diagenode

A Hologic Company

hMeDIP kit

Hydroxymethylated DNA Immunoprecipitation Kit

Cat. No. C02010031

USER GUIDE

Version 4 09_2022

Please read this manual carefully
before starting your experiment

Contents

Introduction

One of the fastest growing fields in biology and cancer research is epigenetics. While the underlying genetic code defines which proteins and gene products are synthesized, it is epigenetic control that defines when and where they are expressed. Epigenetic control is generally mediated by methylation of cytosine to 5-methylcytosine in CpG islands and post-translational modification of histones. Methylation of CpGs near promoters is classically associated with gene silencing or down-regulation, as is deacetylation of histones.

Cytosine hydroxymethylation was recently discovered as another important epigenetic mechanism. This cytosine base modification results from the enzymatic conversion of 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine (5-hmC) by the TET family of oxygenases. Though the precise role of 5-hmC is the subject of intense research and debate, early studies strongly indicate that it is also involved in gene regulation and in numerous important biological processes including embryonic development, cellular differentiation, stem cell reprogramming and carcinogenesis.

The Diagenode hMeDIP kit is designed for enrichment of hydroxymethylated DNA from fragmented genomic DNA samples for use in genome-wide methylation analysis. It features a highly specific monoclonal antibody against 5-hmC for the immunoprecipitation (IP) of hydroxymethylated DNA. Recovered DNA is suitable for many downstream applications to analyze genome-wide DNA hydroxymethylation including PCR, sequencing, and microarrays. The IP efficiency can be monitored with the use of control DNA sequences (5-hmC and unmodified cytosine DNA standards) and control primer pairs (internal and external controls). Performing hydroxymethylation profiling with hMeDIP kit is FAST, RELIABLE and HIGHLY SPECIFIC.

The kit includes:

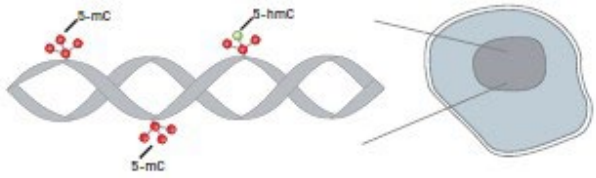
- All the reagents for the immunoprecipitation, including monoclonal antibody against 5-hmC and magnetic beads
- 5-hmC and unmethylated spike-in controls and their associated qPCR primer pairs to verify the efficiency of the IP
- qPCR primer pair against Sfi1 targeting hydroxymethylated gene in mouse to monitor the success of the IP on your DNA samples
- DNA isolation module (after IP): 45 minutes for DNA Isolation Buffer method (DIB)

The kit ensures the use of a low quantity of antibodies and buffers per reaction. The number of steps is reduced and handling is easier which makes this kit cost effective and simple to work with.

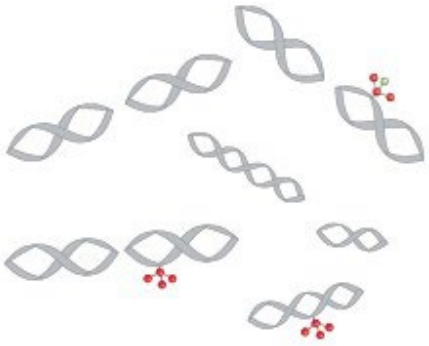
We recommend Diagenode's Magnetic Rack (Cat. No. B04000001) together with our hMeDIP kits. Diagenode's magnetic racks are designed to be used in IP experiments, keeping samples cool longer and allowing the use of small tubes to reduce the reaction volumes and use of reagents. The protocol has been validated with Diagenode's Bioruptor® (Cat. No. B01060010 or B01020001). Nevertheless, DNA can be sheared with any in house protocol and sonication devices as long as efficiency is checked before use. This kit is provided with a manual protocol as well as an automated version for Diagenode IP-Star® Compact Robot (Cat. No. B03000002).

