Supporting Information

for

Simultaneous Detection of 5-Methylcytosine and 5-Hydroxymethylcytosine at Specific Genomic Loci by Engineered Deaminase-Assisted Sequencing

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Preparation of 5mC spike-in DNA

A series of double-stranded 5mC spike-in DNA were synthesized for the calculation of the deamination rates of 5mC in different sequence contexts. Detailed sequences are provided in Tables S2.

For the preparation of DNA-C/5mC spike-in, 0.5 ng of synthetic DNA from Takara was used as a template for PCR amplification. The PCR was performed in a 50- μ L mixture containing 1 unit of Q5 High-Fidelity DNA polymerase, 0.2 mM of dNTP, 5 μ L of 10× reaction buffer, 2 μ L of 10 μ M forward primer, and 2 μ L of 10 μ M reverse primer (Table S4). The PCR reaction consisted of 95°C for 5 min, 30 cycles of 95°C for 1 min, 61°C for 1 min, 68°C for 1 min, followed by an elongation at 68°C for 10 min. The PCR products were separated using agarose gel electrophoresis and extracted with a Gel Extraction kit (Omega Bio-Tek Inc., Norcross, GA, USA).

For the DNA-5mC2 spike-in, 0.5 ng of synthetic DNA from Takara was used as a template for PCR amplification. The PCR was performed in a 50- μ L mixture containing 1 unit of Q5 High-Fidelity DNA polymerase, 0.2 mM of dATP, dTTP, and dGTP, 0.2 mM of 5mdCTP, 5 μ L of 10× reaction buffer, 2 μ L of 10 μ M forward primer, and 2 μ L of 10 μ M reverse primer (Table S4). The PCR reaction consisted of 95°C for 5 min, 30 cycles of 95°C for 1 min, 53°C for 1 min, 68°C for 1 min, followed by an elongation at 68°C for 10 min. Similar protocols with different primers (Table S4) were used for the preparation of *THRA* 5mC spike-in DNA and *ALS2CL* 5mC spike-in DNA. For the DNA-5mC2 spike-in, *THRA* 5mC spike-in DNA, and *ALS2CL* 5mC spike-in DNA, all cytosines were replaced with 5mC, excluding the cytosines in the PCR primers.

Name	Sequence (5' to 3')
DNA-C1	GAGTGACGCTGAGCTTGACGTCGCGCGATGAGAGGTGA TTATGAGTATGTATAGTGTTAGGAAGAGTGTAGTAATAG GATGAAGATGATTATATGATCGATGGTCCGTATGCGTAG AATACGTTGTTGTAGTGATTATAATGGAGTGAGAAATGTA GATGAGTGGAGTAGGTAG
DNA-5mC1	GAGTGACGCTGAGCTTGACGTCGCGCGATGAGAGGTGA TTATGAGTATGTATAGTGTTAGGAAGAGTGTAGTAATAG GATGAAGATGATTATATGAT5mCGATGGT5mC5mCGTAT G5mCGTAGAATA5mCGTTGTTGTAGTGATTATAATGGAG TGAGAATGTAGATGAAGTGGAGTAGGTAAGATGTAG TGGTGAAGAGAGTAATTGTTAGTGGAATGTTGGAGAGG AT
DNA-5hmC1	GAGTGACGCTGAGCTTGACGTCGCGCGATGAGAGGTGA TTATGAGTATGTATAGTGTTAGGAAGAGTGTAGTAATAG GATGAAGATGATTATATGAT5hmCGATGGT5hmC5hmCGT ATG5hmCGTAGAATA5hmCGTTGTTGTAGTGATTATAATG GAGTGAGAATGTAGATGAGTGGAGTAGGTAG
DNA-C2	GATGAGAGGTGATTATGAGTCCGTATAGTGTGGTAGGAT ATGCGTATGTAGTATGATAGTGTACGTGTAGGTTATATG ATTATTCGAGTATGATTATATGATATG
DNA-5mC2	GATGAGAGGTGATTATGAGTC5mCGTATAGTGTGGTAGG ATATG5mCGTATGTAGTATGATAGTGTA5mCGTGTAGGT TATATGATTATT5mCGAGTATGATTATGATATGGTGA GTATGTGTAGAATCAGAGAGTAATTGTTAGTGGAATGTT GG
DNA-5hmC2	GATGAGAGGTGATTATGAGT C5hmC GTATAGTGTGGTAG GATAT <mark>G5hmC</mark> GTATGTAGTAGTATGATAGTGT A5hmC GTGTAG GTTATATGATTATT 5hmC GAGTATGATTATATGATATGGT GAGTATGTGTAGAATCAGAGAGTAATTGTTAGTGGAAT GTTGG
DNA-C/5mC	AGTGACGCTGAGCTTGACGTCGCGCGATGAGAGGTGAT TATGAGTA5mCGTATAGTGTTAGGATAGAGTTCGTGTAG TAATAGGATGATAGTATGATTGATTGAATGGTGAGTATG TGTAGAATCAGAGAGTAATTGTTAGTGGAATGTTGG

