Supplemental Table 1. Sample information and sequencing metrics

datatype	sample name	source	species	brain region	cell-type	sex	age (approx)	library polymerase	raw reads	mapped reads	deduplicated reads	filtered reads	Average coverage (per strand)	Cytosines covered	5mCG: A3A non-conversion (ACE-Seq)	5hmCG: Protection (ACE-Seq)	Aggregate bisulfite non-conversion (TAB-Seq)	5mC: Tet1 non-oxidation + BS non-conversion (TAB-Seq)	5hmC: Protection (TAB-Seq)	5mCG: non-conversion (BS-Seq)	5mCH: non-conversion (BS-Seq)
ACE-Seq	WT mESC 2ng	This study	mouse	-	Embryonic stem cells	M	-	Swift Biosciences	284,958,963	204,664,616	105,352,584	104,657,839	9.7x	92.2%	0.6%	99.6%	-	-	-	-	-
ACE-Seq	WT mESC 20ng	This study	mouse	-	Embryonic stem cells	M	-	Swift Biosciences	476,595,789	342,090,450	286,792,206	283,831,798			0.1%	97.8%	-	-	-	-	-
ACE-Seq	Tet TKO mESC 2ng	This study	mouse	-	Embryonic stem cells	M	-	Swift Biosciences	101,781,304	72,640,617	56,654,212	55,853,778	8.1x	91.8%	0.7%	99.3%	-	-	-	-	-
ACE-Seq	Tet TKO mESC 20ng	This study	mouse	-	Embryonic stem cells	M	-	Swift Biosciences	438,318,291	327,194,866	267,657,791	265,810,623			0.1%	98.0%	-	-	-	-	-
ACE-Seq	WT Neuron 2ng	This study	mouse	frontal cortex	Excitatory neurons	M	6 weeks	Swift Biosciences	308,310,333	221,975,650	136,613,628	135,472,931	11.2x	94.1%	1.2%	99.1%	-	-	-	-	-
ACE-Seq	WT Neuron 20ng	This study	mouse	frontal cortex	Excitatory neurons	M	6 weeks	Swift Biosciences	528,153,977	387,042,417	314,762,979	313,613,561				96.9%	-	-	-	-	-
TAB-Seq	WT mESC	GEO: GSE36173	mouse	-	Embryonic stem cells	M	-	Pfu Turbo Cx	998,337,691	N.A.	N.A.	N.A.	17x	N.A.	-	-	0.38%	2.21%	84.40%	-	-
TAB-Seq	Mm 6 wk	GEO: GSE47966	mouse	frontal cortex	Tissue	M	6 weeks	Kapa HiFi U+	627,702,858	386,364,692	N.A.	N.A.	7.2x	89.2%	-	-	0.52%	2.08%	89.34%	-	-
MethylC-Seq	Exc 1	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks	Pfu Turbo Cx	612,291,601	371,891,916	N.A.	N.A.	6.8x	87.5%	-	-	-	-	-	0.29%	0.33%
MethylC-Seq	Exc 2	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks	Pfu Turbo Cx	595,835,129	354,314,068	N.A.	N.A.	6.5x	87.0%	-	-	-	-	-	0.32%	0.36%
RNA-Seq	Exc 1	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks														
RNA-Seq	Exc 2	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks														
ATAC-Seq	Exc 1	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks														
ATAC-Seq	Exc 2	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks														
ChIP-Seq	H3K27ac 1	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks														
ChIP-Seq	H3K27ac 2	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks														
ChIP-Seq	H3K27me3 1	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks														
ChIP-Seq	H3K27me3 2	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks														
ChIP-Seq	H3K4me1 1	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks														
ChIP-Seq	H3K4me1 2	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks														
ChIP-Seq	H3K4me3 1	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks														
ChIP-Seq	H3K4me3 2	GEO: GSE63137	mouse	frontal cortex	Excitatory neurons	M	8-11 weeks														

Supplemental Table 2. DNA modification levels (5mC and 5hmC) at known imprinted regions (mm10)