Kit method overview

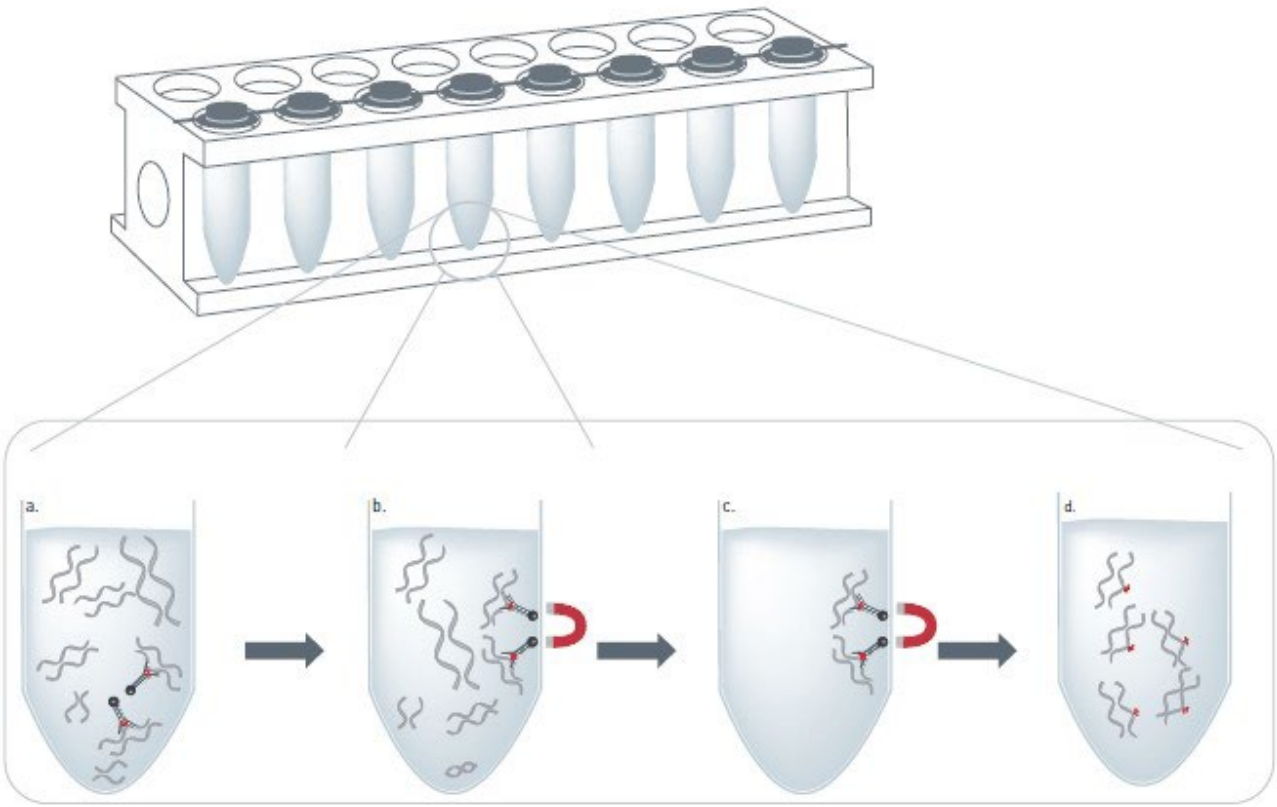
1. Cell collection and lysis



2. DNA Extraction and Shearing using Bioruptor®



3. Immunoprecipitation, washes and DNA isolation



a. Add magnetic beads coated with antibody of interest
b. Magnetic capture of Antibody – DNA complex
c. Washes
d. DNA isolation before qPCR

Kit Materials

The kit content is sufficient to perform 16 hydroxymethylated DNA immunoprecipitations (hMeDIPs).

