Selective Chemical Labeling and Sequencing of 5-Hydroxymethylcytosine in DNA at Single-Base Resolution

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Abstract

5-Hydroxymethylcytosine (5hmC), the oxidative product of 5-methylcytosine (5mC) catalyzed by ten-eleven translocation (TET) enzymes, plays an important role in many biological processes as an epigenetic mediator. Prior studies have shown that 5hmC can be selectively labeled with chemically-modified glucose moieties and enriched using click chemistry with biotin affinity approaches. Besides, DNA deaminases of the AID/APOBEC family can discriminate modified 5hmC bases from cytosine (C)or 5-methylcytosine (5mC). Herein, we developed a method based on ESC whole-genome analysis which could enrich 5hmC-containing DNA by selective chemical labeling and locate 5hmC sites at single-base resolution with enzyme-based deamination. The combination experimental design is an extension of previous methods, and we hope that this cost-effective single base resolution 5hmC sequencing method could be used to promote the mechanism and diagnosis research of 5hmC.

Introduction

5-Hydroxymethylcytosine (5hmC), which exists in various mammalian tissues and cell types, is an oxidative product of 5-methylcytosine (5mC) catalyzed by ten-eleven translocation (TET) enzymes.[1,2] Many researches show that 5hmC is not only an intermediate of DNA demethylation, but also plays an important role in many biological processes and human diseases as an epigenetic mediator.[3-6] The recent development of high-throughput sequencing technology has enabled whole-genome sequencing of 5hmC in mammalian systems. Generally, there are two strategies, which are the selective enrichment-based profiling or deamination based single-base resolution sequencing methods. [7-22] While the current application of these methods provides key information about the distribution and functional insights of 5hmC, there are major shortage for both strategies. The lack of single-base resolution information of the profiling strategy limited its application in detailed 5hmC location context while the single-base resolution method is limited by its high sequencing cost.

Therefore, a method that has both advantage of single-base resolution and enrichment would be highly valued in a broad biological and clinical studies. Recent study developed a bisulfite free strategy to detect 5hmC in single-base resolution using AID/APOBEC family DNA deaminase enzyme as deamination reagent, in which 5hmC is protected by a glucose motif catalyzed by T4 beta-glucosyltransferase (T4-βGT). [23-25] This strategy has also been reported in a well-studied chemical selective profiling method back to year 2011 using a modified UDP-glucose.[7]

Here we introduce a cost-effective single base resolution 5hmC sequencing method which allows genome-wide chemical labeling and enrichment of 5hmC and a bisulfite free single base resolution detection of 5hmC. Our strategy (DIP-CAB-Seq) has three steps,1) label and enrich the 5hmC containing fragments using the N_3-UDP-glucose, click chemistry and biotin-avidin interaction, 2) deaminate unprotected cytosines and 5mC using a deaminase, 3) construct sequencing library in a single-stranded manner. This strategy is compatible with the labeling chemistry, enzyme-based deamination and single-stranded DNA library construction. To demonstrate the superiority and effectiveness of this method, we have applied this approach to compare detected 5hmC signals among our new methods (DIP-CAB-Seq), 5hmC-Seal and ACE-Seq. We found that we can detect reliable single base 5hmC signals using DIP-CAB-Seq method with limited sequencing depth.

Method

Cell Culture and DNA preparation

Cell culture and genomic DNA was prepared as previously described.[23] Briefly, Mouse ESCs (v6.5) were grown on gelatin-coated plates in N2B27 supplemented with 2i/ LIF: PD0325901 (1.0 μM), CHIR99021 (3.0 μM) and leukemia inhibitory factor (1000 unit/ml). The genomic DNA was extracted by SDS/proteinase K digestion, phenol/chloroform extraction and ethanol precipitation.

Library construction

Nano-ACE-Seq Library Construction of 5hmC-containing Genomic DNA

(DIP-CAB-Seq)

500 ng fragmented mESC genomic DNA (average 200 bp) was treated with β-GT in the presence of UDP-6-N_3-Glu and labeled with cyclooctyne-biotin. Subsequently, 5hmC-containing fragments was enriched using Streptavidin Beads. The enriched fragments were subjected to denaturation with NaOH and enzymatic deamination to transform C/mC to U but not hmC. Then the converted DNA was tagged with Illumina compatible adapter and amplified to an appropriate library concentration using Accel-NGS Methyl-Seq DNA Library Kit (Swift Bioscience) combined with NEB Next Multiplex Oligos for Illumina. Libraries were checked for quality and quantified using Agarose Gel Electrophoresis and Qubit3.0 individually.