Table S1. Sequences of DNA substrates with different modifications.

 Table S2. Sequences of spike-ins.

Name	Sequence (5' to 3')
DNA-C/5mC spike-in	AGGAGTCAGCTGGGGCTCACGCGCACTCTGAGAGGTGA
•	TTATGAGTA <mark>5mC</mark> GTATAGTGTTAGGATAGAGTT <mark>C</mark> GTGT
	AGTAATAGGATGATAGTATGATTATATGAATGGTGAG
	TAAGTTATGTGTGAGGTGATGATGA
DNA-5mC2 spike-in	AGGAGTCAGCTGGGCTCACGCGCACTCTAGTGATATG
	TGATGAGAGGTGATTATGAGT <mark>C5mC</mark> GTATAGTGTGGT
	AGGATATG5mCGTATGTAGTATGATAGTGTA5mCGTGT
	AGGTTATATGATTATT5mCGAGTATGATTATATGATAT
	GGTGAGTATGTGTAGAATAGTTAGTAGTAAGTTATGT
	GTGAGGTGATGATGA
THRA 5mC spike-in DNA	AGGAGTTAGTTGGGTTCACGTGCATTTGAGGGTATAG
	TTGTTTAGATTAAGTATGTATTAAG <mark>5mC</mark> GAGGTTTTTT
	TATTTGTGAGTGTAGGTTTTAGTTTAGAAGGGGAAGTT
	ATGTGTGAGGTGATGATGA
ALS2CL 5mC spike-in DNA	AGTGACGCTGAGCTTGACGTCGCGCTGTTTAGTTTTGA
	GTGTTTATGT <mark>5mC</mark> GATGGTTTTTAAATTTAAGGGTTTA
	GTGAAATATAGGTTTTTGGGTTTATTTTTGTATTTTG
	AGAGAGTAATTGTTAGTGGAATGTTGG

Synthetic DNA	Sequence (5' to 3')
DNA-C1, DNA-5mC1 and	Forward: GAGTGACGCTGAGCTTGACGTCGCGC
DNA-5hmC1	Reverse: ATCCTCTCCAACATTCCACTAACAATTA
DNA-C2, DNA-5mC2 and	Forward: GATGAGAGGTGATTATGAGTC
DNA-5hmC2	Reverse: CCAACATTCCACTAACAATTACTC
DNA-C/5mC	Forward:AGTGACGCTGAGCTTGACGTCGCGCGATGAGA GGTGATTATGAGTA <mark>5mC</mark>
	Reverse: CCAACATTCCACTAACAATTACTCTCTG
DNA-C/5mC spike-in	Forward:AGGAGTCAGCTGGGGCTCACGCGCACTCTGAGA GGTGATTATGAGTA <mark>5mC</mark>
	Reverse: TCATCATCACCTCACACATAACTTACTC
DNA-5mC2 spike-in	Forward: AGGAGTCAGCTGGGGCTCACGCGCACTCT
	Reverse: TCATCATCACCTCACACATAACTTACT
THRA 5mC spike-in DNA	Forward: AGGAGTTAGTTGGGTTCACGTGC
	Reverse: TCATCATCACCTCACACATAACTTC
ALS2CL 5mC spike-in DNA	Forward: AGTGACGCTGAGCTTGACGTCGCGC
	Reverse: CCAACATTCCACTAACAATTACTC

Table S3. Sequences of primers used in the synthesis of different DNA.

