ab117134 – Hydroxymethylated DNA Immunoprecipitation (hMeDIP) ChIP Kit

Instructions for Use

For selective enrichment of DNA fragments containing 5-hydroxymethylcytosine in a high throughput format using DNA isolated from various species

This product is for research use only and is not intended for diagnostic use.

Version 3 Last Updated 5 February 2019

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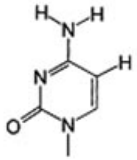
1. BACKGROUND

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.



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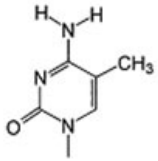
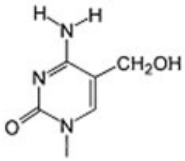


$5^{m}C$



^{hm}C

Unmethylated DNA	Methylated DNA	Hydroxymethylated DNA
T-C-G-T-C-G-A-C-G	T-^{m}C-G-T-^{m}C-G-A-^{m}C-G	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, an innovative method has been developed to capture DNA fragments containing 5-hmC, and this method has been incorporated into the 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.

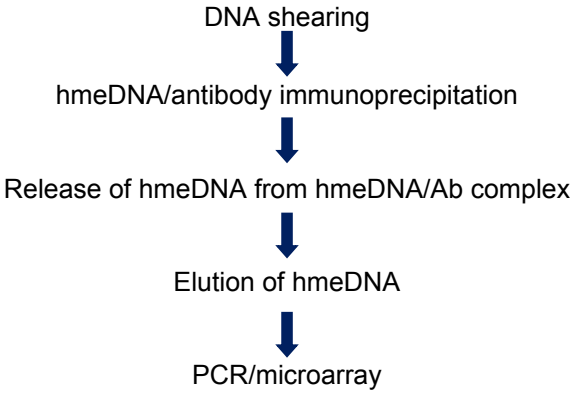
The kit has the following features:

- 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 strip well 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.

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).
ab117134 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).

2. ASSAY SUMMARY



3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit as given in the table upon receipt.

Observe the storage conditions for individual prepared components in sections 9 & 10.

Avoid repeated thawing and re-freezing of temperature sensitive components. It is recommended that you aliquot accordingly ahead of time.

For maximum recovery of the products, centrifuge the original vial prior to opening the cap.

Check if Buffer contains salt precipitates before use. If so, warm at room temperature or 37°C and shake the buffer until the salts are re-dissolved.

5. MATERIALS SUPPLIED

Item	24 Tests	48 Tests	96 Tests	Storage Condition (Before Preparation)
10X Wash Buffer	5 mL	10 mL	20 mL	4°C
Antibody Buffer	4 mL	8 mL	16 mL	RT
hMeDIP Solution	3 mL	6 mL	12 mL	RT
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

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

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Variable temperature waterbath or incubator oven
- Thermacycler 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

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding

8. TECHNICAL HINTS

- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. REAGENT PREPARATION

Prepare fresh reagents immediately prior to use.

9.1 **1X Wash Buffer**

Add 5 mL of 10X Wash Buffer to 45 mL of distilled water (pH 7.2-7.5). The 1X Wash Buffer can now be stored at 4°C for up to six months.

9.2 **Control DNA**

Dilute the Control DNA to 50 ng/ml (50 pg/µl) by adding 1 µL of Control DNA to 9 µL of hMeDIP Solution.

9.3 **DNA Release Buffer\Proteinase K**

Add 1 µL of Proteinase K to every 39 µL of DNA Release Buffer. Mix.

10. SAMPLE PREPARATION

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

DNA Isolation: You can use your method of choice for DNA isolation.

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

10.1 Shearing of Genomic DNA

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

10.1.1 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 microtip probe, followed by a 30-40 sec rest period on ice between each pulse.

10.1.2 Waterbath-based Sonication:
Follow the manufactuerer's user manual for DNA shearing at a size range of 200-600 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.*

11. ASSAY PROCEDURE

11.1 Preparation of Antibody Coated Wells

11.1.1 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)..