Chr	Start	End	Locus	Type	Germline imprints?	GC content	Length (bp)	# of imprinted CGs	Parental origin	5mC signal (%)	5hmC signal (%)	5mC+5hmC signal (%)
chr6	58907401	58907794	*Herc3/Nap1l5*	Known	Y	0.442	394	8	M	23.6	26.7	50.3
chr7	143463662	143463978	*Cdkn1c_upstream*	Known	-	0.513	317	8	P	38.9	22.4	61.4
chr2	152686274	152686927	*Mcts2/H13*	Known	Y	0.485	654	25	M	36.2	17.3	53.4
chr12	109459709	109462033	*Dlk1*	Known	N	0.515	2325	15	P	39.4	16.5	56.0
chr9	89877107	89885234	*Rasgrf1*	Known	Y	0.489	8128	54	P	31.0	12.4	43.5
chr1	63250283	63280525	*Gpr1/Zdbf2*	Known	Y	0.431	30243	94	P	23.9	12.2	36.1
chr7	142577578	142579325	*H19_promoter*	Known	N	0.551	1748	16	P	38.8	11.4	50.2
chr15	72801815	72811184	*Peg13/Trappc9*	Known	Y	0.508	9370	133	M	42.0	10.0	52.0
chr2	174283509	174287894	*Nesp*	Known	N	0.550	4386	73	P	36.3	8.9	45.3
chr6	30735840	30739965	*Mest_(Peg1)*	Known	Y	0.509	4126	57	M	43.4	6.7	50.1
chr10	13090122	13092339	*Plagl1*	Known	Y	0.519	2218	29	M	43.0	5.3	48.3
chr7	128688124	128688233	*Inpp5f*	Known	Y	0.570	110	8	M	32.1	5.3	37.4
chr17	12741760	12742949	*Airn/Igf2r*	Known	Y	0.600	1190	42	M	46.3	4.9	51.3
chr15	97054000	97055741	*Slc38a4*	Known	Y	0.567	1742	29	M	51.8	4.3	56.1
chr11	12023322	12026797	*Grb10*	Known	Y	0.529	3476	25	M	50.7	4.1	54.7
chr11	22971545	22974145	*Zrsr1/Commd1*	Known	Y	0.534	2601	47	M	41.4	3.8	45.3
chr7	6727356	6733209	*Peg3/Usp29*	Known	Y	0.497	5854	55	M	47.9	3.8	51.7
chr7	142579886	142582026	*H19_ICR*	Known	Y	0.503	2141	44	P	47.1	3.6	50.7
chr12	109539603	109543039	*Gtl2*	Known	N	0.529	3437	40	P	56.8	3.6	60.4
chr18	12972847	12974748	*Impact*	Known	Y	0.590	1902	64	M	48.1	3.3	51.4
chr2	174327110	174328002	*Gnas1a*	Known	Y	0.634	893	18	M	42.9	2.8	45.7
chr7	143459335	143461476	*Cdkn1c*	Known	N	0.711	2142	35	P	33.7	2.7	36.5
chr2	174292903	174299786	*Nespas/Gnasxl*	Known	Y	0.535	6884	25	M	49.4	2.0	51.4
chr6	4746303	4749370	*Peg10/Sgce*	Known	Y	0.571	3068	41	M	49.1	1.7	50.8
chr7	143294831	143296101	*Kcnq1ot1*	Known	Y	0.538	1271	45	M	52.1	1.5	53.6
chr7	62348214	62348295	*Ndn*	Known	N	0.640	82	5	M	35.4	1.3	36.7
chr7	62376421	62377281	*Magel2*	Known	NA	0.525	861	15	M	29.4	1.0	30.3
chr7	62463585	62464133	*Peg12*	Known	NA	0.729	549	18	M	34.2	0.9	35.2
chr7	60003140	60005283	*Snurf/Snrpn*	Known	Y	0.533	2144	26	M	64.0	0.5	64.6
chr7	62419126	62420854	*Mkrn3*	Known	N	0.550	1729	13	M	36.1	0.4	36.5

Supplemental Protocol 1: APOBEC3A (A3A) Purification

Introduction:

An *E. coli* codon-optimized version of APOBEC3A (A3A) is expressed as an N-terminal MBP fusion, with a C-terminal His tag. MBP aids in solubility and the C-terminal His tag is used for both purification and to decrease the toxicity in *E. coli*. After purification using the C-terminal His tag, both tags are removed with TEV protease and A3A is isolated by purification over a heparin column.

