

EpiQuik™ Hydroxymethylated DNA Immunoprecipitation (hMeDIP) Kit

Base Catalog # P-1038

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The EpiQuik™ Hydroxymethylated DNA Immunoprecipitation (hMeDIP) Kit is suitable for selective enrichment of DNA fragments containing 5-hydroxymethylcytosine in a high throughput format using DNA isolated from various species. The hydroxymethylated DNA that is enriched with this kit can be used for various downstream applications including PCR (hMeDIP-PCR) and microarray (hMeDIP-chip).

Starting Material and Input Amount of DNA: The starting material should be good quality purified DNA. The amount of DNA for each reaction can be 0.1 µg (approximately 1×10^4 cells) to 1 µg. For an optimal reaction, the input DNA amount should be 0.5 µg per well

DNA Shearing: Genomic DNA should be sheared by sonication before starting hydroxymethylated DNA immunoprecipitation. The sheared DNA fragments should range in size from 200-600 base pairs. For the best results and your convenience, we recommend using the EpiSonic™ Multi-Functional Bioprocessor 1100, which allows for simultaneous sonication of multiple samples (1-384) in sealed vials for consistent and desired size range of DNA (200-600 base pairs).

Internal Control: Negative (Non-Immune IgG) and positive controls (Control DNA) are provided in this kit. The Control DNA is a 200 base pair DNA fragment containing 44 cytosine residues which are hydroxymethylated. The kit also includes control PCR primers that can be used for verifying the enrichment efficiency of hydroxymethylated control DNA

Antibody: The 5-hydroxymethylcytosine rabbit polyclonal antibody used in this kit is highly specific against hydroxymethylated DNA fragments, -both single and double stranded, and is not cross-reactive to methylated and unmethylated DNA fragments.

Precautions: To avoid cross-contamination, carefully pipette the sample or solution into the strip wells. Use aerosol-barrier pipette tips and always change pipette tips between liquid transfers. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

KIT CONTENTS

Component	24 Reactions Cat. #P-1038-24	48 Reactions Cat. #P-1038-48	96 Reactions Cat. #P-1038-96	Storage Upon Receipt
WB (10X Wash Buffer)	5 ml	10 ml	20 ml	4°C
AB (Antibody Buffer)	4 ml	8 ml	16 ml	RT
HS (hMeDIP Solution)	3 ml	6 ml	12 ml	RT
DRB (DNA Release Buffer)	7 ml	14 ml	28 ml	RT
Non-Immune IgG (0.6 mg/ml)*	10 µl	20 µl	40 µl	4°C
5-hmC Antibody (0.6 mg/ml)*	25 µl	50 µl	100 µl	4°C
Control DNA (500 ng/ml)*	5 µl	10 µl	20 µl	-20°C
Proteinase K (10 mg/ml)*	28 µl	56 µl	112 µl	4°C
Control Primer-Forward (20 µM)*	5 µl	10 µl	20 µl	4°C
Control Primer-Reverse (20 µM)*	5 µl	10 µl	20 µl	4°C
8-Well Assay Strips (With Frame)	3	6	12	4°C
Adhesive 8-Well Strip Film	3	6	12	RT
User Guide	1	1	1	RT

* Spin the solution down to the bottom prior to use.

SHIPPING & STORAGE

The kit is shipped in two parts: the first part at ambient room temperature and the second part on frozen ice packs at 4°C

Upon receipt: (1) Store **Control DNA** at -20°C away from light; (2) Store **WB, Non-Immune IgG, 5-hmC Antibody, Proteinase K, Control Primer-Forward, Control Primer-Reverse, and 8-Well Assay Strips** at 4°C away from light; (2) Store remaining components at room temperature away from light.

All components of the kit are stable for 6 months from the date of shipment, when stored properly.

Note: Check if WB (10X Wash Buffer) contains salt precipitates before use. If so, briefly warm at room temperature or 37°C and shake the buffer until salts are re-dissolved.