5hmC-Seal Library Construction of 5hmC-containing Genomic DNA

5hmC-Seal Library was prepared as previously described.[7] Briefly, the fragmented mESC genomic DNA (average 200 bp) was treated with UDP-6-N3-Glu in the presence of β-GT to form chemical modification, followed by label with DBCO-PEG4-Biotin via click reaction. The 5hmC-containing DNA fragments were captured by C1 Streptavidin beads. The beads with enriched DNA fragments were resuspended in water and amplified with 12-17 cycles of PCR using an enzyme mixture in the Nextera kit. The PCR products were purified using AMPure XP beads. Sequencing was performed on the NextSeq instrument.

ACE-Seq Library Construction of 5hmC-containing Genomic DNA

ACE-Seq Library was prepared as previously described. Briefly, the m-ESC genomic DNA was sheared with enzymatic to average 200 base pair and purified. DNA fragment was prepared to a concentration of 15~20ng/μL for using. Assemble the reaction in a total volume of 50μL using the table below as a per reaction. If processing multiple samples, making a master mix of everything except the sample should be recommended.

Placed the reaction in thermocycler and incubated at 37□ for 1 hour. Subsequent reaction was cleaned separately by DNA Clean & Concentrator-5 Kit (ZYMO Research) and eluted in 25μL EB buffer. Glycosylated dsDNA was treated with a

fresh 0.1N NaOH solution, incubated in thermocycler at 50□ for 10 minutes for denaturation. After that, transfer sample into an ice box and stand at least 5 minutes to keep DNA single strand. Followed the ssDNA was subjected to enzymatic deamination to transform C/mC to U but not hmC with NEB Next® Enzymatic Methyl-seq Kit (New England Biolabs). Then the converted DNA was tagged with Illumina compatible adapter and amplified to an appropriate library concentration using Accel-NGS® Methyl-Seq DNA Library Kit (Swift Bioscience) coupled with NEB Next® Multiplex Oligos for Illumina® (New England Biolabs). Libraries were checked for quality and quantified using Agarose Gel Electrophoresis and Qubit3.0 individually.

Sequence alignment and peak identification

Sequencing reads were trimmed by trimmomatic with the trim tail option set true, and mapped to the mouse genome (mm10) by bowtie2. The peaks analysis was conducted by MACS2 call peaks, under the pair-end mode with bam file as input format. Afterward, the peaks were annotated by HOMER annotate Peak, and mm10 was used as reference.

Single base resolution analysis.

The numbers as well as sites information of converted and unconverted cytosines in samples were recognized by bismark, with the criterion that C site cutoff greater than zero, that is, the conversion was valid as long as any read indicates modification of the site.

Definition of enhancer subgroups and motif analysis

Enhancers with evidence showing its interaction with distal genomic regions were considered as the most active enhancer subgroup (interacting enhancers). The genomic locations of these enhancers were obtained from published ChIA-PET dataset. The active and poised enhancers were defined by using histone modification markers. Both H3K4me1 and H3K27ac enriched regions were obtained from previous publication. Liftover was used to convert the genomic location from mm8 to mm10. Regions overlapped with interacting enhancers were discarded. Regions enriched with

H3K27ac were considered as active enhancers. Regions only enriched with H3K4me1 but not H3K27ac were considered as poised enhancers. To detect TF motifs around 5hmC-modified cytosines, we performed de novo motif analysis with HOMER find Motifs Genome around the 5hmC sites in mouse ESCs.

Results

1. DIP-CAB-Seq can generate deaminated DNA library from the pull-downed DNA fragments

We present here a selective chemical labeling coupled bisulfite free sequencing (DIP-CAB-Seq) approach to generate the genome-wide, single-base resolution maps for 5hmC. In the current approach, we first selectively labeled and enriched 5hmC-containing DNA fragments by using the glucosyltransferase and azido-UDP-glucose based DNA profiling prior to the subsequent deamination (Figure 1). We then performed the deamination on the enriched fragments by using deaminase-based bisulfite free reaction. The normal cytosine and 5mC can be deaminated while 5hmC remained as glucosylated 5hmC (N_3-5gmC). Therefore, 5hmC will be read as C after PCR amplification while normal C and 5mC will be read as T. After the single stranded library construction and PCR amplification, the 5hmC containing fragments will be selectively amplified while the 5hmC can be read in single-base resolution and generate the precise genomic locations of 5hmC.

