ACTIVE MOTIF®

Enabling Epigenetics Research

5hmC Profiling Kit

Catalog No. 55023

(Version A1)

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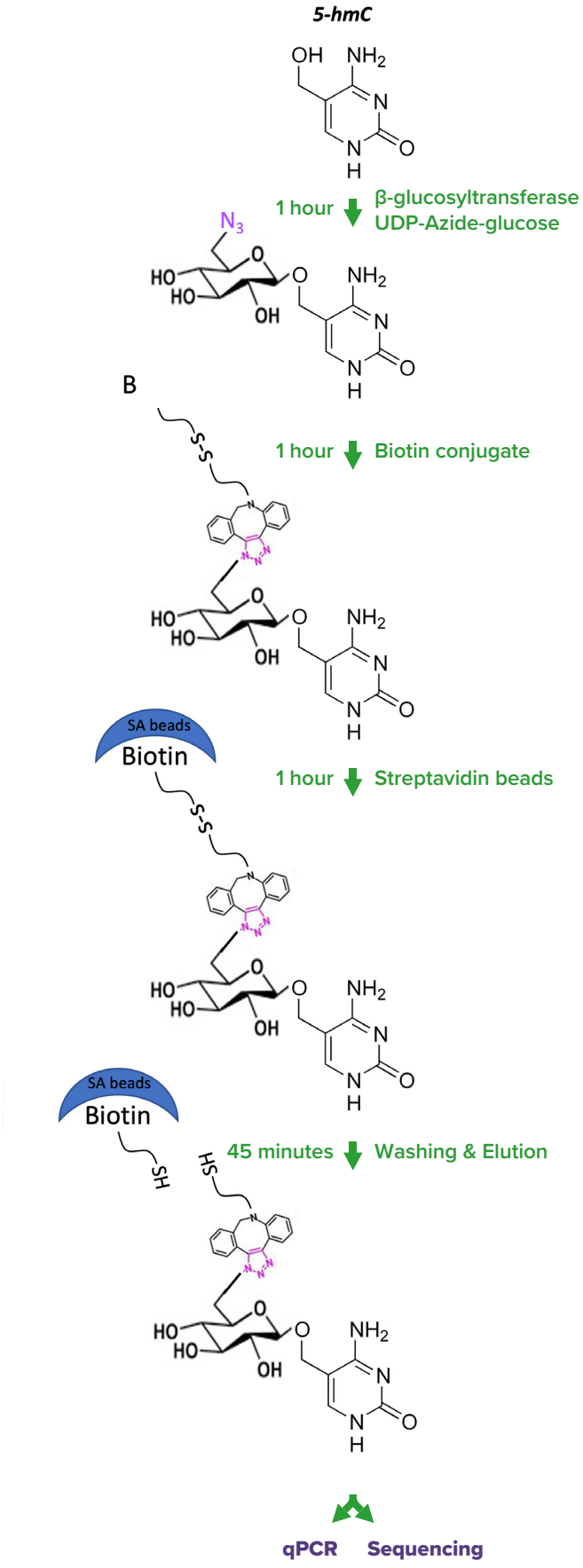
Overview

The 5hmC Profiling Kit is designed to detect and capture DNA fragments containing 5-hydroxymethylcytosine (5hmC) for use in genome-wide analysis. The kit works with double-stranded DNA that has been fragmented to a size range of 100-500 base pairs. The DNA is then incubated in the presence of a β-glucosyltransferase enzyme and modified UDP-glucose donor. The enzyme transfers glucose to 5-hydroxymethylcytosine residues, creating glucosyl-hydroxymethylcytosine. A biotin conjugate is then chemically attached to the modified glucose. Next, magnetic streptavidin beads are used to capture the biotinylated 5hmC DNA fragments. Because the spacer between the biotin and glucose moieties contains a disulfide bond, fragments can be eluted with a reducing agent (elution buffer), therefore enriching fragments containing 5hmC. Following purification with SPRI beads, the enriched DNA can be used in qPCR and/or Next-Generation sequencing for targeted or genome-wide 5-hydroxymethylcytosine analysis, respectively.