A3A expression

1. Transform A3A expression plasmid (Kan resistant, available from Addgene, 75316) into 50 µL of BL21(DE3) cells that also contain a plasmid expressing Trigger Factor (Chlor resistant). Plate onto an LB-Kan/Chlor plate and allow to grow overnight at 37 °C.
   Note: Next day, colonies should be quite small (with possibly some that are larger). Leaky A3A expression is toxic and also prone to mutation of its own plasmid, so larger colonies are likely from bacteria that have inactivated their own A3A. Therefore, in subsequent steps, choose a small colony to pursue for larger-scale expression.
   Note: Co-expression with Trigger Factor is not essential, but improves soluble yields.
2. Inoculate a small colony into an overnight culture of LB including Kan/Chlor, and grow (shaking) overnight at 37 °C.
3. The next day, inoculate a larger culture (e.g. 1 L of LB Kan/Chlor) with a 1:100 dilution of the overnight culture. Grow at 37 °C until culture reaches an OD of ~0.6. Decrease the temperature to 16 °C, allow cultures to equilibrate and grow for an additional 10-20 minutes.
   Note: Given some toxicity associated with leaky A3A expression, the culture grows slower than typically observed in standard protein expression.
4. Induce A3A expression with 0.1 mM IPTG. Allow to grow for 12-16 hours at 16 °C.

Cell Lysis

1. The next day, pellet cells at 8000xg for 15 minutes. Decant supernatant and resuspend cells in 25 mL of “wash buffer” (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 25 mM imidazole) per liter of culture. Also add EDTA-free protease inhibitor tablets (cOmplete EDTA-free protease inhibitor cocktail, Sigma 11873580001) and 150 U of DNase I (Roche 4716728001).
2. Cells can be lysed either by sonication or with a microfluidizer as per manufacturer’s protocols.
3. Spin down cells for 30 minutes at 2700xg. Discard pellet and carry supernatant forward to purification steps.

Cobalt Purification

Necessary buffers:
Wash buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 25 mM imidazole.
Elution buffer 1: 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 150 mM imidazole.
Elution buffer 2: 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 400 mM imidazole.

1. Use wash buffer to wash appropriate volume of resin (Cobalt HisPur resin; ThermoScientific 89964) at least 3 times in a 50 mL falcon tube.
2. Add washed resin to the cell lysate and incubate for at least 1 hour while rocking at 4 °C for batch binding to column.
3. Put the lysis through a gravity column (Bio-Rad 7321010 or similar) and collect the flow-through.
4. Run at least 10 column volumes of wash buffer through the column and collect the wash fractions.
5. Elute in fractions that are the same volume as the resin. Use elution buffer 1 (150 mM imidazole) for the first fraction and elution buffer 2 (400 mM imidazole) for the other three fractions.

6. Analyze fractions on an 8% SDS-PAGE gel stained with Coomassie Blue. Protein should run ~70 kDa (MBP-A3A-His).

Dialysis and TEV cleavage

1. Pool desired elution fractions and dialyze into TEV reaction buffer (50 mM Tris pH 8.0, 0.5 mM EDTA, 1 mM DTT) at 4 °C. Change dialysis buffer once after 4 hours for a total of two rounds of dialysis.
2. After the buffer change, add TEV (available from Addgene, 8827; expression/purification protocol from David Waugh's lab described in PMID 11809930) to a final concentration of ~40 µg/mL (*e.g.* into ~30 mL of pooled elutions, we've added 400 µL of 3 mg/mL TEV stock). TEV will cleave while the sample dialyzes overnight.
   Note: Do not rock or in any way apply shear stress to the protein sample during cleavage step as proteins are likely to precipitate out of solution.
3. After protein is dialyzed and cleaved, but before running over a heparin column, run a small sample on a 12 or 15% SDS-PAGE gel to assess for complete TEV cleavage. If incomplete, add additonal TEV and incubate for longer.

Heparin purification

Necessary buffers:
Buffer A: 50 mM Tris, pH 8.0, 0.5 mM EDTA, 1 mM DTT
Buffer B: 50 mM Tris, pH 8.0, 0.5 mM EDTA, 1 mM DTT, 1 M NaCl
*buffers filtered and degassed.

