Hydroxymethyl Collector™

Catalog No. 55013

(version B2)

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Overview

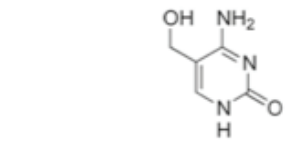
The Hydroxymethyl Collector™ Kit is designed to detect and capture DNA fragments containing 5-hydroxymethylcytosine (5-hmc) DNA methylation*. The kit works with double-stranded DNA that has been fragmented to a size range of 100-500 base pairs. The DNA is then incubated in the presence of a β-glucosyltransferase enzyme and a modified UDP-glucose donor. The enzyme transfers the glucose to 5-hydroxymethylcytosine residues, creating glucosyl-hydroxymethylcytosine. A biotin conjugate is then chemically attached to the modified glucose. Magnetic streptavidin beads and the included bar magnet are used to capture the biotinylated 5-hmC DNA fragments. The elution buffer releases the DNA fragments from the biotin conjugation, leaving you with DNA that is enriched in 5-hmC methylation. Following purification, with the included purification reagents, the enriched DNA can be used for analysis of individual genes by PCR, or in combination with microarrays and sequencing for genome-wide 5-hydroxymethylcytosine analysis.

By utilizing chemical labeling of 5-hmC residues, the Hydroxymethyl Collector Kit is extremely specific in its capture of hydroxymethylated DNA fragments. The biotin-streptavidin binding reaction also allows for more stringent binding and wash conditions, enabling enrichment of 5-hmC residues 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 you enriched 5-hmC DNA in less than 4 hours. For added convenience, the kit also includes a 5-hydroxymethylcytosine DNA control and PCR primers that can be used to verify the efficiency of the enrichment.

product	format	catalog no.
Hydroxymethyl Collector™	25 rxns	55013

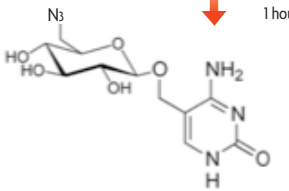
* Patent Pending

Flow Chart of Process



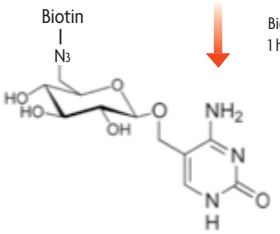
Fragmented DNA
containing 5-hmC

β-Glucosyltransferase enzyme
+ UDP-Azide-Glucose
1 hour at 37 ° C



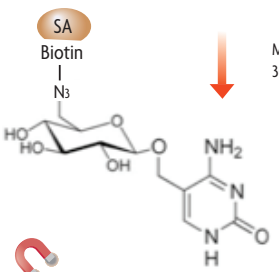
Modified Glucosyl-5-hmC

Biotin Conjugate Solution
1 hour at 37 ° C



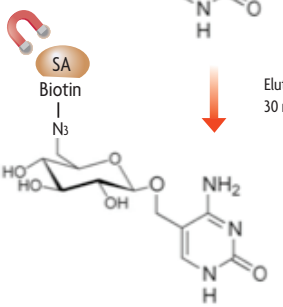
Biotinylated Glucosyl-5-hmC

Magnetic Streptavidin Beads
30 minutes at RT



DNA is captured for washing and elution

Elution of DNA from the Biotin
30 minutes at RT



Eluted DNA is purified using the
included purification reagents

Enriched DNA containing
5-hydroxymethylcytosine

Introduction

5-Hydroxymethylcytosine DNA Methylation (5-hmC)

In mammals and other vertebrates, DNA methylation usually occurs at the C5 position of cytosine (5-mC), mostly within CpG dinucleotides. In 2009, Kriaucionis and Heintz and Tahiliani *et al*. discovered another DNA modification, 5-hydroxymethylcytosine (5-hmC), which was observed to be elevated in neurons and embryonic stem cells[1,2]. The 5-hydroxymethylcytosine modification results from the enzymatic conversion of 5-methylcytosine into 5-hydroxymethylcytosine by the TET family of cytosine oxygenases[2,3]. While the precise function of 5-hmC has yet to be determined, it has been postulated that it could represent a pathway to demethylate DNA, as 5-hydroxymethylcytosine is repaired as mismatched DNA and replaced with unmethylated cytosine[3]. Alternatively, 5-hmC may be produced by the addition of formaldehyde to DNA cytosines by DNMT proteins[4].

One of the difficulties in studying 5-hmC is the fact that many of the traditional techniques employed to study DNA methylation, such as bisulfite sequencing or certain methylation-sensitive restriction enzymes, do not distinguish between 5-mC and 5-hmC residues[3,5,6]. Methyl CpG binding protein enrichment methods only serve to selectively bind 5-mC DNA methylation, but cannot be used to enrich for 5-hmC[5].

In order to overcome some of the difficulties with direct discrimination of 5-mC and 5-hmC residues, a T4 bacteriophage enzyme, β-glucosyltransferase, has been used to modify 5-hydroxymethylcytosine residues. The β-glucosyltransferase enzyme utilizes a UDP-glucose (uridine diphosphoglucose) donor to attach a glucose moiety to 5-hmC which generates glucosyl-hydroxymethylcytosine[7]. With the sugar attached to 5-hmC, the two types of DNA methylation can be discriminated using glucosyl-sensitive restriction enzymes.

Another method used to discriminate between 5-mC and 5-hmC is using antibodies specific for each form of DNA methylation. These antibodies can be used to detect or enrich for DNA fragments containing the methylation of interest. This antibody immunocapture technique is known as Methylated DNA Immunoprecipitation (MeDIP)[8]. One of the drawbacks of the antibody immunoprecipitation method is the enrichment of DNA fragments is limited by the quality of the antibody and the stringency of the binding conditions. High stringency conditions may eliminate weak binding of 5-hmC, while low stringency conditions may increase non-specific binding.

Active Motif's Hydroxymethyl Collector Kit overcomes some of the difficulties with antibody enrichment methods, while still retaining the ability to clearly discriminate between 5-mC and 5-hmC residues. The Kit utilizes the modification properties of β-glucosyltransferase to specifically alter 5-hmC with a modified glucose. A biotin conjugate is chemically introduced and streptavidin magnetic beads are used to enrich for DNA fragments containing the modified 5-hydroxymethylcytosine fragments[9]. The strong binding properties of biotin-streptavidin enable high stringency washes without sacrificing the sensitivity of the assay. Eluted DNA is purified and ready to use in downstream analysis by PCR, microarray or sequencing.