By utilizing chemical labeling of 5hmC residues, the 5hmC Profiling Kit is extremely specific and sensitive in its capture of hydroxymethylated DNA fragments. The biotin-streptavidin binding reaction also allows for more stringent binding and wash conditions, enabling sensitivity and specificity that cannot be detected by antibody immunoprecipitation methods. Active Motif's fast, magnetic protocol has been streamlined to minimize the number of wash and incubation steps, delivering enriched 5hmC DNA in less than 5 hours. For added convenience, the kit also includes optional Spike-In DNA containing an equimolar mixture of two DNA fragments containing a small percentage of either 5-hydroxymethylcytosine or 5-methylcytosine. PCR primers targeting these fragments can be used to verify both the efficiency and specificity of the enrichment.

Product	Format	Catalog No.
5hmC Profiling Kit	24 rxns	55023

Flowchart of Process



Kit Components and Storage

Upon receipt, please store each component at the temperature indicated in the table below. Kit and components are guaranteed for 6 months after receipt when stored properly.

Kit Component	Quantity	Storage
Beta-glucosyltransferase	55 µL	-20°C
UDP-Azide-Glucose	65 µL	-20°C
Biotin Conjugate Solution	500 µL	-20°C
Spike-In DNA (10 ng/µL)	55 µL	-20°C
5hmC Spike-In Primer	160 µL	-20°C
5mC Spike-In Primer	160 µL	-20°C
10X Reaction Buffer	140 µL	4°C
Streptavidin Beads*	620 µL	4°C
Binding Buffer AM13	28 mL	4°C
10X Elution Buffer AM2	140 µL	4°C
SPRI Beads*	4.5 mL	4°C
TT Buffer	3.6 mL	RT
10 mM Tris-HCl, pH 8	11 mL	RT

* Do not freeze the Streptavidin Beads or the SPRI Beads. Upon receipt of this kit, store these beads at 4°C.

Additional Materials Required

- Sample DNA that has been fragmented to between 100-500 bp in size
- End-over-end tube rotator
- 80% Ethanol (make fresh)
- 0.2 mL PCR tubes or 0.2 mL strip tubes
- Magnetic rack for 0.2 mL PCR tubes
- PCR tube centrifuge
- Low nucleic acid binding 1.5 or 2 mL microcentrifuge tubes (such as Costar Product no. 3207)
- Water bath or Heat block set at 37°C
- DNase-free water

Protocols

Buffer Preparation

10X Elution Buffer AM2

Prepare a 1X Elution Buffer by diluting the 10X Elution Buffer AM2 in Binding Buffer AM13. For every 50 µL elution reaction, dilute 5 µL 10X Elution Buffer AM2 into 45 µL Binding Buffer AM13.

SPRI Beads

Bring to room temperature for at least 30 minutes before use. Vortex thoroughly 10 seconds.

Sample Input Preparation

Sample DNA Input Quantity

To ensure enough enriched DNA for library preparation we suggest using an input of 80 ng – 2.5 µg fragmented genomic DNA. It may be possible to use less DNA, depending on the sample type. However, with input less than 80 ng, there may be large numbers of PCR duplicates that arise during library preparation making it difficult to accurately interpret sequencing data. The usage of unique molecular indexes (UMIs) may alleviate the PCR duplication problem by adding unique molecular tags to each molecule before library amplification.

For tissues with very low levels of 5hmC, such as cancer cells, it may be necessary to use more than 2.5 µg of DNA. In this case we suggest setting up multiple enrichment reactions and pooling the DNA together during purification.

Fragments should range in size from 100-500 bp. Use the recommended protocols for preparing fragmented DNA by restriction enzyme digestion or sonication prior to starting the 5hmC Profiling Kit assay.

Fragmentation of Genomic DNA

Prior to starting the 5hmC Profiling Kit assay, genomic DNA should be fragmented using either mechanical fragmentation (*e.g.* sonication) or restriction digestion with a methylation-insensitive restriction enzyme to yield fragments ranging in size from 100 to 500 base pairs. We recommend preparing high-quality genomic DNA using a commercially available kit or a standard established protocol. The quality of the genomic DNA can be assessed by agarose gel electrophoresis and DNA concentration can be determined by UV spectrophotometry.