1. The kit content is described in Table 1. Upon receipt, store the components at the temperatures indicated in Table 1.

Table 1. Components of the hMeDIP kit

Component	Comments	Quantity	Storage
hMeDIP buffer H1	Ion chelator mix included (10x).	3 ml	4°C
hmeDNA control	0.001 ng/µl	40 µl	-20°C
unDNA control	0.1 ng/µl	15 µl	-20°C
5-hmC monoclonal antibody (mouse)	1 µg/µl	32 µl (50 µg)	-20°C
Mouse IgG	1 µg/µl	50 µl	-20°C/4°C
Anti-mouse IgG coated magnetic beads	The beads are supplied for 16 IPs; detergent and 0.02%, sodium azide included.	220 µl	4°C **Do not freeze**
hMeDIP buffer H2	BSA and Ion chelator mix included.	12 ml	4°C
hMeDIP buffer H3	Ion chelator mix included.	4 ml	4°C
DNA isolation Buffer (DIB)	-	5 ml	4°C
Proteinase K	100 x stock solution.	40 µl	- 20°C
hmeDNA primer pairs	5 µM each (Rv & Fw)	50 µl	- 20°C
unDNA primer pairs	5 µM each (Rv & Fw)	50 µl	- 20°C
Mouse Sfi1 primer pairs	5 µM each (Rv & Fw)	50 µl	- 20°C
PCR tube strips	For 1 row of 8 samples each.	4	RT
PCR tube caps	For 1 row of 8 samples each.	4	RT
Water	-	24 ml	4°C

Required Materials Not Provided

Reagents for the IP and qPCR analysis

- Gloves to wear at all steps
- Autoclaved tips
- RNase/DNase-free 1.5-ml (or 2-ml) tubes
- PCR tubes and reagents
- Ultra pure water

Equipment for the IP and qPCR analysis

- DiaMag 0.2ml - magnetic rack (Diagenode, Cat. No. B04000001)
- Centrifuges for 1.5-ml tubes (4°C)
- Rotating wheel (4°C)
- Vortex
- Thermomixer (55°C, 95°C)
- Quantitative PCR facilities

Reagents and equipment for the DNA preparation and shearing

- Tubes: 1.5-ml and 50-ml conical tubes
- Trypsin-EDTA
- Ice-cold PBS buffer
- Agarose and TAE buffer
- DNA molecular weight marker
- Centrifuges for 1.5-ml tubes and 50-ml conical tubes (4°C)
- Agarose gel apparatus
- Cell counter
- Bioruptor Pico (Diagenode, Cat. No. B01060010) or the Bioruptor Plus (Diagenode, Cat. No. B01020001)

Additionnal supplies (included and available separately)

Component	Cat. No.	Format
5-hmC monoclonal antibody (mouse)	C15200200	20 µg/µl 50 µg/µl 100 µg/µl
DiaMag anti-mouse IgG coated magnetic beads	C03010022	220 µl 660 µl 1.5 ml
Mouse IgG antibody	C15400001	100 µg
Mouse Sfi1 primer pairs	C17021046	50 µl
Proteinase K	C06050001 C06050002	100 µl 250 µl

Optional supplies (not included and available separately)

Component	Cat. No.	Format
XL GenDNA Module	C03030020	6 rxns
Mouse gapdh promoter primer pair	C17021045	50 µl 500 µl
5-hmC monoclonal antibody (rat)	C15220001	20 µg 50 µg 100 µg
5-hmC polyclonal antibody (rabbit)	C15410205	20 µg 50 µg
DiaMag protein G-coated magnetic beads	C03010021	220 µl 660 µl 1.5 ml
DiaMag protein A-coated magnetic beads	C03010020	220 µl 660 µl 1.5 ml
Rat IgG	C15420001	25 µl/µg
Rabbit IgG	C15410206	250 µl/µg
DNA hydroxymethylation control package	C02040018	48 rxns
DNA methylation control package V2	C02040019	48 rxns
IPure kit v2	C03010015	100 rxns
DiaMag 0.2ml - magnetic rack	B04000001	

Remarks before starting

DNA extraction

The quality of the DNA to be used in hMeDIP is important. Thus, we recommend using Diagenode XL GenDNA Extraction Module (Cat. No. C03030020) for the DNA extraction. It was optimized for the preparation of genomic DNA (gDNA) from cultured cells. For DNA extraction from blood or tissue, other specific kits should be used.

The XL GenDNA Extraction Module for gDNA isolation provides sufficient volume of reagents for the preparation of 6 gDNA batches, each obtained from 1 to 1.5 million cultured cells.

Starting amount

Concentrations of all the reagents in the hMeDIP Kit are optimized for a starting amount of 1 µg of sheared DNA per IP.

DNA shearing

For an efficient and best resolution IP experiment, the gDNA has to be sheared into fragments around 400 bp on the Bioruptor Pico (Cat. No. B01060010) or on the Bioruptor Plus (Cat. No. B01020001).