Deaminated DNA	Sequence (5' to 3')
DNA-C1, DNA-5mC1 and	Forward: GAGTGATGTTGAGTTTGATGTTGTGT
DNA-5hmC1	Reverse: ATCCTCTCCAACATTCCACTAACAATTA
DNA-C2, DNA-5mC2 and	Forward: GATGAGAGGTGATTATGAGTT
DNA-5hmC2	Reverse: CCAACATTCCACTAACAATTACTC
DNA-C/5mC	Forward: AGTGATGTTGAGTTTGATGTTGT
	Reverse: CCAACATTCCACTAACAATTACTCTCTA
DNA-C/5mC spike-in	Forward: AGGAGTTAGTTGGGGTTTATGTGTATTT
	Reverse: TCATCATCACCTCACACATAACTTACTC
DNA-5mC2 spike-in	Forward: AGGAGTTAGTTGGGGTTTATGTGTATTT
	Reverse: TCATCATCACCTCACACATAACTTACT
5mC sites in THRA gene body	Forward: TTGTTTAGTTTTAGGGTTTGGTTTT
	Reverse: CAAATTCCCTCTCCAAAAAAAAAA
5mC sites in ALS2CL gene body	Forward: ATAATAGGTATTGGTTATTGGGTGT
	Reverse: ACAAATTCTTAAAAACCACCTCAAAC
THRA 5mC spike-in DNA	Forward: AGGAGTTAGTTGGGGTTTATGTGT
	Reverse: TCATCATCACCTCACACATAACTTC
ALS2CL 5mC spike-in DNA	Forward: AGTGATGTTGAGTTTGATGTTGTGT
	Reverse: CCAACATTCCACTAACAATTACTC

Table S4. Sequences of primers used in the amplification of deaminated DNA.

 Table S5. Information of the individual cytosine sites detected in genome.

Genome location	Gene	Gene element
chr17.38222379, CRCh37	THRA	Gene body
chr3.46713993, CRCh37	ALS2CL	Gene body



Figure S1. The schematic illustration of plasmid for the expression of wtA3A or eA3A proteins.

Figure S2. SDS-PAGE analysis of the purified wtA3A and eA3A-M5. "M" represents protein marker. (A) SDS-PAGE analysis of the purified wtA3A. (B) SDS-PAGE analysis of the purified eA3A-M5.



Figure S3. The schematic illustration of analytical strategies for mapping 5mC and 5hmC. (A) The schematic illustration of bisulfite treatment-based mapping methods. (B) The schematic illustration of deaminase treatment-based mapping methods. (C) The schematic illustration of pyridine borane treatment-based mapping methods.



Figure S4. Assessment of wtA3A specificity for C, 5mC, and 5hmC across various sequence contexts. (A) The amino acid compositions of wtA3A and engineered A3A mutants (eA3Av1 to eA3A-M5). (B) Sanger sequencing results for DNA substrates containing C, 5mC, and 5hmC (DNA-C1, DNA-5mC1, and DNA-5hmC1, respectively) as treated by wtA3A. wtA3A converted all the C and 5mC sites to U and T, respectively, which were subsequently read as T during sequencing; 5hmC sites underwent partial deamination, resulting in a mixed read of C and T.



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(A)			(B)	wtA3A	
			DNA-C1	<u>TC</u> GATGGT <u>CC</u> GTAT <u>GC</u> GTAGAAT <u>AC</u> G	<u> </u>
			+wtA3A Sequenced		C │Sequencing T
	α 1 Loop 1 	<u>β6 Loop 7</u> 1 ₃₀	DNA-5mC1	LCGATGGTCCGTATGCGTAGAATACG	5mC
→ wtA3A	HIFTSNFNNGIGRHK	AARIYDYDPL	+wtA3A	MMM Markal MARka	Sequencing
eA3A-M1	HIFTSNFNNEPWVRGRHK	AARIYDYDPL	Sequenced	TTGATGGTTTGTATGTGTAGAATATG	
eA3A-M2	HIFTSNFNNEPWVRGRRQ	AARIYDYDPL			
eA3A-M3	HIFTSNFNNEPWVRGRRQ	AARIYDYDTD			
eA3A-M4	HIFTSNFNNEPWERGRRQ	AARIYDYD <mark>TD</mark>	DNA-5hmC1	<u>TC</u> GATGGTCCGGTATGCGTAGAATACG	5hmC
eA3A-M5	HIFTSNFNNEPWPRGRRQ	AARIYDYD <mark>TD</mark>	+wtA3A		Sequencing
			Sequenced	TTG ATGGT ^{CC} GTATG ^C GTAGAATA ^C G	