Traditional Methods to Study DNA Methylation

To date, there are several methods used for methylation analysis:

1. **Methylation-sensitive restriction enzyme analysis:** Isoschizomers of bacterial restriction endonucleases with different sensitivities for 5-methylcytosine or 5-hydroxymethylcytosine can be used to determine the methylation status of specific CpG dinucleotides[10]. Methylation-sensitive restriction enzymes have several limitations, such as the fact that the methylation-sensitive restriction merely informs on the methylation status of the cytosine residues which are recognized by the restriction enzymes used, but do not provide information about other methylation sites.
2. **Bisulfite conversion:** Bisulfite conversion consists of the treatment of genomic DNA with sodium bisulfite, leading to deamination of unmethylated cytosines into uracil. PCR is then performed with primers that differentiate between methylated and unmethylated sequences[11]. Bisulfite-based techniques can be cumbersome, involving time- and labor-intensive chemical treatments that damage DNA and limit throughput. Additionally, bisulfite conversion doesn't differentiate between 5-methylcytosine and 5-hydroxymethylcytosine[3,5,6].
3. **Methyl-CpG Binding proteins:** This family of proteins takes its definition from the methyl-CpG binding domain (MBD), the minimum portion with specific affinity for a single, symmetrically methylated CpG pair. The MBD2b protein has been found to possess one of the highest affinities for methylated DNA among MBD proteins and has the greatest capacity to differentiate between methylated and unmethylated DNA[12]. The Methylated CpG Island Recovery Assay (MIRA) utilizes the combination of MBD2b with its binding partner MBD3L1, methyl-CpG-binding protein 3-like-1, to generate a higher affinity for methylated DNA than MBD2b protein alone[13, 14]. Methyl-CpG binding proteins are limited to the evaluation of methylated DNA in a CpG context as the proteins do not recognize methylated cytosines that exist outside of a CpG dinucleotide. The MBD proteins are only capable of binding to 5-mC methylation; they cannot be used to enrich for 5-hmC methylation[5].
4. **Methylated DNA Immunoprecipitation (MeDIP):** Methylated DNA Immunoprecipitation is a an immunocapture technique in which an antibody specific for methylated cytosines is used to immunoprecipitate methylated genomic DNA fragments[8]. The affinity of the antibody used in MeDIP enables the detection of methylated cytosines regardless of their context. This means that MeDIP can be used for the detection of any methylated cytosine and is not restricted to analysis of CpG methylation. The enriched DNA can be used for individual analysis of the methylation status of a particular gene by PCR, or in combination with microarrays for genome-wide methylation analysis. MeDIP can also be used to prepare samples for use in high-throughput sequencing (HTS) techniques. Antibodies are available for the specific detection of either 5-methylcytosine or 5-hydroxymethylcytosine.

Kit Performance and Benefits

The Hydroxymethyl Collector kit is designed to specifically enrich for DNA fragments containing 5-hydroxymethylcytosine (5-hmC).

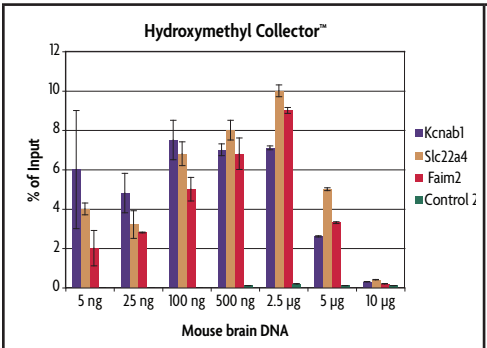
Range of detection: We recommend using between 5 ng and 2.5 µg fragmented genomic DNA per capture reaction for detection by qPCR. Larger quantities of DNA are not efficiently captured (see image below). For downstream analysis by hybridization to a microarray, it may be necessary to pool multiple enrichment reactions or perform whole-genome amplification following 5-hmC DNA enrichment.

For genome-wide analysis by deep sequencing, larger amounts of DNA may be needed. To date Active Motif has not validated the required amount of DNA needed to perform deep sequencing. Suggested input DNA amounts are 8-10 µg from stem cells, 20 µg from cancer cells and 1-3 µg from brain, but optimization may be necessary. To acquire enough sample material for deep sequencing, we recommend performing multiple enrichment reactions and pooling the DNA together.

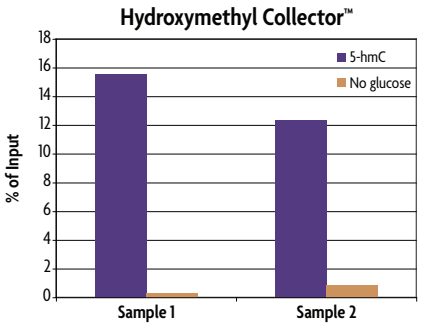
Cross-reactivity: The β-glucosyltransferase reaction is specific to 5-hydroxymethylcytosine and will not react with unmethylated or 5-methylcytosine residues. The chemical conjugation of biotin is specific to the modified glucose moiety included in the kit and will not react with unmodified glucose. This enables precise capture of 5-hydroxymethylcytosine. Hydroxymethyl Collector is suitable with any species (human, mouse, rat, etc.) containing 5-hydroxymethylcytosine residues.

Assay time: 4 hours

Hydroxymethyl Collector™



Hydroxymethyl Collector range of detection.
Mouse brain DNA was assayed at various quantities in the Hydroxymethyl Collector Kit. Enriched DNA was analyzed by qPCR across multiple loci. The enriched DNA was plotted as a percentage of starting material. This graph shows that Hydroxymethyl Collector has a preferred range of detection from 5 ng to 2.5 µg for mouse brain DNA. Larger DNA quantities are not efficiently captured. The Control 2 primer set serves as a negative control in this experiment.



Hydroxymethyl Collector qPCR results using human genomic DNA and "spiked" 5-hmC control.

MseI digested human genomic DNA (500 ng) was "spiked" with 25 pg of the included 5-hmC Control DNA in duplicate. These samples were then processed using the Hydroxymethyl Collector Kit to enrich for 5-hydroxymethylcytosine residues in either the presence (5-hmC) or absence (No glucose) of the UDP-Azide-Glucose donor. Eluted DNA was purified and tested in real time PCR using the included APC PCR primer mix to screen for the enrichment of the 5-hmC Control DNA. The DNA was quantified against the Input standard curve and plotted as a percentage of the starting material. This graph shows that Hydroxymethyl Collector is able to enrich for 5-hmC DNA and that the enrichment only occurs in the presence of the UDP-Azide-Glucose donor.