Before starting with IP experiment, we recommend analyzing the size of the fragments after shearing using a dedicated device such as the BioAnalyzer (Agilent) or the Fragment Analyzer (Agilent).

Example of shearing for hMeDIP using the Bioruptor® Pico

The genomic DNA was diluted in TE buffer to reach a concentration of 100 ng/µl and 100 µl were sheared in a 0.65 ml Bioruptor® Microtube (Cat. No. C30010011). The following program was used:

- Cycles: [15 seconds "ON" & 90 seconds "OFF"]
- 8 cycles

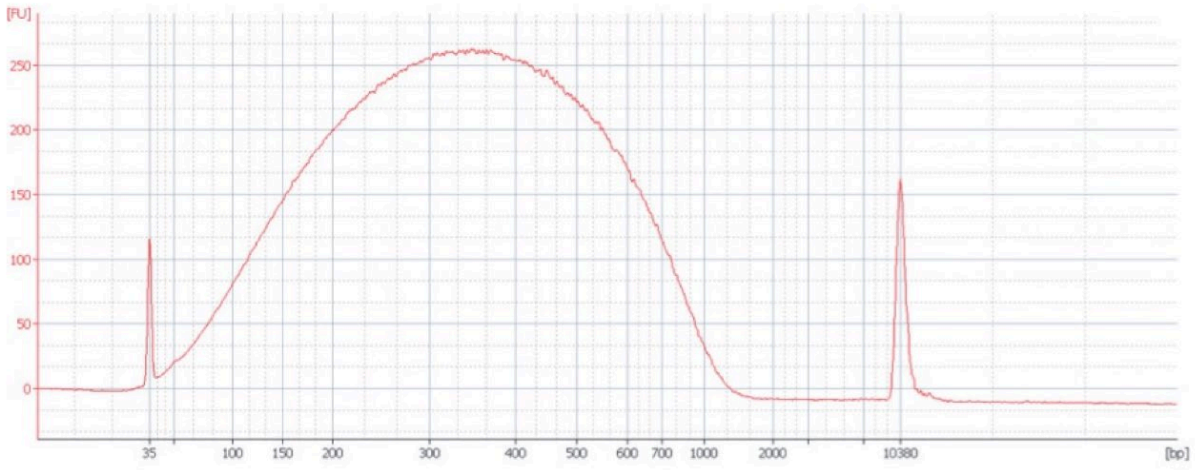


Figure 1: *Agilent High Sensitivity DNA chip profile of sheared genomic DNA: smear around 400 bp.*

Antibody choice

The hMeDIP kit includes a 5-hmC monoclonal mouse antibody (Cat. No. C15200200). However, it has been also validated with a 5-hmC monoclonal rat antibody (Cat. No. C15220001) and a 5-hmC polyclonal rabbit antibody (Cat. No. C15410205). Those antibodies can be used in combination with the hMeDIP kit, as long as the magnetic beads and the negative controls are replaced accordingly.

Please note that:

- the rat antibody (Cat. No. C15220001) should be used in combination with DiaMag protein G-coated magnetic beads (Cat. No. C03010021) and rat IgG (Cat. No. C15420001) as negative control.

- the rabbit antibody (Cat. No. C15410205) should be used in combination with DiaMag protein A-coated magnetic beads (Cat. No. C03010020) and rabbit IgG (Cat. No. C15410206) as negative control.

Magnetic beads

This kit includes magnetic beads (Magbeads). Please make sure the beads do not dry out during the procedure as this may result in reduced performance. Keep the Magbeads homogenous in suspension at all times when pipetting. Variation in the number of beads will decrease reproducibility. Do not freeze the Magbeads.

Hydroxymethylated and unmethylated spike-in controls

The hMeDIP Kit contains one hydroxymethylated and one unmethylated spike-in controls that can be added directly to DNA samples before the IP. Those spike-in controls are not homologous to any model species and will not interfere with the sample of interest. To check the efficiency of the hMeDIP experiment, this kit also includes primer pairs targeting a positive region on mouse genome to calculate their recovery by qPCR. These primer pairs are only suitable for mouse samples.