Figure S5. Assessment of eA3A-M1 specificity for C, 5mC, and 5hmC across various sequence contexts. (A) The amino acid compositions of wtA3A and engineered A3A mutants (eA3A-v1 to eA3A-M5). (B) Sanger sequencing results for DNA substrates containing C, 5mC, and 5hmC (DNA-C1, DNA-5mC1, and DNA-5hmC1, respectively) as treated by eA3A-M1. eA3A-M1 converted all the C and 5mC sites to U and T, respectively, which were subsequently read as T during sequencing; 5hmC sites underwent partial deamination, resulting in a mixed read of T and C.



(B)	eA3A-M1	
DNA-C1	<u>TC</u> GATGGT <u>CC</u> GTAT <u>GC</u> GTAGAAT <u>AC</u> G	
+eA3A-M1 Sequenced		C │Sequencing T
DNA-5mC1 +eA3A-M1 Sequenced		<mark>5mC</mark> ↓Sequencing T
DNA-5hmC1 +etA3A-M1 Sequenced	TCGATGGTCCGTATGCGTAGAATACG	5hmC ↓Sequencing C/T

Figure S6. Assessment of eA3A-M2 specificity for C, 5mC, and 5hmC across various sequence contexts. (A) The amino acid compositions of wtA3A and engineered A3A mutants (eA3A-v1 to eA3A-M5). (B) Sanger sequencing results for DNA substrates containing C, 5mC, and 5hmC (DNA-C1, DNA-5mC1, and DNA-5hmC1, respectively) as treated by eA3A-M2. eA3A-M2 converted all the C and 5mC sites to U and T, respectively, which were subsequently read as T during sequencing; 5hmC sites underwent partial deamination, resulting in a mixed read of C and T.



(B)	eA3A-M2	
DNA-C1	<u>TC</u> GATGGT <u>CC</u> GTAT <u>GC</u> GTAGAAT <u>AC</u> G	
+eA3A-M2 Sequenced		C │Sequencing T
DNA-5mC1 +eA3A-M2 Sequenced	TCGATGGTCCCGTATGCCGTAGAATACG	5mC Sequencing T
DNA-5hmC1 +etA3A-M2 Sequenced	TCGATGGTCCGTATGCGTAGAATACG	<mark>5hmC</mark> ↓Sequencing C/T

Figure S7. Assessment of eA3A-M3 specificity for C, 5mC, and 5hmC across various sequence contexts. (A) The amino acid compositions of wtA3A and engineered A3A mutants (eA3A-v1 to eA3A-M5). (B) Sanger sequencing results for DNA substrates containing C, 5mC, and 5hmC (DNA-C1, DNA-5mC1, and DNA-5hmC1, respectively) as treated by eA3A-M3. eA3A-M3 converted all the C and 5mC sites to U and T, respectively, which were subsequently read as T during sequencing; 5hmC sites remained unaltered and continued to be read as C.

(B)

Sequencing

5mC Sequencing

5hmC Sequencing C



(A)				(B)	eA3	BA-M3	
		R		DNA-C1	TCGATGGTCCGTA	∖T <u>GC</u> GTAGAAT	ACG
				+eA3A-M3 Sequenced	MMM TTGATGGTTTGT/		TATG
	α 1 Loo 20	թ 1 30	<u>β6 Loop 7</u> 130	DNA-5mC1	<mark>ہے</mark> <u>TC</u> GATGGT <u>CC</u> GT	<mark>ہ</mark> ۱۲ <u>GC</u> GTAGAAT	ACG
wtA3A	HIFTSNFNNG.	IGRHK	AARIYDYDPL	+eA3A-M3	<u>^*/////**///</u>		M
eA3A-M1	HIFTSNFNNE	WVRGRHK	AARIYDYDPL	Sequenced	TTGATGGTTTGT	ATGTGTAGAA ⁻	ΓΑ <mark>Τ</mark> G
eA3A-M2	HIFTSNFNNEI	WVRGRRQ	AARIYDYDPL				
→ eA3A-M3	HIFTSNFNNE	WVRGRRQ	AARIYDYD <mark>TD</mark>		ອຍ ຊາມ ຊາມ C	а нш с	-5hmC
eA3A-M4	HIFTSNFNNEI	WERGRRQ	AARIYDYD <mark>TD</mark>	DNA-5hmC1	<u>T¢</u> GATGGT <mark>¢¢</mark> GT/	∖T <u>GĊ</u> GTAGAAT	ī <mark>AĊ</mark> G
eA3A-M5	HIFTSNFNNEI	PWPRGRRQ	AARIYDYDTD	+etA3A-M3	<u>\</u>	MMM	Ŵ
	·			Sequenced	TCGA TGGTCCGT	ATGCGTAGAAT	TACG