1) Centrifuge samples for 10 minutes at ≥4000 rpm at 4 °C before loading on the column to remove potential precipitants.
2) Set up FPLC method according to the following parameters:
   a) For an AKTA FPLC with a 1 mL heparin column (GE HiTrap, 17040601), use flow rates of 0.5 mL/min for passing buffer over the column and up to 5 mL/min for wash not over the column.
   b) After necessary washes, equilibrate column with 10 column volumes of buffer A.
   c) Load samples onto column and pass 10 column volumes of buffer A over the column. Collect flow-through.
   d) To elute, run a gradient of buffer B (up to 100% B) over 15 column volumes. Collect fractions in 250 uL volumes.
3) Afterwards, run samples on a 12 or 15% SDS-PAGE gel, including a sample pre-TEV cleavage, the load onto the column (post-TEV cleavage), and also purified TEV (in order to locate the "TEV" band and differentiate it from cleaved A3A, which are very similar in size:
   a. Band sizes:
   b. MBP-A3A-His: ~70 kD
   c. MBP: ~45 kD
   d. TEV: 27 kD
   e. A3A-His: ~25 kD
   f. A3A: ~23 kD
4) Pool desired fractions and dialyze into A3A storage buffer (50 mM Tris pH 7.5, 50 mM NaCl, 10% glycerol, 0.5 mM DTT, 0.01% Tween-20). After sufficient dialysis, determine protein concentration, aliquot (~25 µL), and store at -80 °C. Minimize freeze-thawing of aliquots.

Supplemental Protocol 2: APOBEC-Coupled Epigenetic Sequencing (ACE-Seq), Full Protocol

Introduction:

APOBEC-Coupled Epigenetic Sequencing (ACE-Seq) has been optimized to provide base-resolution 5hmC mapping with low genomic DNA input. The key to this method is the use of human cytosine deaminase APOBEC3A (A3A). We have previously demonstrated that A3A has efficient deaminase activity on both unmodified and methylated cytosine (C and 5mC, respectively) and minimal activity on hydroxymethylcytosine (5hmC) (Schutsky et al., *NAR*, 2017). In ACE-Seq, A3A activity on 5hmC is further suppressed by first glucosylating the base to fully prevent deamination. As ACE-Seq eliminates the need for bisulfite, which can be destructive to DNA, the amount of input DNA required is drastically decreased, down to low nanogram quantities, and perhaps lower with further optimization.

A detailed protocol for ACE-Seq is provided below.

Materials:

- Samples of interest (1-50 ng gDNA per reaction)
- Sonicator for shearing gDNA (e.g. Covaris M220 Focused-ultrasonicator)
- Spike-in controls (per reaction, use 0.5% (w/w) of each)
    - T4 hmC DNA (as a control for βGT protection of 5hmC from deamination)
        - *Genomic DNA from a mutant T4 phage in which inactivating mutations exist in both the alpha- and beta-glucosyltransferase genes, but the machinery to synthesize dhmCTP remains intact. Therefore, every "C" in this genome is 5hmC (in both CpG and CpH contexts). Control primers are listed in the "Quality control via restriction digestion" section.*
    - Methylated lambda phage DNA (as a control for complete deamination of C/5mC)
        - *Unmethylated lambda phage DNA (Promega- CAT #D1521) was enzymatically methylated at all CpG sites using M.SssI methyltransferase (Thermo - CAT #EM0821). Full CpG methylation was validated using HpaII restriction digest and LC-MS/MS analysis. Control primers are listed in the "Quality control via restriction digestion" section.*
    - Note: Ideally, controls should be added to samples of interest before shearing so that they are processed similar to the sample DNA.
- T4-βGT (New England Biolabs, M0357S), along with 50X UDP-Glucose and CutSmart buffer (provided).
- Two thermocyclers
- PCR cooler rack (e.g. Eppendorf, Z606634) – should be stored at -80 °C for at least a few hours before use.
- 100% DMSO
- 10X A3A reaction buffer (200 mM MES, pH 6.0 + 1% Tween-20)
- Freshly-thawed A3A (50 μM stock)
- Zymo Oligo Clean and Concentrator Kit (D4060) or similar
- Swift Biosciences Accel-NGS Methyl-Seq kit (30024)
- Standard Indexing Kit (e.g. Swift Biosciences Methyl-Seq Set A Indexing Kit – 36024)
- Agilent 2100 Bioanalyzer
- Agilent 2100 Bioanalyzer High Sensitivity DNA Kit (5067-4626)
- ApoI-HF (NEB, R3566S) and HaeIII (NEB, R0108S)