Technique for DNA isolation

The hMeDIP Kit includes a DNA Isolation Buffer for an easy and very fast DNA isolation after hMeDIP providing DNA suitable for qPCR analysis. In case DNA of higher purity is needed for Next-Generation Sequencing (NGS) or other downstream application than PCR, we recommend using the IPure kit v2 (Cat. No. C03010014) for purification.

PCR experiment preparation

Designing your primers

Self-complementarity and secondary structure of the primers can be tested for primer design (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Annealing temperature of 60°C is recommended for qPCR primers.
Short length of amplified DNA fragment (50 - 100 bp) facilitates the PCR efficiency and reduces potential problems in amplification of G/C-rich regions.
Difference in melting temperature between forward and reverse primers should not exceed 2 to 3°C.
G/C stretches at the 3' end of the primers should be avoided.

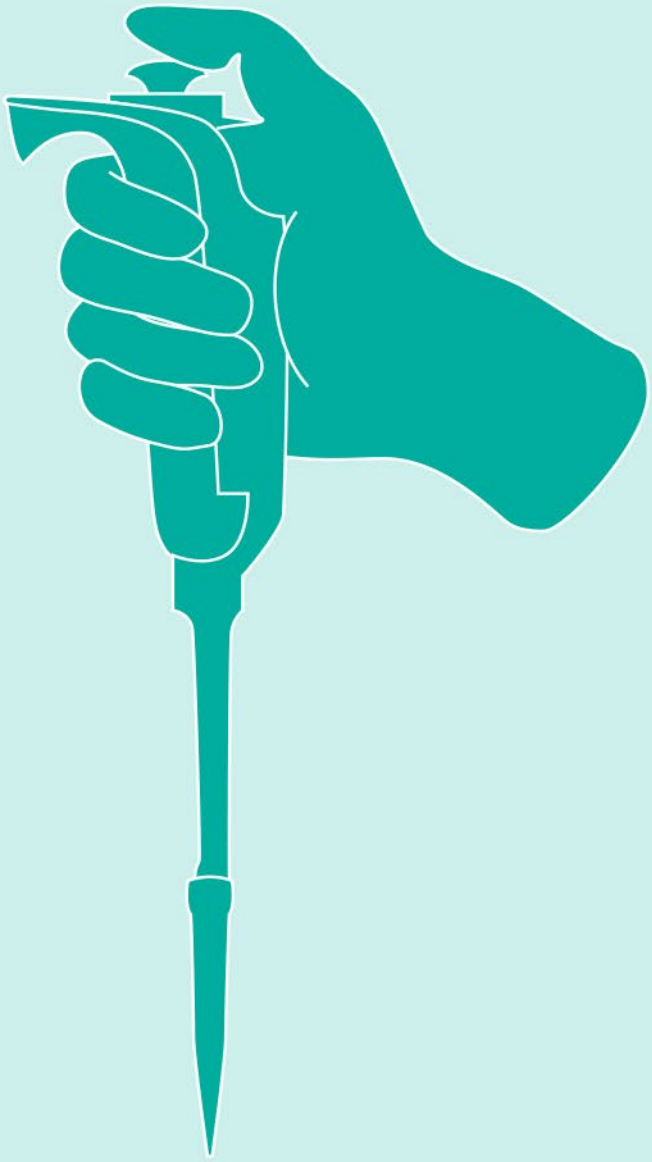
Advantages of the qPCR

qPCR or Real time PCR enable fast, quantitative and reliable results.
The Gene Quantification page (www.gene-quantification.de) describes and summarises all technical aspects involved in quantitative gene expression analysis using real-time qPCR & qRT-PCR. It presents a lot of applications, chemistries, methods, algorithms, cyclers, kits, dyes, analysis methods, meetings, workshops, and services involved.

Validation of your primers

Test primer sets by in silico PCR (http://genome.cse.ucsc.edu/cgi-bin/hgPcr). Primers should amplify unique DNA products from the genome.
Test every primer set in qPCR using 10 fold-serial dilutions of input DNA. Calculate amplification efficiency (AE) of primer set using the following by formula (1): AE= 10^(-1 / slope)
The ideal amplification factor is 2. If it is not the case the qPCR reagents from different brand or new primers should be tested.