Hydroxymethyl Collector Kit Components and Storage

Hydroxymethyl Collector Kits are for research use only. Not for use in diagnostic procedures. All components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity	Storage
β-glucosyltransferase enzyme (200 U/µl)	50 µl	-20°C
UDP-Azide-Glucose (3 mM)	65 µl	-20°C
10X Reaction Buffer AM1	500 µl	-20°C
1 M DTT	100 µl	-20°C
Biotin Conjugate Solution	500 µl	-20°C
Streptavidin Beads*	700 µl	4°C
Binding Buffer AM13	50 ml	4°C
10X Elution Buffer AM2	250 µl	4°C
5-hmC Control DNA (50 ng/µl)	10 µl	-20°C
Human genomic DNA, *MseI* digested (20 ng/µl)	300 µl	-20°C
APC PCR Primer Mix (2.5 µM)	400 µl	-20°C
DNA Purification Binding Buffer	50 ml	RT

DNA Purification Wash Buffer**	50 ml	RT
DNA Purification Elution Buffer	5 ml	RT
3M Sodium Acetate***	500 µl	RT
DNA purification columns	50 ea	RT
DNA column collection tubes	50 ea	RT
0.2 ml PCR tubes	1 bag	RT
Bar magnet and glue dots	1 ea	RT

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

** DNA Purification Wash Buffer must be reconstituted to a final concentration of 80% ethanol prior to use.

*** Do not store the 3M Sodium Acetate at 4°C.

Additional materials required

- Sample DNA that has been fragmented between 100-500 bp in size
- End-to-end rotator (e.g. Labquake from Barnstead/Thermolyne)
- Ethanol, 100%
- 1.5 or 2 ml microcentrifuge tubes
- Microcentrifuge
- DNase-free sterile water

Buffer Preparation and Recommendations

Samples

Hydroxymethyl Collector reactions can be performed using 5 ng - 2.5 µg of fragmented genomic DNA. 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 Hydroxymethyl Collector assay.

Set aside a quantity of fragmented genomic DNA equivalent to 10% of the DNA being used in the capture reactions for use as Input DNA (*e.g.* for 500 ng DNA in capture reaction, set aside 50 ng DNA for Input). Separate Input DNA should be saved for each DNA source tested. The Input DNA can be used in real time PCR to quantify the amount of enriched DNA recovered from the biotin capture reaction. For details on real time PCR analysis and the use of Input DNA, see Appendix C. Store the Input DNAs at -20°C until ready to use.

5-hmC Control DNA

A 338 base pair fragment containing 122 cytosine residues from the APC (adenomatosis polyposis coli) gene promoter was amplified by PCR. The control DNA contains 25% 5-hydroxymethylcytosine residues instead of traditional cytosines.

A separate control reaction can be set up to verify the efficiency of 5-hydroxymethylcytosine DNA capture. For details on setting up a positive control reaction, please refer to Appendix A on page 17. The 5-hmC Control DNA should be "spiked" into sample DNA, or the included human genomic DNA, according to the protocol and the capture reactions performed as indicated. We recommend maintaining a control DNA spike to fragmented DNA ratio of 1:20,000 (*e.g.* 25 pg spike DNA standards in 500 ng fragmented genomic DNA = 1:20,000 ratio). The control DNA is provided at a concentration of 50 ng/µl.

Human genomic DNA, *Mse*I digested

Human genomic DNA that has been digested with *Mse*I is provided as a control for the kit. The 5-hmC Control DNA can be "spiked" into the human genomic DNA to verify the efficiency of the enrichment reactions. Please refer to Appendix A for details on setting up the positive control reaction. The Human genomic DNA is provided at a concentration of 20 ng/µl.

10X Reaction Buffer AM1

The reaction buffer is provided ready to use at a 10X concentration. Please follow the instructions in the protocol.

1M DTT

Prepare a fresh 10 mM working stock of DTT by diluting 5 µl 1M DTT into 495 µl sterile water each time reactions are set up. Vortex to mix. Avoid multiple freeze thaws of DTT as this will diminish its effectiveness. Discard any unused 10 mM DTT.

β-Glucosyltransferase Enzyme

Is provided ready to use. The enzyme is supplied at 200 U/µl concentration.

UDP-Azide-Glucose

The modified glucose moiety is provided at a 3 mM stock concentration and is ready to use.

Biotin Conjugate Solution

Is provided ready to use.

DNA Purification Wash Buffer

The DNA Purification Wash Buffer requires the addition of ethanol before use. The final concentration of ethanol should be 80%. Add 40 ml of fresh 100% ethanol to the DNA Purification Wash Buffer bottle. Invert repeatedly. The buffer can be stored at room temperature after the addition of ethanol. The ethanol only needs to be added before the first use, after that the Wash Buffer is ready for use.

3M Sodium Acetate

It is important to check the sodium acetate before use to ensure that the salts have not precipitated out of solution. Once the sodium acetate is in solution it should be stored at room temperature.

Binding Buffer AM13

Is provided ready to use.

10X Elution Buffer AM2

Prepare a 1X Elution Buffer by diluting the 10X Elution Buffer AM2 in Binding Buffer AM13. For every 100 µl elution, dilute 10µl 10X Elution Buffer AM2 into 90 µl Binding Buffer AM13.

Read the entire protocol before use.

NOTES BEFORE STARTING

Sample Preparation: Fragmentation of Genomic DNA

Prior to starting the Hydroxymethyl Collector 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.

Hydroxymethyl Collector will enrich for 5-hydroxymethylcytosines. If trying to evaluate methylation of CpG dinucleotides, such as in CpG islands, please follow the recommendations below for fragmentation of genomic DNA.

To enable clear interpretation of results, genomic DNA should be prepared such that DNA fragments containing a CpG region of interest do not contain methylated cytosines outside of this region (see "Appendix D. Troubleshooting" for further discussion).

Restriction digestion is especially useful for analysis of individual CpG islands. The genomic DNA is cut with a methylation-insensitive restriction enzyme(s) so that only CpGs of interest are contained within a particular restriction fragment. This fragment should be long enough (75 bp or longer) to allow for PCR analysis. 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.

