 14,000 rpm, RT. Save aqueous phase in a new microfuge (see Note 4).
3. Ethanol precipitation:
   a. Add 0.1 vol Na-Acetate, 2.5 vol ethanol, 20 µg Glycogen.
   b. Vortex briefly.
   c. Incubate 1h minimum at -80°C.
   d. Centrifuge 30 min at 14,000 g, 4°C.
   e. Throw away supernatant and add 500 µL ethanol 75%. Vortex briefly.
   f. Centrifuge 15 min at 14,000 g, 4°C.
   g. Throw away supernatant.
   h. Wait until pellets are dry and resuspend in DNase and RNase free H₂O (see Note 5).

**Lists of materials and reagents**

**Materials**
- Thermomixer
- Refrigerated centrifuge
- Bioruptor sonication system (Diagenode)
- Heating block
- Rotative platform

**Reagents**
- DNeasy® Blood & Tissue kit, Qiagen
- TE: 10 mM Tris-HCl, pH 7.5; 1 mM EDTA
- IP buffer 5X: 50 mM Na-phosphate, pH 7; 0.7 M NaCl, 0.25% Triton X-100

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- 5-hydroxymethylcytosine antibody: we use 2 µg of the rabbit polyclonal antibody from Diagenode (CS-HMC-100). Antibodies from Active Motif (#39769) or BIOTEM (Clone 4D9, Ref # BIO.020.3) give similar results. When using monoclonal antibodies, switch to protein-G Sepharose beads.
- Sepharose beads
- Digestion buffer: 50 mM Tris HCl, pH 8; 10 mM EDTA; 0.5% SDS

Author notes

1. Volumes are given for a 100 mm confluent cell culture dish.

2. To avoid pipetting bias, prepare a mix of antibody qsp 10 µL PBS per sample.

3. When running hMeDIP-seq, it is essential to verify first that the saturated Sepharose beads you are using do not capture DNA non-specifically since this will generate a high amount of non-specific enrichment that will preclude analysis of the results.

4. To get rid of any trace of phenol, it is possible to add a single isoamylic alcohol/chloroform extraction after the phenol-isoamylic alcohol/chloroform extraction.

5. For qPCR analysis, we usually resuspend the pellet in 40 µL of H₂O; however, for deep sequencing, samples need to be more concentrated and we usually pool 10 hMeDIP reactions.