PROTOCOL



STEP 1

Binding antibodies to hydroxymethylated DNA and bead preparation

DAY 1: Binding antibodies to hydroxymethylated DNA

1.1 Prepare the IP incubation mix w/o the antibody and w/o magnetic beads for all your hMeDIP reactions (Table 2).

NOTE: *Make sure when working with hMeDIP buffer H1, that there are no crystals left in solution. Otherwise heat up*

Table 2: IP incubation mix with no antibodies and no beads

Reagent	Volume per IP + INPUT	Volume per additional IP
Water	93.9 µl	78.25 µl
hMeDIP buffer H1	12 µl	10 µl
hmeDNA control	1.5 µl	1.25 µl
unDNA control	0.6 µl	0.5 µl
DNA sample (0.1 µg/µl)*	12 µl	10 µl
TOTAL VOLUME	120 µl	100 µl

** If the DNA sample is at a concentration of 0.1 µ g/µl, use 93.9 µl water per IP. If the concentration of the DNA sample is not at 0.1 µg/ µl, adjust the volumes of DNA and of water to add. Keep the volume of the incubation mix at 120 µ l per IP+input. Caution: in any case 1 µg of DNA is needed per IP!*

1.2 Incubate at 95°C for 10 minutes.

1.3 Quickly chill sample on ice (it is best to use ice-water).

1.4 Perform a pulse spin to consolidate your sample.

1.5 First, take out 10 µl per INPUT (that is 10% input) and transfer to a new labeled tube.

- Keep the input samples at 4°C. The input sample is to be used as a control of starting material and it is therefore not to be used in IP.

1.6 Transfer from what is left: 100 µl per tube into 200-µl tubes using the provided 200-µl tube strips (or individual 200-µl tubes that can fit in our Magnetic Rack).

1.7 Add 2.5 µl of the 5-hmC antibody or 2.5 µl of Mouse IgG per tube.

- The IgG is a negative control antibody. We recommend to include one IgG control for each serie of hMeDIP reactions.

1.8 Incubate on a rotating wheel (40 rpm) at 4°C for 2 hours. During this time proceed to the beads preparation.

Beads preparation

1.9 Wash the magnetic beads with ice-cold hMeDIP buffer H2 as follows: In 1.5 ml tube, add 200 µl of hMeDIP buffer H2 to 12 µl of beads and resuspend the beads. Incubate on a rotating wheel (40 rpm) at 4ºC for 2 hours.

- Keep the beads homogenously in suspension at all times when pipetting. Variation in the amount of beads will lead to lower reproducibility.
- 10 µl of beads are needed per IP.

1.10 After washing, briefly spin the tube containing the beads to bring down liquid caught in the lid. Pellet the beads, discard the supernatant and keep the bead pellet (see below two options to pellet the beads).

Options:

a) Use the magnetic rack for 1.5 ml tubes.

b) Centrifuge for 5 minutes at 1,300 rpm.

1.11 In a new tube, dilute 1:10 in water, the provided hMeDIP buffer H1 to have the diluted hMeDIP buffer H1:10.

- 350 µl of hMeDIP buffer H1:10 are needed per IP.

1.12 Resuspend the beads in diluted hMeDIP buffer H1:10 to the volume then originally used.

- for 1 IP: 12 µl of hMeDIP buffer H1:10

STEP 2

Magnetic immunoprecipitation

2.1 Briefly spin the 8-tube strip containing the incubation mix with antibody or control (from Point 1.8) and add 10 µl of washed beads per tubes (from Point 1.12).

2.2 Incubate on a rotating wheel (40 rpm) at 4ºC for overnight.

DAY 2

2.3 Place the 8 tubes strip in the DiaMag 0.2ml - magnetic rack - (Cat. No. B04000001), wait 1 minute and discard the buffer.

2.4 Wash three times using 100 µl of ice-cold hMeDIP buffer H1:10. Each wash is done as follows: add buffer, close the tube caps, invert the 8-tube strip to resuspend the beads, incubate for 5 minutes at 4°C on a rotating wheel (40 rpm), spin, place in the magnetic rack, wait 1 minute and discard the buffer. Keep the captured beads.

- Do not disturb the captured beads attached to the tube wall.
- Always briefly spin the tubes to bring down liquid caught in the lid prior to positioning in the magnetic rack.