	Recognition Sequence	Number of fragments (per kb) in CpG islands	Number of fragments (per kb) in non-CpG islands
***Mse* I**	TTAA	0.80	**2.88**
***Bfa* I**	CTAG	1.56	1.55
***Tas* I**	AATT	0.80	**2.88**
***Csp6* I**	GTAC	**2.23**	1.41

Mechanical fragmentation is ideal when a single DNA sample will be used for simultaneous analysis of many CpG islands (*e.g.*, when the isolated DNA will be analyzed by microarray methods) or when a CpG region of interest is not flanked by suitable restriction sites. In general, the DNA should be sheared to an average fragment size of less than 500 bp to minimize the number of CpG islands on each fragment.

Example Fragmentation Protocols

Example fragmentation protocols are provided for both restriction digest and mechanical fragmentation. We suggest using 4 µg of purified genomic DNA when performing restriction digestion and 20 µg of purified genomic DNA for sonication. Hydroxymethyl Collector reactions can be performed on 5 ng - 2.5 µg of fragmented genomic DNA.

Restriction digest

This protocol can be modified depending on the amount of isolated genomic DNA or the restriction enzyme being used. 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.

a) Set up the following restriction digest (with *Mse* I as an example, New England Biolabs (NEB)):

Genomic DNA (400 ng/µl)	10 µl
10X NEB Buffer 4	10 µl
100X BSA	1 µl
Mse I (10 U/µl)	1 µl
dH_2O	78 µl
Total volume	**100 µl**

Note 1: The DNA volume may vary depending on its initial concentration.

b) Mix well by pipetting and incubate at 37°C for 2 hours to overnight.

c) Heat-inactivate *Mse* I by incubating the reaction mixture at 65°C for 20 minutes. If using an alternative restriction enzyme that cannot be heat-inactivated, the DNA can be purified by phenol/chloroform extraction and precipitation, or through use of a DNA purification column (such as Active Motif's Chromatin IP DNA Purification Kit, Catalog No. 58002).

Note 1: For greater accuracy, the digested DNA should be quantified.

Note 2: This digested DNA should be stored at -20°C until use.

Mechanical fragmentation (sonication)

Because *Mse* I or other restriction enzymes cannot always be used to fragment and isolate the DNA sequences of interest, sonication of the genomic DNA is an alternative method.

a) Pipette 20 µg genomic DNA into a 1.5 ml microcentrifuge tube and adjust final volume to 300 µl by addition of 10 mM Tris-HCl pH 8.5.

b) Using a tip probe sonicator, sonicate on ice with 15 pulses of 20 seconds (30% amplitude if using Active Motif's EpiShear™ sonicator), with a 20-second pause on ice between each pulse. The sheared DNA can be visualized by ethidium staining after electrophoresis on a 3% agarose gel.

Hydroxymethyl Collector Protocol

Step A: Glucosylation Reaction

1. Set up a 200 µl PCR tube for each glucosylation reaction to be performed. If desired, a control reaction should be set up with the included 5-hydroxymethylcytosine DNA. For notes on using the included control DNA, please see Appendix A on page 17.
2. Calculate the amount of reagent needed for each capture reaction.

   Fragmented DNA: Recommended range between 5 ng and 2.5 µg. Set aside a quantity of fragmented genomic DNA equivalent to 10% of the DNA being used in the glucosylation reaction for use as Input DNA and store at -20°C. Separate Input DNA should be saved for each DNA source.

   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 AM1	5 µl	5 µl
10 mM DTT	5 µl	5 µl
UDP-Azide-Glucose	2.5 µl	–
Fragmented DNA	_____ µl	_____ µl
β-Glucosyltransferase (200 U/µl)	2 µl	2 µl
Total Volume	**50 µl**	**50 µl**

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

Step B: Biotinylation Reaction

1. Quickly spin the PCR tubes to collect the contents at the bottom.
2. Add 20 µl Biotin Conjugation Solution to each reaction.
3. Cap the PCR tubes tightly. Vortex the reaction to ensure complete mixture. Quick spin the tubes to collect the material to the bottom.
4. Incubate the reaction for 1 hour at 37°C.

Step C: Purification of Biotinylated DNA

1. **ADD ETHANOL BEFORE THE FIRST USE ONLY!** The final concentration of ethanol in the DNA Purification Wash Buffer must be 80%. Add 40 ml of fresh 100% ethanol to the DNA Purification Wash Buffer bottle. Invert repeatedly. The buffer can be stored at room temperature after the addition of ethanol.
2. To each biotinylated DNA reaction, add 5 volumes DNA Purification Binding Buffer for every volume of DNA sample. For example, if your reaction is 70 µl, add 350 µl DNA Purification Binding Buffer.
3. Add 5 µl 3M Sodium Acetate and mix. Check that the color of the DNA sample / DNA Purification Binding Buffer mixture is bright yellow, not light orange or violet. If the color is light orange or violet, this indicates that its pH is too high. Add more 3M Sodium Acetate 5 µl at a time, mixing after addition, until the color is bright yellow (see Figure 1). **This step is crucial to the successful binding and purification of your biotinylated DNA sample.**

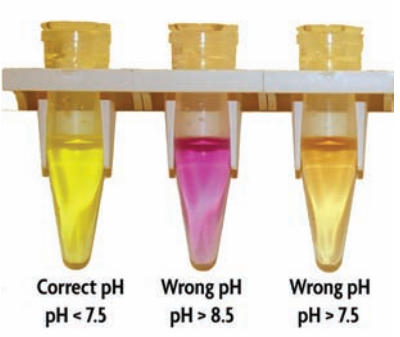


Figure 1: Solution color as a function of pH.
The DNA Purification Binding Buffer has a pH indicator dye, so that the pH of the solution can be easily determined. Only apply the sample to the column when the solution is bright yellow (left), indicating a pH under 7.5. DNA will not bind to the column if the pH is higher than 7.5.

Note: A full-color PDF of this manual can be downloaded from the Active Motif website, which will enable you to see this figure in color.

4. For each sample, place a DNA purification column in the collection tube and add each sample to its own column. Close the cap on each column, place them with the collection tubes in a microcentrifuge and spin them at 14,000 rpm for 1 minute. Alternatively, a compatible vacuum manifold may be used for sample processing.
5. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
6. Add 750 µl of DNA Purification Wash Buffer to the column and cap the column.
7. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
8. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
9. With the column cap open, spin at 14,000 rpm for 2 minutes in a microcentrifuge to remove any residual Wash Buffer from the column.
10. Transfer the column to a clean microcentrifuge tube.
11. Add 50 µl of DNA Purification Elution Buffer to the center of the column matrix and wait for 1 minute.

12. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
13. Discard column and proceed to Step D below.