Procedure:

Preparation of samples

- Purify DNA as directed, including RNase steps. Measure concentration by Qubit (dsDNA).
- Shear samples (including provided spike-in controls) to ~300 bp.
    - Suggested parameters for Covaris M220 Focused-ultrasonicator (130 µL volume): 150s duration, 200 cycles/burst, 50 watts, and 20% duty cycle, but it should be experimentally determined on each machine.
    - Ideally, shearing should be performed to obtain a 1-50 ng/µL sample in water or 10 mM Tris, pH 8.0; if this is not feasible, samples can be concentrated post-shearing using SPRI beads (e.g. Beckman Coulter, B23318).

5hmC Protection with βGT

Note: ACE-Seq has some conceptual similarities to TAB-Seq. While the βGT protection step is utilized both in TAB-Seq and ACE-Seq, the consequences of inefficient glucosylation are different for the readout of each of these methods. In TAB-Seq, if there is inefficient glucosylation, unprotected 5hmCs will be oxidized by Tet and subsequently deaminated by bisulfite; therefore, there will be a high false negative rate and the amount of 5hmCs will be underestimated. In ACE-Seq, however, unprotected 5hmCs will be deaminated at a low rate. Therefore, ACE-Seq limits the undercalling of unprotected 5hmCs relative to TAB-Seq.

- Assemble the reactions in a total volume of 5 µL using the following chart on a per reaction basis.
    - Note: If processing many samples, making a mastermix of everything except the sample DNA is highly recommended.

Sample (1-50 ng/µL)	up to 3.9 µL
10X Cutsmart Buffer	0.5 µL
50X UDP-Glc	0.1 µL
T4 βGT	0.5 µL
Water	Total volume up to 5 µL
Total Volume	*5 µL*

- Incubate in thermocycler at 37 °C for 1 hour.
    - Note: Samples can be kept at 4 °C or -20 °C if necessary until ready to proceed.

Snap cooling

Note: Should be performed immediately preceding A3A treatment procedure (below). We recommend performing "Snap cooling" and "Conversion with A3A" in quick succession, including making sure necessary thermocyclers are preheated to their starting temperatures (and lid temperatures) before starting this section.

- Preheat one thermocycler to 95 °C (with heated lid). On the other thermocycler, preheat with the “ramp” method in the “A3A treatment” section.
- Add 1 µL of 100% DMSO to each reaction (mixing thoroughly and spinning down). Keep on ice until ready to proceed.
- Remove PCR cooler rack from -80 °C freezer and put on dry ice near the thermocycler.
- Put samples in preheated thermocycler at 95 °C for 5 minutes.
- After 5 minutes, immediately move tubes from 95 °C thermocycler to PCR cooler rack on dry ice. Leave in the PCR rack for 30-60 seconds so they freeze thoroughly. Be careful as lids might pop open. Proceed to "A3A treatment" procedure as soon as possible.

Conversion with A3A

Note: The rationale for the ramping conditions is based on balancing the kinetic tendency for reannealing with A3A deamination efficiency. When the ramp starts at a low temperature, the strands should not favor reannealing and A3A deamination will start. As the temperature slowly increases, A3A deamination efficiency will increase, leading to more deamination events; even though the thermal fluctuations may increase and the tendency of the DNA to reanneal will be higher, the fact that some deamination will already have occurred can disrupt base pairing and disfavor reannealing. The protocol thus aims to increase single-stranded-ness and promote more efficient deamination.