2.5 Wash one time with 100 µl ice-cold hMeDIP buffer H3 (as described above: Point 2.4), spin, place in the Magnetic Rack, wait 1 minute and discard the buffer. Keep the captured beads for Point 3.3.

STEP 3

DNA isolation

NOTE: *This kit includes a DNA isolation buffer (DIB) for easy and very fast DNA isolation after hMeDIP providing DNA suitable for qPCR analysis (STEP 4).*
If you need DNA of higher purity for next generation sequencing or other downstream application than PCR, we recommend using the IPure kit v2 (Cat. No. C03010014) for purification.

	DNA isolation buffer (DIB)	IPure v2
Time	45 minutes	1h30
DNA concentration	+	++ (possible to concentrate)
DNA purity	+	++
Subsequent analysis	qPCR	Next generation sequencing, microarray, qPCR amplification

3.1 Take the input samples, centrifuge briefly and from now onwards treat the input DNA samples and IP samples in parallel.

3.2 Prepare 100 µl complete DIB per sample as follows. Add 1 µl of Proteinase K per 100 µ l of DIB. Scale accordingly knowing that 100 µl are needed per IP'd DNA sample and 90 µl, per input DNA sample.

3.3 Remove the tubes from the magnetic rack and add 100 µ l of complete DIB buffer per IP'd DNA sample. Resuspend the beads.

3.4 Add 90 µl of complete DIB to 10 µl of input DNA sample.

3.5 Incubate at 55°C for 15 minutes both IP'd DNA sample and input DNA sample.

3.6 Incubate at 100°C for 15 minutes all the samples.

3.7 Label new 1.5 ml tubes:

3.8 Place the samples in the magnetic rack and wait 1 minute or until the supernatant is clear.

3.9 Transfer the supernatants in new labeled tubes. That is the DNA ready for qPCR analysis. Store at -20°C.

STEP 4

Quantitative PCR & Data analysis

This last step consists in amplifying and analysing the IP'd DNA.

4.1 Prepare your qPCR mix using SYBR® PCR Green master mix and start out qPCR.

dqPCR mix (total volume of 25 µl/reaction):

- 6.50 µl of water
- 12.50 µl of master mix (e.g.: iQ SYBR® Green supermix)
- 1.00 µl of provided primer pair (stock: 5 µM each: reverse and forward)
- 5.00 µl of isolated DNA or INPUT

	Temperature	Time	Cycle
PCR Amplification	95°C	7 minutes	x1
	95°C	15 seconds	x40
	60°C	60 seconds	
	95°C	1 minute	x1
Melting Curve	65°C and increment of 0.5°C per cycle	1 minute	X60

4.2 When the PCR is done, analyse the results. Some major advices are given below.

Data interpretation

The efficiency of hydroxymethyl DNA immunoprecipitation of particular genomic locus can be calculated from qPCR data and reported as a percentage of starting material: % recovery (hmeDNA-IP/ Total input).

% recovery = 2^[(Ct(10%input) - 3.32) - Ct(hmeDNA-IP)]x 100%

Here 2 is the AE (amplification efficiency); Ct (hmeDNA-IP) and Ct (10%input) are threshold values obtained from exponential phase of qPCR for the hydroxymethyl DNA sample and input sample respectively; the compensatory factor (3.32) is used to take into account the dilution 1:10 of the input.