Step D: Capture Reaction

1. Resuspend the Streptavidin Beads by vortexing or flicking the tube. Make sure the beads are fully resuspended before use. If preparing multiple reactions, ensure beads do not settle by occasional pipetting to resuspend the beads before addition to the capture reactions.
2. In fresh 0.2 ml PCR tubes prepare the following reactions:

Reagents	Sample	Negative Control	(Optional) Positive Control
Streptavidin Beads	25 µl	25 µl	25 µl
Binding Buffer AM13	25 µl	25 µl	25 µl
Purified Biotinylation reaction	50 µl	50 µl	50 µl
Total Volume	**100 µl**	**100 µl**	**100 µl**

3. Incubate the reaction for 1 hour at room temperature with end-to-end rotation.

Step E: Washing and Elution

1. 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. To use the magnet provided in the kit, please see Appendix B.
2. Wash beads five times with 200 µl Binding Buffer AM13. Pipette 2-3 times gently to resuspend.
    a. Place tubes on magnetic stand and allow beads to pellet on the side of the tube.
    b. Carefully remove the supernatant and any residual bubbles.
    c. Add 200 µl Binding Buffer AM13 and resuspend the pellet completely by pipetting several times. 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.
    d. Repeat steps a-c.
3. After the final wash, place tubes on magnetic stand and allow beads to settle to the side. Remove and discard supernatant without disturbing the beads.
4. Resuspend the washed beads with 100 µl 1X Elution Buffer AM2 by pipetting 2-3 times.

5. Incubate for 30 minutes at room temperature with end-to-end rotation.
6. Briefly centrifuge the tubes to collect liquid from the cap.
8. Place tubes in magnetic stand and allow beads to pellet onto tube sides.
9. Transfer the supernatant, which contains the enriched DNA, to a fresh tube.

Step F: Purification of Enriched DNA

1. To each enriched DNA reaction, add 5 volumes DNA Purification Binding Buffer for every volume of DNA sample. For example, if your reaction is 100 µl, add 500 µl DNA Purification Binding Buffer.
3. Add 5 µl 3M Sodium Acetate and mix. Check that the color of the DNA sample / DNA Purification Binding Buffer mixture is bright yellow, not light orange or violet. If the color is light orange or violet, this indicates that its pH is too high. Add more 3M Sodium Acetate 5 µl at a time, mixing after addition, until the color is bright yellow (see Figure 1). **This step is crucial to the successful binding and purification of your 5-hmC DNA sample.**

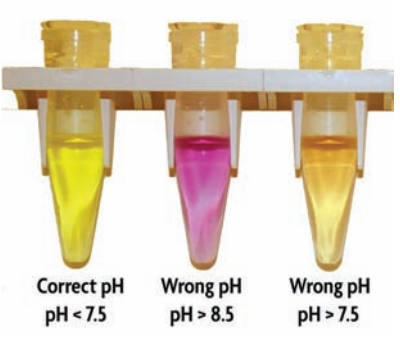


Figure 1: Solution color as a function of pH.
The DNA Purification Binding Buffer has a pH indicator dye, so that the pH of the solution can be easily determined. Only apply the sample to the column when the solution is bright yellow (left), indicating a pH under 7.5. DNA will not bind to the column if the pH is higher than 7.5.

Note: A full-color PDF of this manual can be downloaded from the Active Motif website, which will enable you to see this figure in color.

4. For each sample, place a DNA purification column in the collection tube and add each sample to its own column. Close the cap on each column, place them with the collection tubes in a microcentrifuge and spin them at 14,000 rpm for 1 minute. Alternatively, a compatible vacuum manifold may be used for sample processing.
5. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
6. Add 750 µl of DNA Purification Wash Buffer to the column and cap the column.
7. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
8. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
9. With the column cap open, spin at 14,000 rpm for 2 minutes in a microcentrifuge to remove any residual Wash Buffer from the column.
10. Transfer the column to a clean microcentrifuge tube.

11. Add 50 µl of DNA Purification Elution Buffer (or purified water with a pH equal to or greater than 7) to the center of the column matrix and wait for 1 minute.
12. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
13. Discard column. The DNA eluted into the microcentrifuge tube is purified and ready to use in your downstream application.

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Appendix

Section A. Use of Included 5-hMC Control DNA

Control 5-hydroxymethylated DNA is included as an optional validation of the enrichment efficiency. When control 5-hydroxymethylcytosione DNA is "spiked" into genomic DNA, it will undergo the glucosylation and biotinylation reactions and will be captured by the streptavidin beads. Following elution and purification, the DNA can be analyzed in real time PCR using the provided APC PCR primer mix to verify that the efficiency of the capture method. The APC locus in not methylated in normal, human genomic DNA, therefore if the source of the genomic DNA used is normal human, the only enrichment you should expect is from the "spiked" 5-hmC DNA. *Mse*I digested human genomic DNA is provided for use as a control in setting up the spiked reaction. Alternatively, sample genomic DNA can be used to set up the spiked reactions. For sources of genomic DNA other than normal human, you will need to run a background reaction containing only the sample DNA, without any control DNA, alongside the spiked reactions to determine the amount of enrichment from naturally occurring APC methylation in your genomic DNA sample versus the amount of enrichment due to the control DNA spike. There should be an increase in enrichment in spiked samples as compared to genomic DNA alone.

1. Set up a 200 µl PCR tube for each glucosylation reaction to be performed.
2. Calculate the amount of reagent needed for each glucosylation reaction.

   Fragmented DNA: Recommended range between 5 ng and 2.5 µg. Set aside a quantity of fragmented genomic DNA equivalent to 10% of the DNA being used in the capture reaction for use as Input DNA and store at -20°C. Separate Input DNA should be saved for each DNA source.

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

   Control 5-hmC DNA: The amount of spiked control 5-hmC DNA should be determined based on the amount of DNA used in the capture reaction. We recommend maintaining a spiked control DNA to fragmented DNA ratio of 1:20,000.

   Prepare a 10% excess of spiked control 5-hmC DNA in fragmented genomic DNA. Mix well by vortexing. Remove 10% of the spiked genomic DNA reaction and set aside for use as Input DNA for quantification during real time PCR. Input DNA can be stored at -20°C until ready to use. Some example calculations are shown below for reference.