MATERIALS REQUIRED BUT NOT SUPPLIED

- Variable temperature waterbath or incubator oven
- Thermalcycler with 48- or 96-well block
- Sonication device
- Orbital shaker
- Adjustable pipette and multiple-channel pipette

- Aerosol resistant pipette tips
- Parafilm M
- 0.2 ml or 0.5 ml PCR vials

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of the EpiQuik™ Hydroxymethylated DNA Immunoprecipitation (hMeDIP) Kit is tested against predetermined specifications to ensure consistent product quality. Epigentek guarantees the performance of all products in the manner described in our product instructions.

Product Warranty: If this product does not meet your expectations, simply contact our technical support unit or your regional distributor. We also encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

Safety: Suitable lab coat, disposable gloves, and proper eye protection are required when working with this product.

Product Updates: Epigentek reserves the right to change or modify any product to enhance its performance and design. The information in this User Guide is subject to change at any time without notice. Thus, only use the User Guide that was supplied with the kit when using that kit.

Usage Limitation: The EpiQuik™ Hydroxymethylated DNA Immunoprecipitation (hMeDIP) Kit is for research use only and is not intended for diagnostic or therapeutic applications.

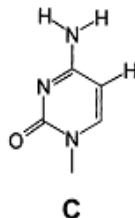
Intellectual Property: The EpiQuik™ Hydroxymethylated DNA Immunoprecipitation (hMeDIP) Kit and the method of processing contain proprietary technologies by Epigentek.

A BRIEF OVERVIEW

DNA methylation occurs by the covalent addition of a methyl group at the 5-carbon of the cytosine ring, resulting in 5-methylcytosine (5-mC). In somatic cells, 5-mC is found almost exclusively in the context of paired symmetrical methylation of the dinucleotide CpG, whereas in embryonic stem (ES) cells, a substantial amount of 5-mC is also observed in non-CpG contexts. The biological importance of 5-mC as a major epigenetic modification in phenotype and gene expression has been widely recognized.

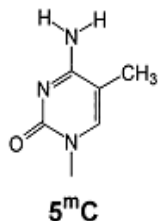
Quite recently, a novel modified nucleotide called 5-hydroxymethyl-cytosine (5-hmC) has been detected to be abundant in mouse brains and embryonic stem cells. In mammals, it can be generated by the oxidation of 5-methylcytosine, a reaction mediated by the Tet family of enzymes and DNMT proteins. It is a hydroxylated and methylated form of cytosine.





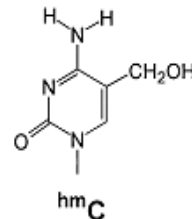
Unmethylated DNA

T-C-G-T-C-G-A-C-G



Methylated DNA

T-^mC-G-T-^mC-G-A-^mC-G



Hydroxymethylated DNA

T-^{hm}C-G-T-^{hm}C-G-A-^{hm}C-G

A line of evidence showed that 5-hmC also plays an important and different role from 5-mC in regulation of DNA methylation, chromatin remodeling, and gene expression, particularly in brain-specific gene regulation. For example, it was shown that 5-hmC inhibits the binding of the methyl-CpG binding domain proteins to DNA, suggesting a potential gene regulatory function of 5-hmC. 5-hmC was observed to be linked with epigenetic reprogramming in mammalian zygotes. However, the exact functions of 5-hmC have not yet been fully identified since gene-specific distribution of 5-hmC is unknown due to the inability of currently used DNA methylation analysis methods in distinguishing 5-hmC from 5-mC.

Because of the presence of 5-hmC in DNA with unclear functions in gene regulation and because of the discovery of enzymes that produce 5-hmC, it is crucial to identify hydroxymethylation status in specific gene loci, which would help to better understand methylation-based epigenetic regulation of gene functions. To achieve this, Epigentek has developed an innovative method to capture DNA fragments containing 5-hmC, and incorporated this method into its EpiQuik™ Hydroxymethylated DNA Immunoprecipitation (hMeDIP) Kit. This kit uses a high affinity 5-hmC antibody to selectively capture double-stranded or single stranded DNA fragments containing 5-hmC. The kit has the following features:

- Extremely fast and convenient protocol with a total procedure time (from input sample to ready-to-use hydroxymethylated DNA) of less than 3 hours, which includes a minimal handling time of less than 20 minutes.
- Flexible 96 stripwell microplate format makes the assay very easy to handle: manual method with one reaction at a time or high throughput method with 96 reactions at a time.
- Highly efficient enrichment ratio of positive/negative control > 1000.
- Low DNA input requirement of as low as 0.1 µg per reaction.
- High reproducibility through pre-optimized hMeDIP conditions.
- Compatible with various downstream analysis workflows including hMeDIP-PCR and hMeDIP-chip.