Restriction Digest

Restriction digestion can be used to fragment the genomic DNA by cutting with a methylation-insensitive restriction enzyme. This fragment should be long enough (75 bp or longer) to allow for PCR analysis. Restriction enzyme digestion is recommended if working with limited sample amounts due to the smaller volumes that may be used. Restriction enzyme digestion also offers a more reproducible fragmentation pattern. Some useful methylation-insensitive restriction enzymes are shown in the below table. As might be

expected, the enzymes whose recognition sites contain G and C bases cut more frequently in CpG islands than enzymes whose sites are composed only of A and T bases.

Restriction Enzyme	Recognition Sequence	No. of fragments (per kb) in CpG Islands	No. of fragments (per kb) in non-CpG Islands
Mse I	TTAA	0.8	2.88
Bfa I	CTAG	1.56	1.55
Tas I	AATT	0.8	2.88
Csp6 I	GTAC	2.23	1.41

Follow the recommendations of the enzyme provider to set up the restriction digestion. The following is an example protocol. This protocol can be modified depending on the amount of isolated genomic DNA or the restriction enzyme being used. The example below demonstrates a restriction digestion of 1 µg DNA with Mse I (New England Biolabs (NEB)). Scale up the reaction as needed for larger DNA quantities.

Genomic DNA	1 µg
10X rCUTSmart Buffer	5 µL
Mse I (10 U/µL)	1 µL
dH_2O	to 50 µL final volume
Total volume	**50 µL**

1. Mix well by pipetting and incubate at 37°C for 1 hour to overnight.
2. Heat-inactivate *Mse I* by incubating the reaction mixture at 65°C for 20 minutes.
3. Purify the DNA using either phenol/chloroform extraction and precipitation or using a commercial DNA Purification Kit such as the QIAGEN MinElute Reaction Cleanup Kit (Cat No. 28204).

Mechanical Fragmentation (sonication)

Mechanical fragmentation is ideal when using a large quantity of genomic DNA. In general, the DNA should be sheared to an average fragment size of less than 500 bp. A multi-sample sonicator like Active Motif's PIXUL® or a probe sonicator like Active Motif's EpiShear™ can be used for that purpose.

A protocol optimized with the EpiShear™ is shown below.

1. Transfer genomic DNA into a 1.5 mL microcentrifuge tube and adjust final volume to 300 µL by addition of 10 mM Tris-HCl, pH 8. We suggest using 20 µg or more DNA per sonication reaction.
2. Using a tip probe sonicator, sonicate on ice with 6 pulses of 30 seconds "on" (45% amplitude if using Active Motif's EpiShear™ sonicator), and a 30-second pause on ice between each pulse. The sheared DNA can be visualized by electrophoresis on a 2% agarose gel.

Spike-In DNA (10 ng/µL)

The optional positive control Spike-In DNA (10 ng/ µL) is meant to be added to the fragmented sample DNA before adding to the glucosylation reaction, in order to verify efficient and selective capture of 5hmC. We recommend maintaining a ratio of Spike-In DNA to fragmented sample DNA of 1:10,000 (*e.g.*, 2.5 ng Spike-In DNA (10 ng/ µL) in 25 µg fragmented genomic DNA = 1:10,000 ratio). For more detailed instructions, please refer to the **Appendix, Section A. Use of Included Spike-In DNA** on page 13.

The Spike-In DNA (10 ng/µL) contains an equimolar mixture of two different dsDNA fragments which have been amplified using a small admixture of either 5-methylcytosines or 5-hydroxymethylcytosines.

These fragments were separately amplified from the rabbit IgH locus, pooled at equimolar concentrations, and stored at 10 ng/µL. They should be diluted just before use and the stock solution should always be kept frozen.

The two Spike-In DNA fragment sequences are shown below.

