5hmC TAB-Seq Kit
Catalog no. K001

The 5hmC TAB-Seq Kit can be used to prepare genomic DNA to differentiate 5-hydroxylmethylcytosine (5hmC) from 5-methylcytosine (5mC) for subsequent sequencing applications by converting 5-methylcytosine (5mC) to 5-carboxylcytosine (5caC) while keeping 5hmC protected.

Kit Contents:

10×βGT protection buffer	30 µl
10 mM UDP-Glucose	15 µl
T4-βGT (18 µM)	15 µl
Tet1 oxidation reagent 1	50 µl
Tet1 oxidation reagent 2	180 µl
Tet1 protein (5.0 mg/ml)	48 µl
Proteinase K (20 mg/ml)	15 µl

Additional Reagents May be Needed:

- 5mC spike-in control DNA and primers
- 5hmC spike-in control DNA and primers
- QIAquick Nucleotide Removal Kit (Qiagen)
- Micro Bio-Spin 30 Columns (BioRad)
- QIAquick PCR Purification Kit (Qiagen)
- EpiTect Bisulfite Kit (Qiagen)
- Zero Blunt TOPO PCR Cloning Kit (Invitrogen)
- PfuTurbo Cx Hotstart DNA Polymerase (Agilent) or any high fidelity polymerase

Protocol

Important notes before starting

- Aliquot Tet1 and store at -80 °C or lower upon arrival. Avoid repeated freezing and thawing (up to **THREE** times). Multiple freeze/thaw cycles may result in decreased performance.
- Store Tet1 oxidation reagents 1 and 2 at -80°C.
- The reaction solution should turn from transparent to brown after mixing Tet oxidation reagents 1 and 2.

Procedure

Preparation of genomic DNA

1. **For each 1 µg genomic DNA, add 5 ng (0.5%) 5mC control DNA and sonicate the mixed genomic DNA to 300 bp.**
2. **After sonication, for each 1 µg genomic DNA, add 30 ng (3%) 5hmC control DNA.**

βGT-based 5hmC protection

3. **Prepare the β-glucosyltransferase (βGT)-based reaction.**

	Amount	Final Concentration
Genomic DNA from Step 2	2-5 µg, up to 16.5 µl	100-250 ng/µl
10 mM UDP-Glucose	1 µl	200 µM
10×βGT protection buffer	2 µl	1×
T4-βGT (18 µM)	1.1 µl	1 µM
Milli-Q water	to 20 µl	
Final volume	20 µl	

Typically, 2-3 µg genomic DNA to start with in Step 3 should be sufficient.

4. **Mix well and allow the reaction to proceed at 37 °C for 1 h.**
5. **Purify DNA using QIAquick Nucleotide Removal Kit (Qiagen). Elute DNA in 30 µl water.**

70-80% DNA from Step 3 can be recovered after Step 5.

mTet1-based 5mC oxidation

6. **Prepare the Tet1-based oxidation reaction. Add each component in the order listed below.**

	Amount	Final Concentration
Genomic DNA	500 ng, up to 26.1 µl	10 ng/µl
Tet oxidation reagent 1	3.5 µl	
Tet oxidation reagent 2	15 µl	
Tet1 protein (5.0 mg/ ml)	4.8 µl	0.48 µg/ µl
Milli-Q water	to 50 µl	
Final volume	50 µl	

For each 500 ng DNA to start with in Tet1 oxidation reaction, 200-300 ng of DNA can be recovered after purification (Step 5).
Note: If a different amount of genomic DNA is applied, change the volume of the reaction accordingly (from 20 µl to 50 µl) with the same final concentration of all reagents.
Note: If there is more than 3% of 5mC in the genomic DNA, increase the concentration of Tet1 accordingly.

7. **Mix well and allow the reaction to proceed at 37 ^{o}C for 1 h.**

8. **Add 1 µl Proteinase K (20 mg/ml) to the reaction mixture and incubate at 50 ^{o}C for 1 h.**

9. **Purify the oxidized DNA with Micro Bio-Spin 30 Columns (Bio-Rad) first and then apply to QIAquick PCR Purification Kit (Qiagen). Elute DNA in 30 µl water.**

Test conversion efficiency of 5mC to 5caC of the 5mC spike-in control DNA

10. **Apply 50 ng Tet1-oxidized DNA from Step 9 to EpiTect Bisulfite Kit (Qiagen) following the manufacture's instruction.**

11. **Use the table below as guidance to prepare 50 µl PCR.**

	50 µl PCR Reaction	Final Concentration
10×PfuTurbo C_x reaction buffer	5 µl	1×
10 mM dNTP Mix	1 µl	200 µM
5mC control primers (10 µM)	2 µl	0.2 µM each
Bisulfite treated DNA (from Step 10)	1 µl	
PfuTurbo C_x DNA Polymerase (2.5 U/ µl)	1 µl	2.5 U/50 µl PCR
Milli-Q water	to 50 µl	
Final volume	50 µl	

Set up and run the PCR program as follows:

	Cycles	Temp	Time
Initial activation step	1	95 °C	2 min
Denaturation	40	95 °C	0.5 min
Annealing		57 °C	0.5 min
Extension		72 °C	1 min
Final Extension	1	72 °C	10 min

12. Verify PCR amplification by running 5-10 µl of PCR reaction on agarose gel (single band at ~300 bp)

13. Use 1 µl of PCR reaction in Zero Blunt TOPO PCR Cloning Kit (Invitrogen) to isolate individual clones for sequencing.