- Thermocycler should already be pre-loaded with the following method so that the temperature of the block is at 4 °C when reactions are prepared:
    - *Note: Lid temperature should be 37-50 °C.*

Temperature	Duration
4 ºC	10 minutes
4 ºC - 50 ºC	ramp: 2:15 min per degree of the ramp (~2 hours total)
50 ºC	10 minutes
4 ºC	Hold

- Make a mastermix of 10X A3A reaction buffer, water, and A3A:
    - Per reaction, use 1 µL 10X buffer, 2 µL water, and 1 µL 50 µM A3A.
- Add 4 µL of the mastermix to the side wall of snap frozen reactions. Spin down briefly.
    - Note: Mastermix may freeze when pipetted onto the sides of the tubes, especially if there are many samples. Take caution when tubes are spun down that the samples mix adequately.
- Transfer samples quickly to the thermocycler preheated with the temperature ramp (above). Immediately begin the method.
- Return A3A aliquot to -80 °C freezer.
    - Note: In general, a fresh aliquot of A3A is recommended, as multiple freeze-thaw cycles could negatively impact the efficiency of conversion.

Library preparation and Illumina sequencing

Note: This protocol is compatible with any post-bisulfite adaptor tagging (PBAT) library preparation method. After comparing many kits, we achieved the most success generating high complexity libraries with the Swift Biosciences Accel-NGS Methyl-Seq kit.

- After conversion, ACE-Seq reactions should be purified using Zymo Oligo Clean and Concentrator Kit and eluted in 15 µL of EB (10 mM Tris, pH 8.0).
- Follow the procedure for the Accel-NGS Methyl-Seq kit from Swift Biosciences for library preparation.
    - Note: Before final library amplification step, use qPCR to empirically determine the correct number of cycles for library amplification to obtain highly-complex libraries.
- After final purification of the libraries, 1-5 ng should be run on an Agilent Bioanalyzer High Sensitivity DNA Chip to confirm proper library fragment sizes.

Quality control on phage spike-ins

Note: The following protocol describes a quick and qualitative assay for assessing overall levels of C/5mC conversion and 5hmC protection utilizing restriction enzyme cleavage of amplified

fragments of spike-in DNA as a readout. Near-complete cleavage of fragments for each lambda DNA and T4 DNA are indicative of efficient conversion and protection, respectively.

Note: This protocol utilizes the final Illumina library as the template for amplification. Alternatively, this protocol can also be performed directly after the "Conversion with A3A" protocol (before or after purification).

- Set up PCRs according to the table below. For quality control, set up one PCR reaction for amplifying the lambda spike-in (with lambda forward and reverse primers below) and one for amplifying the T4 spike-in (with T4 forward and reverse primers below):

Sample (directly from deamination reaction)	1 µL
For primer (10 µM)	1 µL
Rev primer (10 µM)	1 µL
Water	22 µL
Taq 2X Mastermix	25 µL

  - *Note: While the spike-in control PCRs were optimized with Taq, the procedure could be optimized with a uracil-tolerant high-fidelity polymerase.*

Primers for spike-ins:
Lambda forward: 5' aaaggccactatcaggcagctttgt 3'
Lambda reverse: 5' gaagaaatgtttcctgtaatggaagatg 3'
T4 forward: 5' agcttatttgaaattaactgggttg 3'
T4 reverse: 5' tcttctccagaataaatgaatttacct 3'

PCR conditions:
95 °C for 5 min
95 °C for 15 s
50 °C for 30 s
60 °C/72 °C for 15 s (60 °C for lambda; 72 °C for T4-hmC)
60 °C/72 °C for 5 min (60 °C for lambda; 72 °C for T4-hmC)
4 °C hold

- Assess controls either by standard TA cloning and Sanger sequencing of individual clones or by restriction digest. The protocol for restriction digest analysis is described below
  - For lambda controls, set up two reactions using 10 µL each of the lambda control PCR reaction:
    (1) uncut - as a control for amplification efficiency
    (2) ApoI-HF - recognizes RAATTY (underlined T being the deamination product). If C/5mC were deaminated efficiently, the PCR product should cleave to near completion. Full length = 122 bp; cleavage products = 79 bp + 43 bp

  - For T4 controls, set up two reactions using 10 µL each of the T4 control PCR reaction:
    (1) uncut - as a control for amplification efficiency
    (2) HaeIII - recognizes GGCC sequences. If deaminated, this site will be protected from cleavage. If 5hmC was efficiently protected, near-complete cleavage would be predicted. Full-length = 107 bp; cleavage products = 54 bp + 53 bp

  - After digestion for at least one hour at 37 °C, reactions should be run on a 20% polyacrylamide gel and stained with SybrSafe or analogous visualization reagent.