5mC DNA fragment 266 bp sequence:

5'TTCTCTCCACAGGTGTCCACTCCCAGGTCCAAGTTTAAACTCTAGGAAGGAGGGCCACCAT-GACCGAGTGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTACGAATTCGCAGTCG-GTGGAGGAGTCCGAGGGAGGCCTGATCAGGCCTGGAGGAACCCTGACACTCACCTGCACAG-CCTCTGGATTCTCCCTCAGTAGGTATGGAGTGACCTGGGTCCGCCAGGCTCCAGGGAACGGGCT-GGAATGGATCGGATTC3'

5hmC DNA fragment 207 bp sequence:

5'TTGCCTTTCTCTCCACAGGTGTCCACTCCCAGGTCCAAGTTTAAACTCTAGGAAGGAGGGC-CACCATGACCGAGTGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTACGAATTCG-GCCCTTGTGATGACCCAGACTCCACATTCCACGTCTGCGGCCGTGGGAGGCACAGTCACCAT-CAACTGCCAGTCCAGTCAGA3'

5hmC Profiling Protocol

Glucosylation Reaction (60 minutes)

1. Calculate the volumes of reagents needed for each glucosylation reaction using the table below.
2. Set up a 200 µL PCR tube for each glycosylation reaction to be performed.

   Sample: The sample reactions will contain all reagents required for the beta-glucosyltransferase to selectively transfer glucose to 5hmC in the DNA fragments.

   Negative Control: We recommend a negative control reaction alongside the samples which does not contain the UDP-Azide-Glucose donor. There should be no enrichment of 5hmC in the negative control.

   Fragmented Sample DNA + Spike-In DNA*: The recommended range for fragmented sample DNA is between 0.08 µg and 2.5 µg. We suggest a starting quantity of 2.5 µg per sample. For instructions on adding the optional Spike-In DNA to the fragmented sample please see the **Appendix, Section A. Use of the Included Spike-In DNA** on page 13.

Note: The combined total of Spike-In DNA + fragmented sample DNA should not exceed 40 µL.

Sterile water: Determine the amount of sterile water needed to bring the reaction to a final volume of 50 µL.

3. Add reagents in the order listed below to each PCR tube.

Reagents	Sample	Negative Control
Sterile water	_____ µL	_____ µL
10X Reaction Buffer	5 µL	5 µL
UDP-Azide-Glucose	2.5 µL	–
Fragmented sample DNA + Spike-In DNA*	_____ µL	_____ µL
Beta-glucosyltransferase	2 µL	2 µL
Total Volume	**50 µL**	**50 µL**

*The Spike-In DNA is optional.

4. Pipette up and down to ensure complete mixture. Cap the PCR tubes tightly. Quickly spin the tubes to collect the material to the bottom.
5. Incubate the reaction at 37°C for 1 hour.

Biotinylation Reaction (60 minutes)

6. Quickly spin the PCR tubes to collect the contents at the bottom.
7. Add 20 µL Biotin Conjugation Solution to each reaction.

8. Cap the PCR tubes tightly. Vortex the reaction to ensure complete mixture. Quickly spin the tubes to collect the material to the bottom.
9. Incubate the reaction at 37°C for 1 hour.

Purification of Biotinylated DNA (30 minutes)

Perform SPRI clean-up of biotinylated DNA using 105 µL SPRI bead solution, 1.5X the sample volume.

Note: The SPRI beads should be brought to room temperature for 30 minutes and then vortexed for 10 seconds before using.

10. Add 105 µL of well-mixed, room temperature SPRI beads to each tube containing the biotinylated DNA.
11. Pipet up and down 5-10 times.
12. Incubate the tubes for 5 minutes.
13. Place on magnet to collect beads for 10 minutes.
14. Aspirate the supernatant and discard.
15. Repeat the following wash step 2 times.
    a. With the magnet still applied, add 200 µL of freshly prepared 80% ethanol.
    b. Incubate for 30 seconds at room temperature.
    c. Aspirate the supernatant and discard.
16. Allow tubes to sit at room temperature so that residual ethanol can evaporate. Once the beads transition from shiny to matte (2-5 minutes) proceed to the next step.
17. With the tubes off the magnet, resuspend the beads using 55 µL of 10 mM Tris HCl, pH 8.0. Wait 2 minutes.
18. Place the tubes on the magnet for 2 minutes.
19. For future sequencing or qPCR analysis, a small amount of the supernatant will be put aside as Input and stored at -20°C by doing the following:
    a. Transfer 5 µL of the supernatant into a clean PCR tube.
    b. To this add 15 µL of TT Buffer for a final volume of 20 µL.