The first challenge is whether we can generate high quality next-generation sequencing library from the trace amount pull-downed DNA. We validated that approach by generating 5hmC library in genomic DNA of mECS. We constructed ACE-Seq libraries and DIP-CAB-Seq libraries from two replicated mECS gDNA, respectively. The ACE-Seq library generate 35067558 reads with 68% mapping ratio, while the DIP-CAB-Seq library generate 32769029 reads with 61% mapping ratio, not far from the former result. The DIP-CAB-Seq methods have slightly lower mapping ratio than ACE-Seq method which is fully acceptable

considering the ultra-low input DNA amount in the library construction step in DIP-CAB-Seq method. Both DIP-CAB-Seq libraries and regular ACE-Seq libraries have similar total sequencing reads which guaranteed us a fair comparison between ability of the two methods in detecting the 5hmC sites in genome. These data indicate that we can successfully construct the 5hmC single-base resolution library from the enriched DNA fragments.

Figure 1 Schematic diagram of selectively labeling and deaminase-based sequencing of 5hmC in DNA (DIP-CAB-Seq).

2. DIP-CAB-Seq can selectively enrich 5hmC-containing DNA fragments

Using the DIP-CAB-Seq strategy, we performed both regular hmC-Seal profiling and single-base resolution mapping of 5hmC on mESCs genomic DNA. The profiling analysis indicated that 5hmC from both libraries accumulate at intergenic and intron regions in mouse ESCs (Figure 2a). This observation is consistent with previous findings.[7] We barely observed any abnormal 5hmC signals nor did we observe any noticeable changes of 5hmC at these genomic element regions between the two methods. Therefore, the DIP-CAB-Seq can totally inherit the profiling information that the traditional hmC-Seal method carried.

Figure 1 Enrichment of peaks at various genomic regions

3. DIP-CAB-Seq can detect single base information with limited sequencing depth

We next analyzed 5hmC enriched regions using the base-resolution data in ACE-Seq and DIP-CAB-Seq so as to evaluate the repeatability of both methods. We observed 5936200 5hmC sites in CpG context in the union set of two ACE-Seq samples, while there're only 291527 5hmC sites, that is, only 4.9% of the union set in the intersection set of both samples. On the other hand, we found 7821453 5hmC sites in CpG context in the union set of the DIP-CAB-Seq samples, and there are 1273953 5hmC sites in the intersection set of both samples, accounting for 16.3% of the union set. These data indicate that the DIP-CAB-Seq

method has higher detection ability and consistency than ACE-Seq with same sequencing depth. Considering the total sequencing depth is much lower compared to a regular expensive whole genome 5hmC sequencing, the DIP-CAB-Seq method shows much better application potential in large-scale sample studies. We also detect 5hmC signals in CHH and CHG, which may have 5hmC sites according to the previous study. As expected, DIP-CAB-Seq methods showed higher consistency than ACE-Seq. We observed lots of CHH and CHG sites from the ACE-Seq library, which may come from the false positive signal of the ACE-Seq due to the non-perfect deamination. Therefore, the enrichment of the 5hmC fragments can also decrease the false positive signals even if the deamination reagents is not perfect in a bisulfite free system. We further analyzed the single-base resolution signals from both method in genomic elements.

Based on the above findings, we observed much better consistency from DIP-CAB-Seq method than ACE-Seq, indicating the DIP-CAB-Seq method could have better understanding of the biology with limited sequencing depth (Fig. 2b).

Figure 2 Repeatability of 5hmC sites with ace, DIP-CAB-Seq and nano-seal methods

4. DIP-CAB-Seq can detect detailed preferential occurrences of 5hmC

With single-base resolution information available, we continued to investigate the detailed preferential occurrences of 5hmC sites in the genome to understand the potential of this method in biology study.

The previous base-resolution mapping of 5hmC allowed for the determination of the surrounding base composition of 5hmC sites in mouse ESCs.[22] We aligned our 5hmC sites in CG context and examined the base compositions (Fig. S1). Compared to reported results, our data possesses a similar local sequence context, having increased guanine abundance with a depletion of thymine.