Background determination

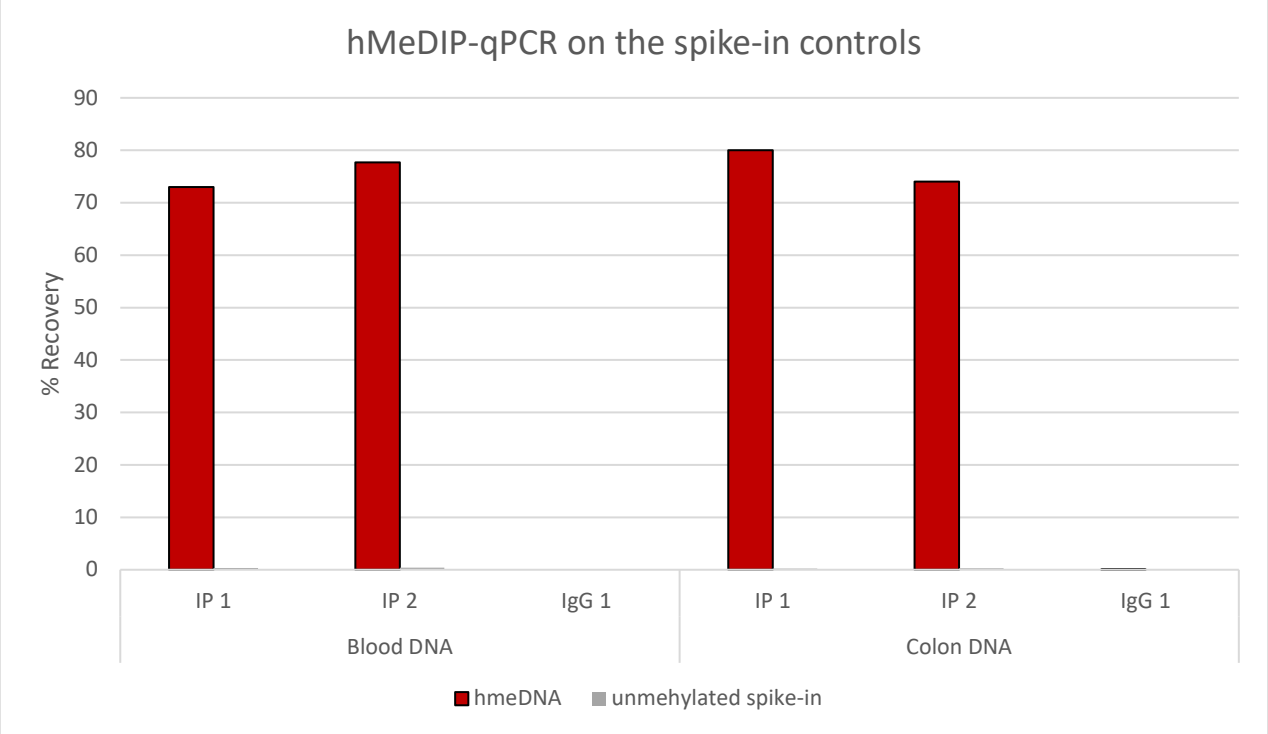
The final goal of IP is to calculate the enrichment in the same IP sample of the specific DNA fragments (corresponding to the hydroxymethylated DNA) in comparison with background (corresponding to unmethylated DNA).
This enrichment can be calculated as a ratio of specific signal over background.

enrichment = % recovery (specific locus) / % recovery (background locus)

Results

hMeDIP results obtained using the hMeDIP kit (mouse monoclonal antibody), including:

- 5-hmC mouse monoclonal antibody
- 5-hmC and cytosine spike-in controls
- Mouse IgG



hMeDIP-qPCR was performed using the hMeDIP kit (mouse monoclonal antibody)

One µg of gDNA from human whole blood and colon has been sonicated with Bioruptor to obtain DNA fragments of 300-500 bp. The hMeDIP has been performed using Diagenode hMeDIP kit and protocol in duplicates. The IgG isotype antibody from mouse was also included as negative control. Unmethylated and hydroxymethylated spike-in controls have been added to the samples and then analyzed by qPCR using specific corresponding primer pairs.

Related products

Product	Cat. No.
5-hmC monoclonal antibody (rat)	C15220001
5-hmC polyclonal antibody (rabbit)	C15410205
DiaMag protein G-coated magnetic beads	C03010021
DiaMag protein A-coated magnetic beads	C03010020
Rat IgG	C15420001
Rabbit IgG	C15410206
MagMeDIP kit	C01020021
XL GenDNA Extraction Module	C03030020
DNA hydroxymethylation control package	C02040018
DNA methylation control package V2	C02040019
DiaMag 0.2ml - magnetic rack	B04000001
DiaMag rotator	B05000001
Bioruptor® Pico sonication device	B01060010
Bioruptor® Plus sonication device	B01020001

Revision history

Version	Date of modification	Description of modifications
Version 4 09_2022	September 2022	- Correction of the antibody concentration in the table of content - Removal of discontinued product - Removal of mDNA control and modification of the corresponding protocol (Step 1.1)
Version 4 03_2022	March 2022	Page 7 - Correction of the storage temp. - hMeDNA control

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