Control spike DNA	Fragmented Genomic DNA	Quantity removed for Input DNA	Final control spike DNA per rxn	Final genomic DNA per rxn	Ratio of spike to genomic
5.5 pg	110 ng	10% volume	5 pg	100 ng	1:20,000
27.5 pg	550 ng	10% volume	25 pg	500 ng	1:20,000
55 pg	1.1 µg	10% volume	50 pg	1 µg	1:20,000

5-hmC Control DNA is provided at a concentration of 50 ng/µl. Please follow the recommendations in the table below for preparing the "spike" control DNA into the provided human genomic DNA or your sample genomic DNA. Example calculations are shown below for different genomic DNA concentrations. The included Human genomic DNA is provided at a concentration of 20 ng/µl. We recommend performing the first dilution of control DNA in sterile water (Tube 1), followed by subsequent dilutions into genomic DNA at the appropriate stock concentration. The dilutions are performed in genomic DNA in order to avoid over-diluting the genomic DNA concentration used in the final capture reactions. Use the control DNA at the provided concentration. Mix each dilution step by vortexing.

Sample genomic DNA concentration	**4 ng/µl**	**20 ng/µl**	**40 ng/µl**
Spike + genomic DNA quantity	**5 pg in 100 ng**	**25 pg in 500 ng**	**50 pg in 1 µg**
Tube 1	5 µl control DNA into 5 ml H_20 = 50 pg/µl	5 µl control DNA into 995 µl H_20 = 0.25 ng/µl	5 µl control DNA into 495 µl H_20 = 0.5 ng/µl
Tube 2	2 µl Tube 1 into 18 µl genomic DNA = 5 pg/µl	2 µl Tube 1 into 18 µl genomic DNA = 25 pg/µl	2 µl Tube 1 into 18 µl genomic DNA = 50 pg/µl
Tube 3	2 µl Tube 2 into 48 µl genomic DNA = 0.2 pg/µl	2 µl Tube 2 into 48 µl genomic DNA = 1 pg/µl	2 µl Tube 2 into 48 µl genomic DNA = 2 pg/µl
Input DNA volume	2.5 µl Tube 3	2.5 µl Tube 3	2.5 µl Tube 3
Reaction volume	25 µl Tube 3	25 µl Tube 3	25 µl Tube 3

3. Set up glucosylation reactions by adding reagents in the order listed to each PCR tube. Sample and Negative control reactions should just include fragmented DNA, while the Positive control sample will use the spiked DNA prepared in Step 2 above.

Reagents	**Sample**	**Negative Control**	**Spiked Positive Control**
10X Reaction Buffer AM1	5 µl	5 µl	5 µl
10 mM DTT	5 µl	5 µl	5 µl
β-Glucosyltransferase (200 U/µl)	2 µl	2 µl	2 µl
UDP-Azide-Glucose	2.5 µl	–	2.5 µl
Fragmented DNA	_____ µl	_____ µl	25 µl
Sterile water	_____ µl	_____ µl	10.5 µl
Total Volume	**50 µl**	**50 µl**	**50 µl**

4. Cap the PCR tubes tightly. Vortex the reaction to ensure complete mixture. Quick spin the tubes to collect the material to the bottom.
5. Incubate the reaction at 37°C for 1 hours with end-to-end rotation.
6. Proceed with Step B: Biotinylation Reaction on page 12.

Notes about Input DNA

- **Sample IP Input:** To be used with the PCR primers designed to analyze the sample DNA. This standard curve will be used to determine the amount of enriched 5-hydroxymethylcytosine DNA present in the final elution. This Input can be used to quantify DNA in the Sample IP reactions and the Negative control reactions.
- **5-hmC DNA spike Input:** To be used with the provided APC PCR primer set. This standard curve will be used to determine the amount of 5-hmC DNA captured in the final elution of the positive control reaction.

Section B. Use of Magnetic Beads and Included Bar Magnet

Caution: The included neodymium bar magnet is extremely powerful and is easily broken if handled incorrectly.

1. The magnet should be stored in the provided tube.
2. Be careful when working near metal objects or surfaces. A free magnet will jump great distances onto nearby metal surfaces with surprising speed. This can break the magnet.
3. If the magnet becomes attached to a flat metal surface, it should be removed by sliding it off the edge of surface. The magnet may be broken if you attempt to pull one end away from the metal.

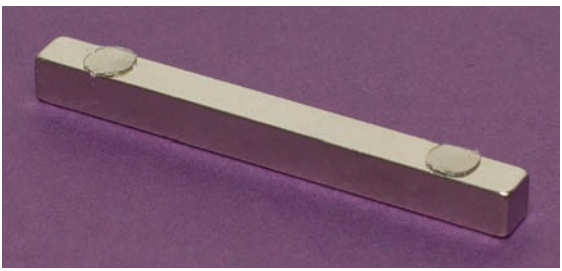
Assembly of Magnetic Stands

The provided Mini Glue Dots can be used to attach the bar magnet to an empty tip box to create an effective magnet stand.

Creating a magnetic stand for 8-well PCR strips:

Note: 8-well strip tubes for use with standard 96-well PCR cyclers are appropriate.

1. Remove the covering tape from one side of two glue dots.
2. Place a strip of PCR tubes in the wells of an empty tip box (200 µl tips) and place the magnet directly against the tubes. This is the way the magnet will be positioned when the glue dots are used to affix it to the box.
3. Attach the glue dots on the bar magnet (the uncovered face of the dot is placed on the magnet) as shown below.



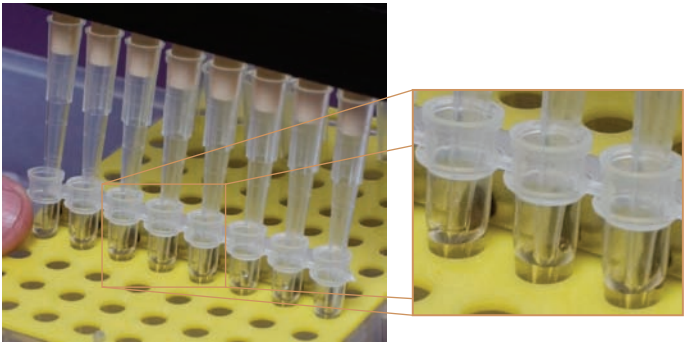
4. Remove the covering tape from the exposed side of the glue dots. Fix the magnet to the tip box so that it is against the PCR tubes. The magnetic stand is now ready for use.