PRINCIPLE & PROCEDURE

The EpiQuik™ Hydroxymethylated DNA Immunoprecipitation (hMeDIP) Kit contains all reagents required for carrying out a successful hMeDIP procedure using DNA isolated from mammalian cells or tissues. This kit includes a positive control DNA fragment, a negative control non-immune IgG, and control primers that can be used with the positive control to demonstrate the enrichment efficacy for hydroxymethylated DNA with the kit reagents and protocol. The positive control DNA containing 5-hmC can be immunoprecipitated by a 5-hmC antibody but not by a non-immune IgG. In this hMeDIP, immunoprecipitation of 5-hmC-enriched DNA fragments is processed in a microplate under optimized reaction conditions, which enables hMeDIP to be completed within 3 hours with high efficiency. Immunoprecipitated hydroxymethylated DNA is then cleaned, released, and eluted. Eluted DNA can

be used for various downstream applications including PCR (hMeDIP-PCR) and microarray (hMeDIP-chip).

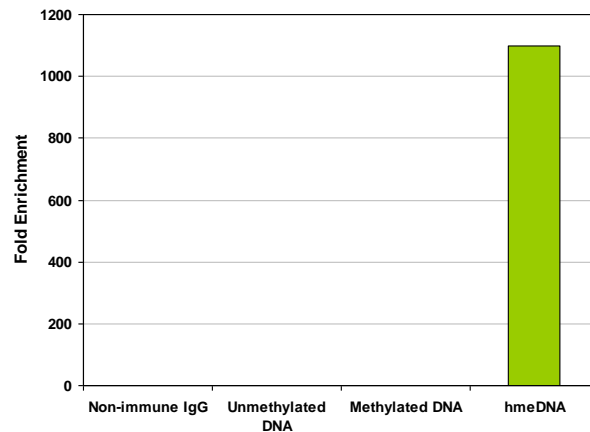
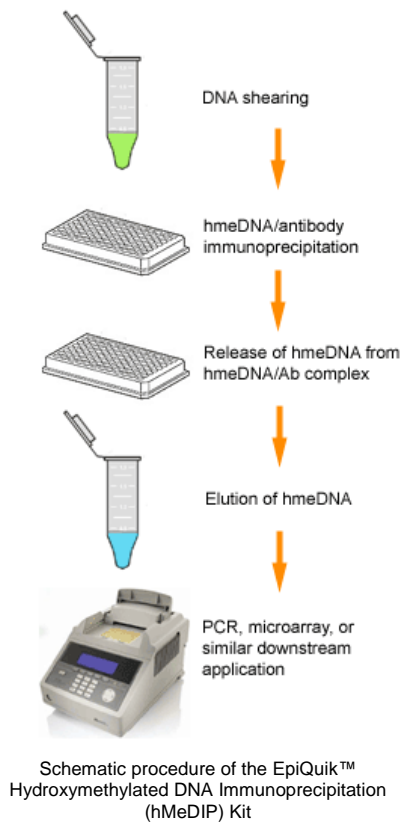


Fig. 1. Selective enrichment of hydroxymethylated DNA with the EpiQuik™ hMeDIP Kit | 50 pg of unmethylated, methylated, and hydroxymethylated DNA control were each spiked into fragmented human genomic DNA (500 ng). hMeDIP was processed with the 5-hmC antibody and non-immune IgG included in the kit. Eluted DNA was analyzed by real time PCR with the control primers included in the kit to detect the presence of spiked control DNA. Fold-enrichment represents the amount of recovered control DNA and was calculated based on the Cts.