Figure S8. Assessment of eA3A-M4 specificity for C, 5mC, and 5hmC across various sequence contexts. (A) The amino acid compositions of wtA3A and engineered A3A mutants (eA3A-v1 to eA3A-M5). (B) Sanger sequencing results for DNA substrates containing C, 5mC, and 5hmC (DNA-C1, DNA-5mC1, and DNA-5hmC1, respectively) as treated by eA3A-M4. After the treatment of eA3A-M4, C and 5mC sites underwent partial deamination, resulting in a mixed read of C and T; 5hmC sites remained unaltered and continued to be read as C.

(A)		9		
	<u>α1</u>	Loop 1	30	<u>β6</u> Loop 7
wtA3A	HIFTSN	FNNGI	GRHK	AARIYDYDPL
eA3A-M1	HIFTSN	FNNEPWVR	GRHK	AARIYDYDPL
eA3A-M2	HIFTSN	FNNEPWVR	gr <mark>rq</mark>	AARIYDYDPL
eA3A-M3	HIFTSN	FNNEPWVR	gr <mark>rQ</mark>	AARIYDYD <mark>TD</mark>
→ eA3A-M4	HIFTSN	FNNEPWER	gr <mark>rq</mark>	AARIYDYDTD
eA3A-M5	HIFTSN	FNNEPWPR	GR <mark>RQ</mark>	AARIYDYD <mark>TD</mark>



Figure S9. Evaluation of the deamination rates of C and 5mC in the same sequence context from different DNA under eA3A-M5 treatment. (A) DNA-C/5mC and DNA-C/5mC spike-in were mixed up with the content of DNA-C/5mC spike-in being 0.1% in the mixture followed by eA3A-M5 treatment and sequencing. (B) The deamination rate of 5mC from DNA-C/5mC and DNA-C/5mC spike-in treated by eA3A-M5 for different time. (C) The deamination rate of C from DNA-C/5mC and DNA-C/5mC spike-in treated by eA3A-M5 for different time.



Figure S10. Quantitative assessment of 5mC level at GC sites containing C and 5mC by EDA-seq. (A) DNA-C2 and DNA-5mC2 were mixed at varying ratios, with DNA-5mC2 ranging from 0% to 100%. A 0.1% DNA-5mC2 spike-in was introduced to determine the deamination rate of 5mC. (B) Sanger sequencing results of GC sites in the DNA-C2 and DNA-5mC2 mixture, along with the DNA-5mC2 spike-in.



(B) Spike-in TAGGATATGCGTATGTAGTATG TAGGATATGCGTATGTAGTATG CGTATGTAGTATG CGTATGTAGTATG	DNA mixture TAGGATATGCGTATGTAGTATG TAGGATATGTGTATGTAGTATG	Theoretical P _{5mC}	Measured P _{5mC}
<u> </u>	MAMAAAAAAA 100%	0%	0%
TA G G A TA T G C G T A T G T A G T A T G	TA G G A TA T G C TA T G TA G TA T G		
<u>/////////////////////////////////////</u>	<u>/////////////////////////////////////</u>	33.0%	30.7%
T A G G A T A T G <mark>C</mark> G T A T G T A G T A T G	TA G G A TA T G T G T A T G T A G T A T G		
<u> </u>	<u></u>	66.0%	63.4%
T A G G A T A T G <mark>C</mark> G T A T G T A G T A T G	TA G G A TA T G C G TA T G TA G TA T G		
MMM 41.2%	MMMMM 42.4%	100%	98.0%

Figure S11. Quantitative assessment of 5mC level at AC sites containing C and 5mC by EDA-seq. (A) DNA-C2 and DNA-5mC2 were mixed at varying ratios, with DNA-5mC2 ranging from 0% to 100%. A 0.1% DNA-5mC2 spike-in was introduced to determine the deamination rate of 5mC. (B) Sanger sequencing results of AC sites in the DNA-C2 and DNA-5mC2 mixture, along with the DNA-5mC2 spike-in.