Note: Familiarize yourself with using the magnetic stand before performing with PCR tubes for the first time. Add 5 µl of magnetic beads to 100 µl Binding Buffer AM13 in one tube of an 8-well strip of PCR tubes. Use this tube with the assembled bar magnet stand to become familiar with use of the beads and magnet. It is difficult

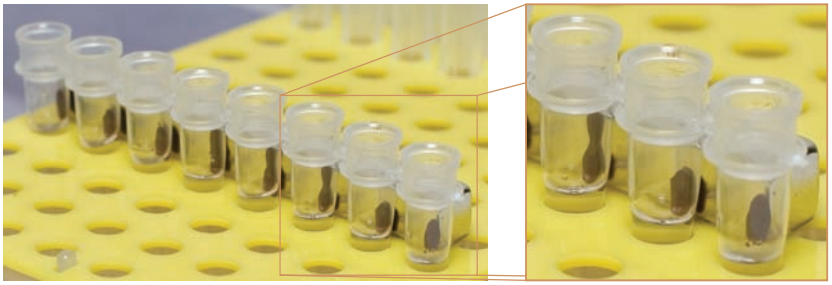
to re-suspend the beads if the tubes are directly adjacent to the magnet, so it is usually best to move the tubes away from the magnet for resuspension.

Washing should be performed as follows:

a. Place the tubes in the rack against the magnet and allow the beads to be pinned to the side of the tube, as shown below.



b. Remove supernatant with a 200 µl pipetteman or a 200 µl eight-channel pipetteman.



c. Move the tube strip into a row that is not adjacent to the magnet.

d. Add wash buffer and pipette up and down to fully resuspend the beads. Ensure that a minimal amount of beads cling to the tips when the resuspension is complete.

e. Repeat steps a-d until desired washing steps are complete.

Centrifugation of 8-well PCR strip tubes:

When working with 8-well PCR strip tubes, it may be desirable to centrifuge the tubes to collect the liquid and beads from the inside of the caps. This is easily accomplished in a centrifuge fitted with adaptors for spinning microtiter plates. In this case, a standard 96-well plate can be placed in the adaptor to hold the tubes in place. Take care to ensure the rotor is balanced (*e.g.*, place a microtiter plate and tubes of appropriate mass in the rotor's opposing 96-well plate adaptor). Spin the plates briefly to let the rotor reach a speed of 1000 x *g* before allowing the rotor to stop.

Section C. PCR Analysis

PCR Primer Design

The Hydroxymethyl Collector Kit includes an APC PCR primer mix for use with the provided control DNA. If possible, real time PCR is recommended for analysis of DNA enriched by Hydroxymethyl Collector. To design primers specific to the CpG region of interest in your sample, please follow the recommendations below.

Primer design considerations

i. Primers should flank the CpG region of interest and produce an amplicon between 100-350 bp in length for end point PCR or an amplicon of 100-150 bp for real time PCR.

ii. Each primer should be approximately 18-22 nucleotides long, contain 50% GC content and have a Tm between 55°C-60°C.

iii. Restriction-digested DNA: PCR primer pairs should amplify a restriction fragment (or portion of a restriction fragment) that contains the CpG-rich region of interest. Each amplicon must also be free of internal sites for the restriction enzyme.

iv. Sonicated DNA: PCR primers should flank the CpG-containing region of interest and the amplicon should not contain any CpG-dinucleotides that are outside of this region. This will minimize amplification of fragments that are isolated as a result of methylated CpGs that are near, but not within, the CpG-rich region of interest.

v. PCR primers should be designed with the aid of a reliable primer design computer program (*e.g.*, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Due to the technical limitations of PCR, it is sometimes necessary to design more than one primer pair for a given fragment of interest. Primers that dimerize should be avoided as they compromise accurate quantitation.

vi. Potential primer pairs can be evaluated via computer simulation using a program such as UCSC Genome Browser (http://genome.ucsc.edu/) to ensure the primers selected will produce a single amplicon in a CpG region of the species being amplified.

Determining Primer Efficiency

It is advised to determine the efficiency of the PCR primers being used. Primers with an efficiency less than 90% will have poor reproducibility. To calculate primer efficiency:

$$\text{Primer efficiency (\%)} = [10^{(-1/\text{slope})}-1] \times 100\%$$

To obtain the slope value, follow the instructions for generating and graphing a standard curve in the Data Analysis and Use of Input DNAs section on page 24. Use the slope of the plotted standard curve in the primer efficiency equation above.

Real Time PCR Analysis

It is important to use purified DNA prior to use in downstream applications. The samples and Input DNAs should be subjected to a DNA clean-up step prior to real time PCR analysis. Below is an example PCR reaction. Please follow the specific instructions for your real time PCR instrument.

1. For notes on preparing standard curves with the Input DNA, please see page 24.
2. For one PCR Reaction:

Reagent	10 µl PCR reactions	20 µl PCR reactions
Fast SYBR Green master mix	5 µl	10 µl
Forward primer* (5 pmol/µl)	0.5 µl	1 µl
Reverse primer* (5 pmol/µl)	0.5 µl	1 µl
Sterile water	1 µl	3 µl
DNA sample (eluted or Input)	3 µl	5 µl
Total volume	**10 µl**	**20 µl**

* The provided APC PCR Primer Mixes contain both Forward and Reverse primers for use with the provided control DNA. Use 1 µl of the PCR Primer Mix in the 10 µl reaction or 2 µl of the PCR primer mix in the 20 µl reaction for the PCR protocol described above.

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

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

   95°C for 2 minutes
   (95°C for 3 seconds, 60°C for 30 seconds) for 40 cycles

4. Analyze the results. Data analysis varies depending on the instrument used. Obtain the standard curve from the Input samples. Use the standard curve to quantify the DNA in each sample.

Data Analysis and Use of Input DNAs

5-Hydroxymethylcytosine DNA isolated using Hydroxymethyl Collector can be analyzed by PCR amplification of the loci of interest. However, if the goal is to compare the methylation status of particular loci in different DNA samples, it is essential that Hydroxymethyl Collector be performed on the same amount of each DNA sample. In addition, Input DNA should be prepared for each of the different DNA samples to clearly indicate the relative concentrations of the DNA samples.

For real time PCR, generating a standard curve using the Input DNA enables accurate determination of the enriched DNA concentration.