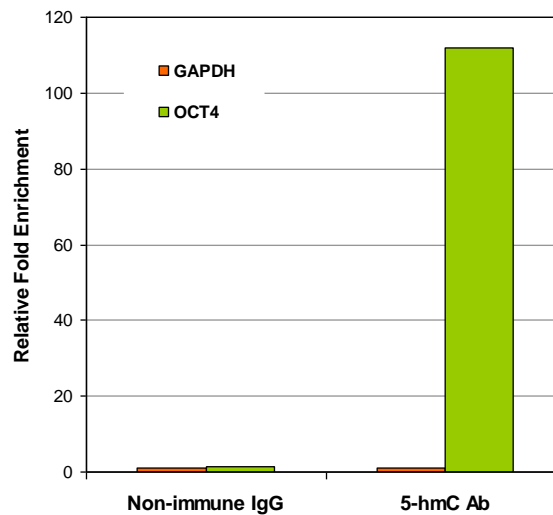


Fig. 2. Sensitive detection of gene-specific hydroxymethylation by hMeDIP-QPCR | Human brain DNA (500 ng) was fragmented to 200-600 bps with an EpiSonic 1000. The fragmented DNA was used for hydroxymethylated DNA enrichment with the EpiQuik™ hMeDIP Kit. Eluted DNA was analyzed by real time PCR with primers specifically for OCT4 or GAPDH sequences in the promoter regions. Results show that the promoter region is hydroxymethylated in OCT4 but not in GAPDH. Fold-enrichment represents the amount of recovered DNA and was calculated based on the Cts.



PROTOCOL

For the best results, please read the protocol in its entirety prior to starting your experiment.

Starting Materials

Input DNA Amount: DNA amount can range from 100 ng to 1 µg per reaction. An optimal amount is 500 ng per reaction.

DNA Isolation: You can use your method of choice for DNA isolation. Epigentek offers a series of genomic DNA isolation kits for your convenience.

DNA Storage: Isolated genomic DNA can be stored at 4°C (short term) or -20°C (long term) until use.

1. Preparation of 1X Wash Buffer

24-Reaction Kit: Add 5 ml of **WB** 10X Wash Buffer to 45 ml of distilled water (pH 7.2-7.5).

48-Reaction Kit: Add 10 ml of **WB** 10X Wash Buffer to 90 ml of distilled water (pH 7.2-7.5).

96-Reaction Kit: Add 20 ml of **WB** 10X Wash Buffer to 180 ml of distilled water (pH 7.2-7.5).

This **Diluted WB** 1X Wash Buffer can now be stored at 4°C for up to six months

2. Preparation of Antibody Coated Wells

- Predetermine the number of strip wells required for your experiment. Carefully remove un-needed strip wells from the plate frame and place them back in the bag (seal the bag tightly and store at 4°C).
- Add 100 µl of **AB** to each well and then add the following antibodies: 1 µl of **Non-Immune IgG** to the negative control well, 1 µl of **5-hmC Antibody** to the sample wells, and 1 µl of **5-hmC Antibody** to the positive control wells.
- Cover the wells with parafilm M and incubate at room temperature for 60 min. Meanwhile, prepare fragmented DNA as described in the next step.

3. Shearing of Genomic DNA

For the best results, DNA should be fragmented by a suitable sonication method:

Probe-based Sonication: You will need to optimize the sonication settings. For example, DNA of 200-1000 bp size can be obtained by sonicating 3-4 pulses of 10-12 sec each at level 2 using a Branson Microtip probe, followed by a 30-40 sec rest period on ice between each pulse.

Waterbath-based Sonication: Follow the pre-optimized protocol in the EpiSonic™ Multi-Functional Bioprocessor 1100 user manual for DNA shearing at a size range of 100-600 bp.

Note: If desired, remove 10 µl of sheared DNA for purification and agarose gel analysis along with a DNA marker on a 1-2% agarose gel, stained with ethidium bromide. Visualize it under ultraviolet light.

4. Preparation of hMeDIP Reaction

- Remove **AB** from the wells and wash the wells two times with 200 µl of **Diluted WB** each time.