(B)	U		
Spike-in g	DNA mixture	Theoretical	Measured
ATAGTGTACGTGTAGGTTATAT R _{5mC}	ATAGTGTACGTGTAGGTTATAT P_{T}	P _{5mC}	P _{5mC}
A T A G T G T A T A <mark>C</mark> G T G T A G G T T A T A T	A T A G T G T A T G T G T A G G T T A T A		
<u> </u>	MAMMAMAMMM 100%	0%	0%
A T A G T G T A <mark>C</mark> G T G T A G G T T A T A T	A T A G T G T A <mark>T</mark> G T G T A G G T T A T A T		
44.4%	<u> </u>	33%	30.1%
A T A G T G T A <mark>C</mark> G T G T A G G T T A T A T	ATAGTGTA <mark>C</mark> GTGTAGGTTATAT		
		669/	64 60/
43.9%	05.5%	00 %	01.5%
A T A G T G T A C T G T G T A G G T T A T A T	A T A G T G T A C G T G T A G G T T A T A T		
<u> </u>	Ann 43.8%	100%	99.5%

Figure S12. Quantitative assessment of 5mC level at CC sites containing C and 5mC by EDA-seq. (A) DNA-C2 and DNA-5mC2 were mixed at varying ratios, with DNA-5mC2 ranging from 0% to 100%. A 0.1% DNA-5mC2 spike-in was introduced to determine the deamination rate of 5mC. (B) Sanger sequencing results of CC sites in the DNA-C2 and DNA-5mC2 mixture, along with the DNA-5mC2 spike-in.



Figure S13. Quantitative assessment of C, 5mC, and 5hmC levels at GC sites containing C, 5mC, and 5hmC. (A) DNA-C2, DNA-5mC2, and DNA-5hmC2 were mixed at proportions of 30%, 30%, and 40%, respectively. A 0.1% DNA-5mC2 spike-in was included in the mixture to determine the deamination rate of 5mC. (B) Sanger sequencing results of GC sites in the DNA-C2, DNA-5mC2, and DNA-5hmC2 mixture, as well as the DNA-5mC2 spike-in, after treatment with eA3A-M5 for varying durations.



Figure S14. Quantitative assessment of C, 5mC, and 5hmC levels at AC sites containing C, 5mC, and 5hmC. (A) DNA-C2, DNA-5mC2, and DNA-5hmC2 were mixed at proportions of 30%, 30%, and 40%, respectively. A 0.1% DNA-5mC2 spike-in was included in the mixture to determine the deamination rate of 5mC. (B) Sanger sequencing results of AC sites in the DNA-C2, DNA-5mC2, and DNA-5hmC2 mixture, as well as the DNA-5mC2 spike-in, after treatment with eA3A-M5 for varying durations.



Figure S15. Quantitative assessment of C, 5mC, and 5hmC levels at CC sites containing C, 5mC, and 5hmC. (A) DNA-C2, DNA-5mC2, and DNA-5hmC2 were mixed at proportions of 30%, 30%, and 40%, respectively. A 0.1% DNA-5mC2 spike-in was included in the mixture to determine the deamination rate of 5mC. (B) Sanger sequencing results of CC sites in the DNA-C2, DNA-5mC2, and DNA-5hmC2 mixture, as well as the DNA-5mC2 spike-in, after treatment with eA3A-M5 for varying durations.



Figure S16. The schematic illustration of BS-seq and ACE-seq. (A) In BS-seq, all the C sites are deaminated and read as T; 5mC and 5hmC are not deaminated and read as C. The sequence results of BS-seq provides the total proportion of 5mC and 5hmC in the individual cytosine sites. (B) In ACE-seq, all the 5hmC sites are first glycosylated by β -glucosyltransferase. After wtA3A treatment, all the C and 5mC sites are deaminated and read as T; the glycosylated 5hmC (5gmC) are not deaminated and read as C. The sequence results of ACE-seq provides the proportion of 5hmC in the individual cytosine sites.