1. For Hydroxymethyl Collector DNA, produce a standard curve at 3.3, 0.33, 0.033 and 0.0033 ng/µl in triplicate using the Input DNAs captured in Step A instruction #2. We recommend running a standard curve every time the PCR amplification is performed. An example standard curve calculation is provided on page 26.
2. Run each sample with the appropriate Input DNA standard (*i.e.* prepare a separate standard curve for each DNA source or spiked control tested.
   - **Sample IP Input:** To be used with the PCR primers designed to analyze the sample DNA. This standard curve will be used to determine the amount of enriched 5-hydroxymethylcytosine DNA present in the final elution. This Input can be used to quantify DNA in both the Sample IP reactions and the Negative control reactions.
   - **(Optional) 5-hmC DNA spike Input:** To be used with the provided APC PCR primer set. This standard curve will be used to determine the amount of 5-hmC DNA captured in the final elution of the Positive control reaction.
3. Every gene and/or primer set will generate a different amplification profile.
4. CT = Threshold Cycle and is the cycle number where the signal exceeds the background threshold level. CT values should be plotted for each gene to create a linear regression plot.
5. Plot CT versus log DNA concentration. See Figure 1 on page 25. The slope of the standard curve can be used to determine primer efficiency in the equation on page 22.

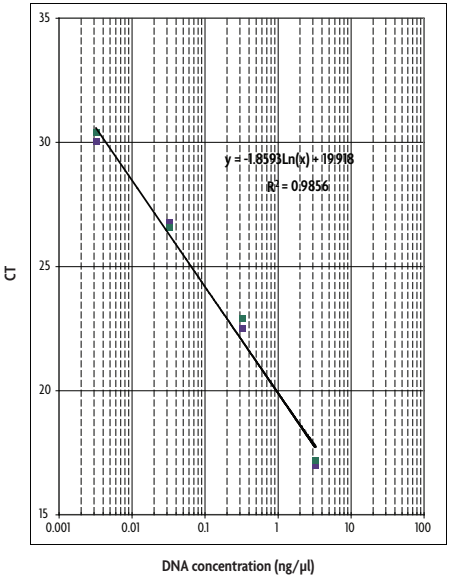


Figure 1: Example standard curve linear regression plot.

A standard curve for Input DNA is provided as a reference only. Ten-fold dilutions of Input DNA were tested in triplicate and plotted against the CT value. A new standard curve should be generated each time the assay is performed.

6. Using the CT value of the sample, extrapolate the DNA concentration of the sample DNA using the appropriate standard curve plot. To determine the total amount of enriched DNA in the sample, simply multiple the DNA concentration by the volume of enriched DNA.
7. Calculate the percent enrichment. Use the sample DNA quantity calculated above and compare it with the amount of DNA used in the initial glucosylation reaction (Step A).

$$\textbf{\% of Input} = \frac{\text{Amount of enriched Sample DNA material (ng)}}{\text{Amount of fragmented DNA used in the initial reaction (ng)}} \times 100\%$$

$$\textbf{Fold enrichment} = \frac{\text{Amount of enriched Sample DNA material (ng)}}{\text{Amount of enriched Negative control material (ng)}}$$

Example Standard Curve Calculations

To illustrate how to set up a standard curve at the recommended concentrations of 3.3, 0.33, 0.033 and 0.0033 ng/µl, example calculations are shown below for the preparation of a standard curve using Input DNA from the optional spike reaction containing 25 pg spike DNA in 500 ng genomic DNA. It is critical to adjust DNA concentrations and DNA quantities based on the actual amount of spike DNA and genomic DNA used in the reactions in order to obtain accurate quantification of eluted material. Perform the following serial dilutions to generate the DNA standards listed in the table below.

Input: Contains 10% of initial reaction quantity. (2.5 pg spike DNA in 50 ng genomic DNA)

Tube 1: Take the 2.5 µl Input DNA and add 12.5 µl sterile water. Vortex to mix.

Tube 2: Make a 1:10 dilution by transferring 5 µl DNA from Tube 1 into 45 µl sterile water. Vortex to mix.

Tube 3: Make a 1:10 dilution by transferring 5 µl DNA from Tube 2 into 45 µl sterile water. Vortex to mix.

Tube 4: Make a 1:10 dilution by transferring 5 µl DNA from Tube 3 into 45 µl sterile water. Vortex to mix.

Tube #	Genomic DNA conc.	Spike DNA conc.	Final volume	Volume per PCR	Genomic DNA qty.	Spike DNA qty.
Input	50 ng	2.5 pg	2.5 µl	N/A	N/A	N/A
1	3.3 ng/µl	0.166 pg/µl	15 µl	3 µl	10 ng/well	0.5 pg/well
2	0.33 ng/µl	0.0166 pg/µl	50 µl	3 µl	1 ng/well	0.05 pg/well
3	0.033 ng/µl	0.00166 pg/µl	50 µl	3 µl	0.1 ng/well	0.005 pg/well
4	0.0033 ng/µl	0.000166 pg/µl	50 µl	3 µl	0.01 ng/well	0.0005 pg/well

Use 3 µl of DNA from Tubes 1-4 to set up PCR reactions for the standard curve.

Plot the CT vs. log genomic DNA concentration as shown on page 25.

Section D: Troubleshooting Guide

Problem/question	Recommendation
Little or no enrichment of methylated DNA	The Hydroxymethyl Collector kit is optimized for use with 5 ng - 2.5 µg of fragmented (<500 bp) DNA per reaction. Using different DNA concentrations will alter the amount of β-glucosyltransferase enzyme available per DNA fragment and decrease the efficiency of 5-hmC modification. Incomplete glucosylation will reduce the recovery of 5-hmC 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.
Are there alternative incubation temperature and times?	The glucosylation reaction is incubated at 37°C for 1 hour, but alternatively, it can be incubated overnight at 30°C. The biotinylation reaction is incubated at 37°C for 1 hour, but alternatively, it can be incubated overnight at 30°C The streptavidin capture reaction is incubated at RT for 1 hour, but alternatively, it can be incubated overnight at 4°C.
PCR amplification	Follow the recommendations for PCR primer design on page 22 to prepare specific primers for your sample. The included APC PCR primers are designed to work with the included 5-hmC Control DNA provided in the kit.
Storage of DNA	Once DNA is enriched with Hydroxymethyl Collector, samples may be stored at -20°C prior to PCR analysis. However, we recommend heating the frozen material to 37°C for 10 minutes before use in PCR, as heat-treatment releases any DNA bound to the tube during storage.
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 (microarray), but it may not be possible to shear DNA small enough to isolate CpG islands of interest. In addition, results may vary from shearing to shearing depending on sonicator used. Also it is difficult to prepare DNA from a small number of cells.
Heat inactivation or removal of restriction enzyme 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 MeDIP and hMeDIP 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 Active Motif Technical Service at one of the numbers listed below.

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