We then performed motif analysis with HOMER32 at +/- 100 bp region around the 5hmC sites. We studied the well-known 5hmC containing motif Klf4, Oct4, Hif1a, Esrrb, and Sox2, our motif analysis successfully identified these motifs around the 5hmC sites in mouse ESCs (Fig.3). Our data indicated that our method

can obtain these detailed 5hmC signals with very low sequencing depth compared to the reported method.

Figure 3 Motif analysis of 5hmC sites in mouse ESCs

Discussion

The current dilemma between cost and sequencing resolution has hamper lots of biological and clinical studies of 5hmC. Generally, 5hmC has 10 times lower genome percentage than 5mC and 5hmC is distributed broadly across the genome, which requires very deep sequencing for detecting low abundant 5hmC sites. Although the profiling methods provides a cheap manner to map the 5hmC peak, it is much more difficult to quantify the 5hmC signals than the single-base resolution method. DIP-CAB-Seq method we developed can solve this problem, although we cannot quantify the 5hmC abundance for a specific 5hmC site, it provides much better resolution than the regular profiling method. We showed that the base-resolution data from the very limited sequencing depth can provide detailed 5hmC loci information. It is well known that 5hmC profiling has the potential to be a clinical tool in different clinical questions. The current profiling can only calculate the 5hmC score based on 5hmC reads density, which needs lots of adjustment between the samples and cohorts. To further study the role of 5hmC in clinical application, our method can provide better quantification information and could be more reliable than the profiling method.

Conclusions

Herein, we developed a method based on ESC whole-genome analysis which could enrich 5hmC-containing DNA by selective chemical labeling and locate 5hmC sites at single-base resolution with enzyme-based deamination. The combination experimental design is an extension of previous methods, and we hope that this cost-effective single base resolution 5hmC sequencing method could be used to promote the mechanism and diagnosis research of 5hmC.

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Footnote

Conflicts of Interest: All authors have no conflicts of interest to declare.

Ethical Statement: None required.

References

1 S. Kriauc˘ ionis and N. Heintz, The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science, 2009, 324, 929–930.

2 M. Tahiliani, K. P. Koh, Y. H. Shen, W. A. Pastor, H. Bandukwala, Y. Brudno, S. Agarwal, L. M. Iyer, D. R. Liu, L. Aravind and A. Rao, Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1 Science, 2009, 324, 930–935.

3 K. E. Szulwach, X. Li, Y. Li, C. X. Song, H. Wu, Q. Dai, H. Irier,A. K. Upadhyay, M. Gearing, A. I. Levey, A. Vasanthakumar,L. A. Godley, Q. Chang, X. Cheng, C. He and P. Jin, 5-hmC-mediated epigenetic dynamics during postnatal neurodevelopment and aging. Nat. Neurosci., 2011, 14, 1607–1616.

4 M. Mellen, P. Ayata, S. Dewell, S. Kriaucionis and N. Heintz, MeCP2 binds to 5hmC enriched within active genes and accessible chromatin in the nervous system. Cell, 2012, 151, 1417–1430.

5 B. M. Colquitt, W. E. Allen, G. Barnea and S. Lomvardas, Alteration of genic 5-hydroxymethylcytosine patterning in olfactory neurons correlates with changes in gene expression and cell identity. Proc. Natl. Acad. Sci. U. S. A., 2013, 110, 14682–14687.

6 S. E. Taylor, Y. H. Li, P. Smeriglio, M. Rath, W. H. Wong and N. Bhutani, Stable 5□Hydroxymethylcytosine (5hmC) Acquisition Marks Gene Activation During Chondrogenic Differentiation. J. Bone Miner. Res., 2016, 31, 524–534.

7 Song CX, Szulwach KE, Fu Y, Dai Q, et al. 2011. Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. Nat Biotechnol 29: 68–72.

8. Cui L, Chung TH, Tan D, Sun X, et al. 2014. JBP1-seq: a fast and efficient method for genome-wide profiling of 5hmC. Genomics 104: 368–75.

9. Pastor WA, Huang Y, Henderson HR, Agarwal S, et al. 2012. The GLIB technique for genome-wide mapping of 5-hydroxymethylcytosine. Nat Protocols 7: 1909–17.