- b. Dilute the **Control DNA** to 50 ng/ml (50 pg/ μ l) by adding 1 μ l of **Control DNA** to 9 μ l of **HS hMeDIP Solution** and dilute your sample DNA with **HS hMeDIP Solution** to 10 μ g/ml (10 ng/ μ l). Setup the hMeDIP reactions by adding the appropriate reagents to each corresponding well according to the following chart:

Reagent	Sample Well	Positive Control Well	Negative Control Well For Sample	Negative Control Well For Control DNA
HS Solution	50 μ l	99 μ l	50 μ l	99 μ l
Sample DNA	50 μ l	N/A	50 μ l	N/A
Control DNA	0 μ l	1 μ l	0 μ l	1 μ l

Note: (1) The final amount of each component should be 500 ng/well for sample DNA and 50 pg/well for control DNA; (2) An input DNA control is only used for estimating enrichment efficiency of hMeDIP and is generally not needed as the included positive and negative controls can be used for estimating the same objective more accurately; (3) If an input DNA control is to also be included, remove 5 μ l of the sonicated DNA solution prepared at Step 3 to a 0.5 ml vial, label as "input DNA", and place on ice.

- c. Cover the wells with parafilm M and incubate at room temperature for 90 min on an orbital shaker at 50-100 rpm.

5. Wash of the Reaction Wells

- Carefully remove and discard the solution containing the reagents by pipetting out each well.
- Thoroughly wash each well five times with 200 μ l of the **Diluted WB** each time. This can be done by simply pipetting **Diluted WB** in and out of the wells.
- Wash each well with 200 μ l of **DRB** one time by pipetting **DRB** in and out.

6. Release and Elution of DNA

- Prepare **DRB-PK Solution** by adding 1 μ l of **Proteinase K** to every 39 μ l of **DRB**. Mix.
- Add 40 μ l of the **DRB-PK Solution** to each well.
- Separate and insert the wells into a thermalcycler with a 48 well block.
- Tightly seal the wells with **Adhesive 8-Well Strip Film** and incubate at 60°C for 15 min, followed by incubation at 95°C for 3 min.

Note: If only a thermalcycler with a 96 well block is available, then (1) incubate the wells at 65°C for 20 min and quickly transfer the DNA solution from each well to 0.2 ml strip PCR tubes. Cap the PCR tubes and then incubate the PCR tubes containing the DNA solution at 95°C for 3 min in the thermalcycler; then (2) place the PCR tubes in room temperature. If liquid is collected on the inside of the caps, briefly centrifuge the liquid down to the bottom.

DNA is now ready for use or storage at -20°C.

For real time PCR analysis, we recommend using 1-2 μ l of eluted DNA in a 20 μ l PCR reaction. Input DNA can be added directly to a PCR reaction after appropriate dilution. For end point PCR, the number of PCR cycles may need to be optimized for better PCR results. In general, the amplification difference between "non-immune IgG control" and "5-hmC antibody" may vary from 3 to 8 cycles, depending on experimental conditions.

For hMeDIP-chip, additional DNA cleanup/concentration and whole genome amplification (WGA) steps may be needed. For your convenience, Epigenetek offers the DNA Concentrator Kit (Cat. No. P-1006) for DNA cleanup and concentration.

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
Little or no PCR products generated from samples	Poor DNA quality due to insufficient cell amounts, extraction, or degradation.	To obtain the best results, the amount of DNA per hMeDIP should be 0.1-1 µg with 260/280 ratio >1.6.
	Inappropriate DNA fragmenting conditions.	DNA fragment size should be between 200-1000 bp with an optimal size range of 200-600 bp. Oversized DNA fragments may reduce targeted DNA capturing via antibody and undersized DNA fragments may decrease PCR efficiency.
	Incorrect temperature and/or insufficient time during DNA release.	Ensure the incubation time and temperature described in the protocol are followed correctly.
	Improper PCR program settings.	Ensure PCR program settings are properly programmed.
	Inappropriate PCR reaction solution.	If using a homemade PCR reaction solution, check if each component is correctly mixed. If using a PCR Fast Kit, check if it is suitable for your PCR.
	Inappropriate primers.	Confirm the species specificity of your primers. Primers should be designed to cover a short sequence region (70-150 bp) for more efficient and exact amplification of target DNA regions.
	Improper sample storage.	DNA samples should be stored at -20°C (3-6 months).
No difference in signal intensity between negative control and positive control	Insufficient washing of wells.	<p>Check if washing recommendations at each step is performed according to the protocol. If the signal intensity in the negative control is still high, washing stringency can be increased in the following ways:</p> <ol style="list-style-type: none"> 1. Increase wash time at each wash step: after adding Diluted WB, leave it in the tubes/wells for 2-3 min before removing it. 2. Add an additional one or two wash steps: The volume of Diluted WB is sufficient for at least two extra washes for each sample.
	Too many PCR cycles.	Pleateau phase of amplification caused by over-increased number of PCR cycles in endpoint PCR may mask the difference in