Note: The TT Buffer contains 0.05% Tween-20 to minimize the loss of DNA.

    c. Store this DNA at -20 °C.

20. Pipette off the remaining supernatant (~50 µL) from the magnetic beads into a second clean PCR tube.
21. This DNA can be safely stored in tubes at -20°C until use in the capture reaction.

Capture Reaction (60 minutes)

Be sure the Streptavidin Beads are fully resuspended before adding to each capture reaction by vortexing. If working with multiple reactions, the beads may need to be resuspended more than once.

22. In a fresh set of 0.2 mL PCR tubes prepare the following reactions:

Reagents	Sample	Negative Control
Streptavidin Beads	25 µL	25 µL
Binding Buffer AM13	25 µL	25 µL
Purified Biotinylation Reaction	50 µL	50 µL
Total Volume	**100 µL**	**100 µL**

23. Incubate the reactions for 1 hour at room temperature with end-to-end rotation.

Washing and Elution (60 minutes)

Before washing and elution be sure that you've prepared 1X Elution Buffer by diluting the 10X Elution Buffer AM2 into Binding Buffer AM13. For every 50 µL elution reaction, dilute 5µL 10X Elution Buffer AM2 into 45 µL Binding Buffer AM13.

24. After the capture step is complete, spin the PCR tubes briefly and place tubes on a magnetic stand to pellet beads to the side of the tube. If further analysis of the unbound fraction will be performed, place supernatant in a microcentrifuge tube and store at -20°C. Otherwise, carefully remove and discard the supernatant.

25. Repeat the following wash steps (a-c) five times:

- **a.** Add 200 µL Binding Buffer. Pipette 2-3 times gently to resuspend. Ensure that the beads do not stick to the pipette tips. [Depending on the strength of the magnet being used, it may be necessary to remove the tubes from the magnet and place in a separate rack to fully resuspend the beads.]
- **b.** Place tubes on magnetic stand and allow beads to pellet on the side of the tube.
- **c.** Carefully remove the supernatant and any residual bubbles.

26. After all five washes resuspend the beads with 50 µL 1X Elution Buffer by pipetting 2-3 times.

27. Incubate for 30 minutes at room temperature with end-to-end rotation.

28. Briefly centrifuge the tubes to collect liquid from the cap.

29. Place tubes in magnetic stand and allow beads to pellet onto tube sides.

30. Transfer the supernatant, which contains the enriched DNA, to a fresh tube.

31. Proceed to Purification of Enriched DNA.

Purification of Enriched DNA (30 minutes)

Perform SPRI clean-up of enriched DNA using 75 µL SPRI bead solution, 1.5X the sample volume.

Note: The SPRI beads should be brought to room temperature for 30 minutes and then vortexed for 10 seconds before using.

32. Add 75 µL of well-mixed, room temperature SPRI beads to each tube containing the biotinylated DNA.

33. Pipet up and down 5-10 times.

34. Incubate the tubes for 5 minutes.

35. Place on magnet to collect beads for 10 minutes.

36. Aspirate the supernatant and discard.

37. Repeat the following wash steps 2 times.

- **a.** With the magnet still applied, add 200 µL of freshly prepared 80% ethanol.
- **b.** Incubate for 30 seconds at room temperature.
- **c.** Aspirate the supernatant and discard.

38. Allow tubes to sit at room temperature so that residual ethanol can evaporate. Once the beads transition from shiny to matte (2-5 minutes) proceed to the next step.