10. Han D, Lu X, Shih AH, Nie J, et al. 2016. A highly sensitive and robust method for genome-wide 5hmC profiling of rare cell populations. Mol Cell 63: 711–9.

11. Pastor WA, Pape UJ, Huang Y, Henderson HR, et al. 2011. Genomewide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature 473: 394–7.

12. Tan L, Xiong L, Xu W, Wu F, et al. 2013. Genome-wide comparison of DNA hydroxymethylation in mouse embryonic stem cells and neural progenitor cells by a new comparative hMeDIP-seq method. Nucleic Acids Res 41: e84.

13. Thomson JP, Hunter JM, Nestor CE, Dunican DS, et al. 2013.Comparative analysis of affinity-based 5-hydroxymethylation enrichment techniques. Nucleic Acids Res. techniques. Nucleic Acids Res 41: e206

14. Robertson AB, Dahl JA, Ougland R, Klungland A. 2012. Pull-down of 5-hydroxymethylcytosine DNA using JBP1-coated magnetic beads. Nat Protocols 7: 340–50.

15. Yu M, Hon GC, Szulwach KE, Song CX, et al. 2012. Base-resolution analysis of 5-hydroxymethylcytosine in the mammalian genome. Cell 149:1368–80.

16. Booth MJ, Branco MR, Ficz G, Oxley D, et al. 2012. Quantitative sequencing of 5-methylcytosine and 5-hydroxymethylcytosine at single base resolution. Science 336: 934–7.

17. Petterson A, Chung TH, Tan D, Sun XG, et al. 2014. RRHP: a tag-based approach

for 5-hydroxymethylcytosine mapping at single-site resolution. Genome Biol 15: 456.

18. Sun Z, Dai N, Borgaro JG, Quimby A, et al. 2015. A sensitive approach to map genome-wide 5-hydroxymethylcytosine and 5-formylcytosine at single-base resolution. Mol Cell 57: 750–61.

19. Yu M, Hon GC, Szulwach KE, Song CX, et al. 2012. Tet-assisted bisulfite sequencing of 5-hydroxymethylcytosine. Nat Protocols 7: 2159–70.

20. Booth MJ, Ost TW, Beraldi D, Bell NM, et al. 2013. Oxidative bisulfite sequencing of 5-methylcytosine and 5-hydroxymethylcytosine. Nat Protocols 8: 1841–51

21. Hu L, Liu Y, Han S, Yang L, et al. 2019 Jump-seq: Genome-Wide Capture and Amplification of 5-Hydroxymethylcytosine Sites. J Am Chem Soc. 141(22):8694-8697.

22. Gibas P, Narmontė M, Staševskij Z, et al. 2020 Precise genomic mapping of 5-hydroxymethylcytosine via covalent tether-directed sequencing. PLoS Biol. 18(4): e3000684.

23. Schutsky EK, DeNizio JE, Hu P, Liu MY, Nabel CS, Fabyanic EB, Hwang Y, Bushman FD, Wu H, Kohli RM. 2018 Nondestructive, base-resolution sequencing of 5-hydroxymethylcytosine using a DNA deaminase. Nat Biotechnol.

24. Yibin Liu, Paulina Siejka-Zielińska, Gergana Velikova, Ying Bi et al. 2019 Bisulfite-free direct detection of 5-methylcytosine and 5-hydroxymethylcytosine at base resolution. Nature Biotechnology, 37(4).

25. Wray, J., Kalkan, T., Gomez-Lopez, S., Eckardt, D., et al. 2011. Inhibition of glycogen synthase kinase-3 alleviates Tcf3 repression of the pluripotency network and increases embryonic stem cell resistance to differentiation. Nat. Cell Biol. 13, 838-845.