		signal intensity between negative control and positive control. Decreasing the number of PCR cycles (ex: 32-35 cycles) to keep amplification at exponential phase will reduce high background in endpoint PCR and allow differences in amplification to be seen. Real time PCR is another alternative in such cases.
Little or No PCR products generated from positive control only	PCR conditions are not optimized.	Make sure the PCR conditions include correct and appropriate temperature, cycles, and solutions.

PCR ANALYSIS

Real Time PCR

Primer Design

Primers designed should meet the criteria for real time PCR. For example, the covered sequence region should be 50-150 bp in length. G/C stretches at 3' ends of primers should be avoided.

PCR Reaction

Real time PCR can be performed using your own proven method. For your convenience and best results, Epigentek offers the EpiQuik™ Quantitative PCR Fast Kit (Cat #P-1029) which is optimized for fast qPCR reactions. As an example, the protocol is presented below:

Prepare the PCR Reactions

Thaw all reaction components including master mix, DNA/RNA free water, primer solution and DNA template. Mix well by vortexing briefly. Keep components on ice while in use, and return to -20°C immediately following use. Add components into each well according to the following:

Component	Size (µl)	Final Concentration
Methylamp Master Mix (2X)	10 µl	1X
Forward Primer	1 µl	0.4-0.5 µM
Reverse Primer	1 µl	0.4-0.5 µM
DNA Template	1-2 µl	50 pg-0.1 µg
DNA/RNA-free H ₂ O	6-7 µl	
Total Volume	20 µl	

For the negative control, use DNA/RNA-free water instead of DNA template.

Program the PCR Reactions

Place the reaction plate in the instrument and set the PCR conditions as follow:

Cycle Step	Temp	Time	Cycle
Activation	95°C	7 min	1
Cycling	95°C	10 sec	40
	55°C	10 sec	
	72°C	8 sec	
Final Extension	72°	1 min	1

Fold Enrichment Calculation

Fold enrichment (FE) can be calculated by simply using a ratio of amplification efficiency of the ChIP sample over that of non-immune IgG. Amplification efficiency of Polymerase RNA II can be used as a positive control.

$$FE \% = 2^{(IgG\ CT - Sample\ CT)} \times 100\%$$

For example, if CT for IgG is 38 and the sample is 34, then...

$$FE \% = 2^{(38 - 34)} \times 100\% = 1600\%$$

Endpoint PCR

Primer Design

Primers designed should meet the criteria for endpoint PCR. For example, the covered sequence region should be 100-400 bp in length. PCR primer design tools (e.g., Primer3Plus) can be used to help in the selection of appropriate primer pairs.

PCR Reaction

Endpoint PCR can be performed using your own proven method. It is important to stop the PCR reaction at the exponential phase by setting up an appropriate number of PCR cycles in order to make a reliable comparison of enrichment efficiency obtained from different ChIP reactions. Thus, the optimized number of PCR cycles should be determined empirically.

PCR Product Analysis

Endpoint PCR products can be analyzed by separating amplicons on a 1-2% agarose gel followed by staining with ethidium bromide and visualizing with UV-illumination.

RELATED PRODUCTS

Methylated DNA Immunoprecipitation

- P-1015 *Methylamp*TM Methylated DNA Capture Kit
- P-2019 *EpiQuik*TM Methylated DNA Immunoprecipitation Kit
- P-2020 *EpiQuik*TM Tissue Methylated DNA Immunoprecipitation Kit

DNA Hydroxymethylation Analysis

- P-1036 *MethylFlash*TM Hydroxymethylated DNA Quantification Kit (Colorimetric)
- P-1037 *MethylFlash*TM Hydroxymethylated DNA Quantification Kit (Fluorometric)

Sonication Instruments

- EQC-1100 EpiSonicTM Multi-Functional Bioprocessor 1100

PCR Analysis

- P-1029 EpiQuikTM Quantitative PCR Fast Kit