39. With the tubes off the magnet, resuspend the beads using 20 µL of TT Buffer. Wait 2 minutes.

40. Place the tubes on the magnet for 2 minutes.

41. Pipette the supernatant from the magnetic beads into a clean PCR tube.

42. For long term storage transfer to a low nucleic acid binding tube at -20 °C until use in downstream analysis.

Downstream Analysis

1. Quantify the 5hmC enriched DNA using a fluorescent quantitation system such as a Qubit. Depending on the level of 5hmC in the sample, Nanodrop or other quantitation systems may not be able to accurately quantify the DNA. Verify that the sample falls within the detectable range of the quantification method selected.

2. If desired, perform real time PCR analysis as a check for enrichment before library preparation. We suggest using 2µL of pulldown for qPCR and 18 µL of pulldown for library construction. If the Optional Spike-In DNA was used, run qPCR with the provided Primer Mix according to the instructions in the **Appendix, Section A. Use of the Included Control Spike-In DNA** on page 13 in order to determine if enrichment was successful. Alternatively, you could amplify your own targets of interest in the DNA samples. The amplification conditions will need to be optimized for each target locus, master mix, and instrument.

3. Follow the recommendation for library preparation based on the sequencing platform to be used. This process involves adding adapters to the ends of the dsDNA fragments. The library is then PCR amplified and validated prior to sequencing. Use a commercially available library preparation kit and follow the manufacturer's recommendations. Enriched DNA from Active Motif's 5hmC Profiling Kit has been extensively validated using the Illumina® sequencing platforms. The Input DNA set aside can also be submitted for sequencing to determine the baseline peaks for each sample.

Appendix

Section A. Use of Included Control Spike-In DNA

A control containing both 5-methylated (5mC) and 5-hydroxymethylated (5hmC) DNA is included as an optional validation of the enrichment efficiency and specificity. This Spike-In DNA is provided at 10 ng/µL. When the Spike-In DNA is added to the sample DNA, the fragments containing 5hmC will undergo the glycosylation and biotinylation reactions and will be captured by the streptavidin beads. In contrast, the 5mC containing fragments will not undergo these reactions and provide only background signal. Following elution and purification, the DNA can be analyzed using real time PCR with the provided primer mix to verify the efficiency of the capture method. Because the fragment sequences in the Spike-In DNA come from rabbit genomic DNA, they will not interfere with capture reactions on human and mouse genomic DNA.

We recommend using a 1:10,000 ratio of Spike-In DNA (ng) to fragmented sample DNA (ng) for each glycosylation reaction. The Spike-In DNA will need to be pre-diluted first before adding to the fragmented sample DNA. For convenience below are some recommendations for pre-dilution of the Spike-In DNA for different sample ranges.

Input is 20 – 200 ng fragmented sample DNA

1. First create a 1:10,000 diluted stock of Spike-In DNA.
    a. Label three 0.2 mL PCR tubes 1:100, 1:1,000, and 1:10,000 respectively.
    b. Into the 1:100 tube, dispense 198 µL 10mM Tris HCl, pH 8 and 2 µL of Spike-In DNA (10 ng/µL).
    c. Vortex tube for 3 seconds.
    d. Into the 1:1000 tube, dispense 90 µL 10mM Tris HCl, pH 8 and 10 µL of the previous 1:100 dilution.
    e. Vortex tube for 3 seconds.
    f. Into the 1:10,000 tube, dispense 90 µL 10mM Tris HCl, pH 8 and 10 µL of the previous 1:1,000 dilution.
    g. Vortex tube for 3 seconds.
    h. This tube now contains Spike-In DNA at a concentration of 0.001 ng/µL.

2. The equation below can be used to calculate the volume (V) of this diluted Spike-In DNA (0.001 ng/µL) to add to the sample DNA (S) to create a 1:10,000 ratio of Spike-In DNA to sample DNA:

$$V = \frac{\left(\frac{S}{10{,}000}\right)}{0.001\ ng/\mu L}$$

For example, if using 25 ng fragmented sample DNA (S) in the glycosylation reaction we recommend adding 2.5 µL of diluted (0.001 ng/µL) Spike-In DNA (2.5 pg) for a 1:10,000 ratio.