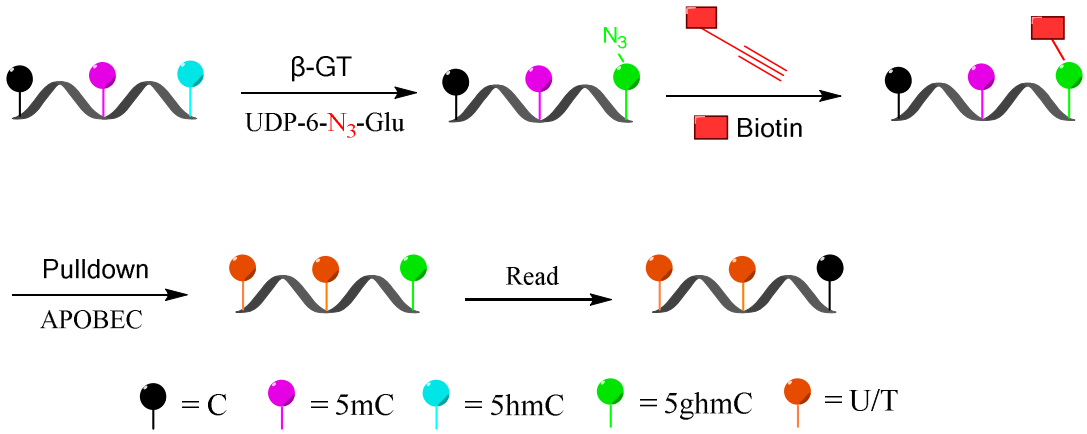
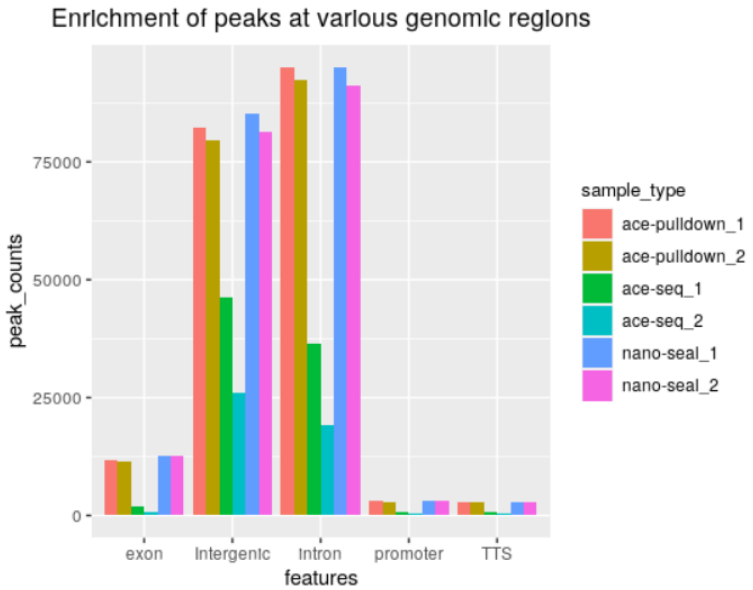


Figure 1 Schematic diagram of selectively labeling and deaminase-based sequencing of 5hmC in DNA (DIP-CAB-Seq).

a)



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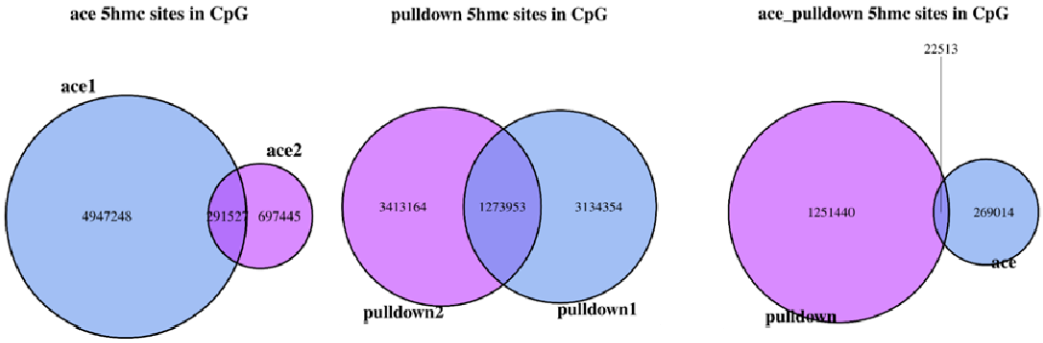

Figure 2 Enrichment of 5hmC-containing DNA fragments with ace, DIP-CAB-Seq and nano-seal methods a) Enrichment of peaks at various genomic regions b) Repeatability of 5hmC sites with ace, DIP-CAB-Seq and nano-seal methods

Name	SEQ19588 P-value	SEQ19589 P-value
Hif1b	1e-16	1e-14
Esrrb	1e-25	1e-22
Oct4	1e-10	1e-4
Sox2	1e-18	1e-14

Figure 3 Motif analysis of 5hmC sites in mouse ESCs