Recommended: Pick and sequence at least 20 individual clones.

14. Check the sequencing results to estimate the conversion efficiency of 5mC at CpG positions.

DNA methylated with CpG methyltransferase selectively at CpG sites is recommended as the spike-in control. Over 95% conversion of 5mC should be obtained.

Test 5hmC protection efficiency of 5hmC spike-in control DNA

15. Use the table below as guidance to prepare 100 µl PCR.

	100 µl PCR Reaction	Final Concentration
10×HotStarTaq PCR buffer	10 µl	1×
10 mM dNTPs	2 µl	200 µM
5hmC control primers (10 µM each)	2.5 µl	0.25 µM each
Bisulfite treated DNA (from Step 10)	2 µl	
HotStarTaq DNA Polymerase (5U/ µl)	0.5 µl	2.5 U/100 µl PCR
Milli-Q water	to 100 µl	
Final volumn	100 µl	

16. Set up and run the PCR program as follows:

Cycle step	Cycles	Temp	Time
Initial activation step	1	95 °C	16 min
Denaturation	48	95 °C	0.5 min
Annealing		45 °C	0.5 min
Extension		72 °C	1 min

Final Extension	1	72 °C	7 min

17. Verify PCR amplification by running 10 µl of PCR reaction on agarose gel (single band at 140 bp)

18. Purify the PCR product with QIAquick PCR Purification Kit (Qiagen). Elute DNA in 30 µl water.

19. Concentrate it to ~10-15 µl and send for Sanger sequencing.

Over 80% of 5hmC should be read as C. The real protection efficiency should be over 95% due to contamination of most commercially available 5hmdCTP. If experiments are run alongside conventional bisulfite treatments, dCTP contamination can be adjusted for by direct measurement of bisulfite converted cytosine in the 5hmC spike-in control.

The Genomic DNA prepared from Step 9 is now ready for genome-wide or loci-specific 5hmC sequencing analysis following the following recommended protocol.

Recommended Protocol For Genome-Wide 5hmC Sequencing Analysis

1. **500 ng-900 ng treated genomic DNA (from Step 9) was end-repaired, adenylated, and ligated to methylated (5mC) adapters (Illumina TruSeq Genomic DNA adapters) according to standard Illumina protocols for genomic DNA library construction, maintaining the proper molar ratios of adapter to insert.**

2. **Adapter ligated fragments with 200-600 bp inserts were gel purified by 2% agarose gel electrophoresis and sodium-bisulfite treated using the MethylCode kit (Invitrogen).**

3. **Bisulfite treated adapter-ligated DNA was amplified by PCR with PfuTurbo Cx Hotstart DNA polymerase.**

   The number of PCR cycles used was determined by quantification of bisulfite treated adapter-ligated DNA by qPCR (KAPABiosystems library quant kit for Illumina libraries) such that the final library concentration obtained was approximately 20 nM.

4. **Final sequencing libraries were purified with AMPure XP beads or 2% agarose gel electrophoresis and quantified by qPCR (KAPABiosystems library quant kit for Illumina libraries).**

   Up to 3 separate PCR reactions were performed per sample.

Recommended Protocol For Loci-Specific 5hmC Sequencing Analysis

1. **Use the table below as a guide to prepare 50 µl PCR.**

	50 µl PCR Reaction	Final Concentration
10×HotStarTaq PCR buffer	5 µl	1×
10 mM dNTPs	1 µl	200 µM
primers of interested loci (12.5 µM each)	1 µl	0.25 µM each
Bisulfite treated DNA (from Step 10)	2 µl	
HotStarTaq DNA Polymerase (5U/ µl)	0.25 µl	1.25 U/50 µl PCR
Milli-Q water	to 50 µl	
Final volumn	50 µl	

Protocol is provided for HotStarTaq DNA Polymerase. Other polymerase or PCR protocols can be substituted. However, Hot Start polymerase is recommended.

2. **Set up and run the PCR program as follows:**

	Cycles	Temp	Time
Initial activation step	1	95 °C	16 min
Denaturation	35	95 °C	0.5 min
Annealing		50-68 °C	0.5 min
Extension		72 °C	1 min
Final Extension	1	72 °C	7 min

3. **Verify PCR amplification by running 5-10 µl of PCR reaction on agarose gel (clear single band)**

4. **Purify the PCR product with QIAquick PCR Purification Kit (Qiagen). Elute DNA in 30 µl water or EB.**

5. **Apply the purified PCR products to Sanger sequencing analysis. The presence of 'C' represent the positions of 5-hmC.**