Note: The total volume of Spike-In DNA + fragmented sample DNA should not exceed 40 µL per glycosylation reaction.

Input is 200 – 2000 ng fragmented sample DNA

1. First create a 1:1,000 diluted stock of Spike-In DNA.
   - **a.** Label two 0.2 mL PCR tubes 1:100 and 1:1,000 respectively.
   - **b.** Into the 1:100 tube, dispense 198 µL 10mM Tris HCl, pH 8 and 2 µL of Spike-In DNA (10 ng/µL).
   - **c.** Vortex tube for 3 seconds.
   - **d.** Spin down the liquid for 3 seconds with benchtop minicentrifuge. This tube now contains Spike-In DNA at a concentration of 0.1 ng/µL.
   - **e.** Into the 1:1000 tube, dispense 90 µL 10mM Tris HCl, pH 8 and 10 µL of the previous 1:100 dilution.
   - **f.** Vortex tube for 3 seconds.
   - **g.** Spin down the liquid for 3 seconds with benchtop minicentrifuge. This tube now contains Spike-In DNA at a concentration of 0.01 ng/µL.
2. The equation below can be used to calculate the volume (V) of this diluted Spike-In DNA (0.01 ng/µL) to add to the sample DNA (S) to create a 1:10,000 ratio of Spike-In DNA to sample DNA:

$$V = \frac{\left(\frac{S}{10{,}000}\right)}{0.01\ ng/\mu L}$$

For example, if using 250 ng fragmented sample DNA (S) in the glycosylation reaction we recommend adding 2.5 µL of diluted (0.01 ng/µL) Spike-In DNA (25 pg) for a 1:10,000 ratio.

Note: The total volume of Spike-In DNA + fragmented sample DNA should not exceed 40 µL per glycosylation reaction.

Section B. qPCR recommendations on the Spike-In DNA pool

The Spike-In DNA (10 ng/µL) contains an equimolar mixture of two different dsDNA fragments which have been amplified using a small admixture of either 5-methylcytosines or 5-hydroxymethylcytosines. Post-assay enrichment efficiency before proceeding to library preparation and sequencing can be determined with qPCR performed on these Spike-In targets.

To use the Spike-In QC in conjunction with generated libraries for sequencing, follow the protocol below. You'll be using 10% (by volume) of the Input and enriched pulldown material for qPCR analysis. Use the remaining material for library construction.

Note: It is recommended to prepare triplicates of each sample and Input reaction.

1. Add 48 µL TT Buffer into a PCR tube along with 2 µL of the 20 µL Input DNA (set aside in after Purification of Biotinylated DNA, Step 19).
2. Vortex and spin down.
3. Add 48 µL of TT Buffer into a PCR tube along with 2 µL of the 20 µL enriched DNA (from Purification of Enriched DNA, Step 42).
4. Vortex and spin down.
5. Assemble a master mix for the number of samples required, for either the 5mC or the 5hmC Primer Mix, according to the reaction table below.

Reagent	20 µL PCR reactions
Fast SYBR Green master mix	10 µL
5mC or 5hmC Primer Mix*	2 µL
Sterile water	3 µL
DNA sample (eluted or Input)	5 µL
Total Volume	**20 µL**

*The provided PCR Primer Mixes contain both Forward and Reverse primers for use with the provided control DNA. Use 2 µL of the appropriate PCR Primer Mix (either 5mC or 5hmC) in the 20 µL reaction for the PCR protocol described above.

6. Place tubes in a Real Time PCR instrument and program as shown below. The amplification conditions should be optimized for each target locus, master mix reagent and PCR instrument. A suggested starting point is:

Step	Protocol	
1	95 °C for 2 minutes	
2	95 °C for 10 seconds	Repeat 40x
3	65 °C for 30 seconds	

Section C. % Input Calculation

To calculate % Input:

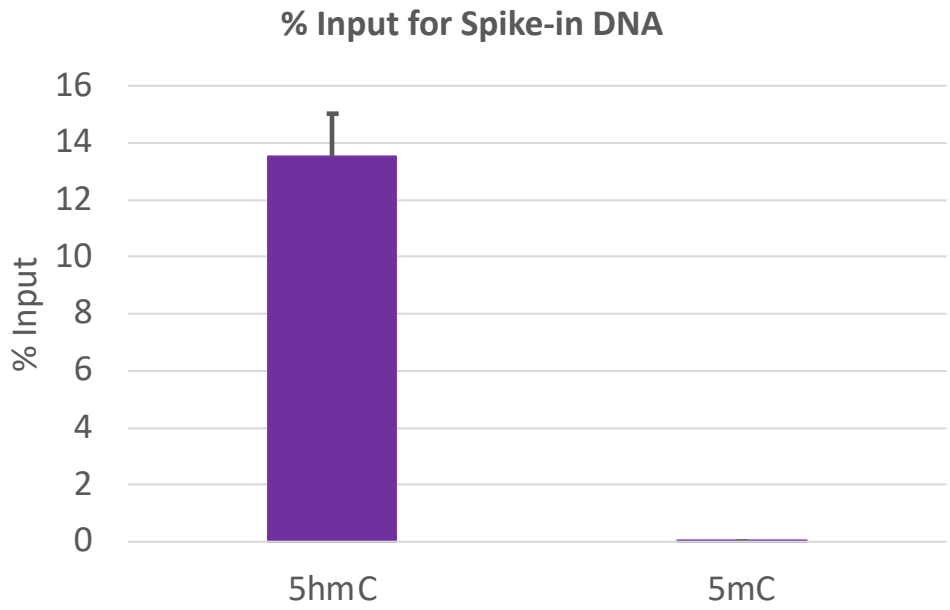
Take the average of the PCR triplicate CTs (n=3) together for each sample. Subtract -3.32 from Input CT value to correct for input amount relative to its pulldown. Use the following calculation for % Inputs per target:

$$\% \text{ input} = (2^{-(\text{Avg Pulldown Ct-Avg Input Ct})}) \times 100$$

Our suggested passing criteria for the Spike-In DNA:

% Input 5hmC >9.5%
% Input 5mC <1%

Below is example data for Spike-In DNA enrichment. In this experiment 31 pg of Spike-In DNA was added to 310 ng of mouse brain gDNA and run through the 5hmC Profiling Kit protocol in triplicate. RT-PCR was done using either the 5mC or the 5hmC primer mix for both the Input and the post-enrichment DNA. The % Input was calculated using the average CT values for each reaction and plotted below. The enrichment was 13.52 for 5hmC and 0.02 for 5mC.



Troubleshooting Guide

Problem/Question	Recommendation
Little or no enrichment of methylated DNA	The 5hmC Profiling Kit is optimized for use with 0.08 – 2.5 µg of fragmented (<500 bp) DNA per reaction. For samples with low levels of 5hmC it may be necessary to use more sample material per reaction. If using more than 2.5 µg, set up multiple reactions and pool samples together during the Purification of Enriched DNA.
	Make sure reagents were prepared as stated in the Buffer Preparation and Recommendation section prior to starting the assay. Reagents should be added in the order listed.
Should I use Restriction Digest or Sonication to fragment my DNA?	Restriction Digest is very precise and reproducible; however, the DNA must be well purified, and analysis of several loci may also require use of different enzymes. In addition, the region of interest may not be flanked by suitable restriction sites and single-nucleotide polymorphisms (SNPs) between different cell types may confound results. In contrast, Sonication is random, which enables analysis of many loci simultaneously.
Should I use heat inactivation to remove the restriction enzymes used to fragment DNA?	After restriction digest, we recommend that samples be treated for 20 minutes at 65°C. Some enzymes (such as Mse I) will be inactivated by this treatment, while those that are not will be forced off the DNA. In most cases (even when using enzymes that are not heat-inactivated), DNA treated in this fashion should be suitable for use in the 5hmC Profiling Kit protocols. In some situations (*e.g.*, when the DNA used in a digest is contaminated with cellular proteins or when a large amount of restriction enzyme is required for the digest) it may be desirable to purify the digested DNA by purification columns or through phenol extraction/ethanol precipitation.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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