11.1.2 Add 100 µL of Antibody Buffer to each well and then add the following antibodies: 1 µL of Non-Immune IgG (Sample DNA Negative Control and Control DNA Negative Control Wells) or 1 µL of 5-hmC Antibody (Sample DNA and Control DNA Positive Control Wells).

11.1.3 Cover the wells with parafilm M and incubate at room temperature for 60 min.

11.2 Preparation of hMeDIP Reaction

11.2.1 Remove Antibody Buffer from the wells and wash the wells two times with 200 µL of 1X Wash Buffer each time.

11.2.2 Dilute your DNA with hMeDIP Solution to 10 µg/mL (10 ng/µL). Setup the hMeDIP reactions by adding the appropriate reagents to each corresponding well according to the following chart.

Component	Sample DNA Well (µL)	Control DNA Positive Control Well (µL)	Sample DNA Negative Control Well (µL)	Control DNA Negative Control Well (µL)
hMeDIP Solution	50	99	50	99
Sample DNA	50	0	50	0

Control DNA	0	1	0	1

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 µL of the sonicated DNA solution prepared at Step D to a 0.5 mL vial, label as "input DNA", and place on ice.

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

11.3 **Washing of Reaction Wells**

11.3.1 Carefully remove and discard the solution containing the reagents by pipetting out each well.

11.3.2 Thoroughly wash each well five times with 200 µL of the 1X Wash Buffer each time. This can be done by simply pipetting Diluted 1X Wash Buffer in and out of the wells.

11.3.3 Wash each well with 200 µL of DNA Release Buffer one time by pipetting DNA Release Buffer in and out.

11.4 **Release and Elution of DNA**

11.4.1 Add 40 µl of the DNA Release Buffer\Proteinase K Solution to each well.

11.4.2 Separate and insert the wells into a thermalcycler with a 48 well block.

11.4.3 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;(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.

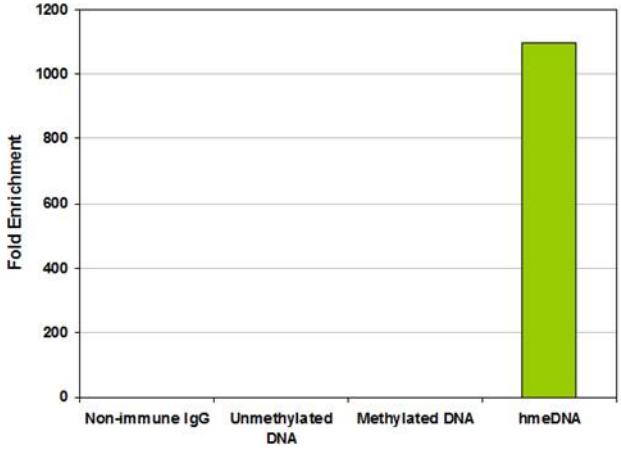
DNA is now ready for use or storage at -20°C.

12. ANALYSIS

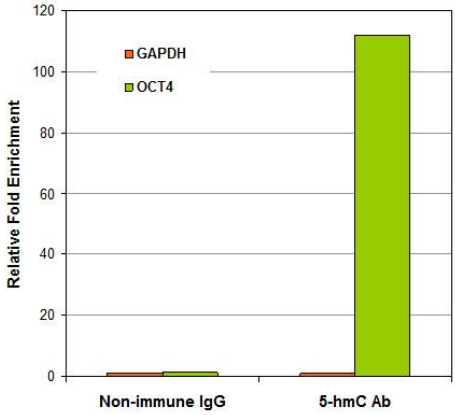
For real time PCR analysis, we recommend using 1-2 µL of eluted DNA in a 20 µL PCR reaction. Input DNA can be added directly to a PCR reaction after appropriate dilution. For end point PCR, the number of PCR cycles may need to be optimized for better PCR results. In general, the amplification difference between “Non-Immune IgG” 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.

Typical Results:



Selective enrichment of hydroxymethyated DNA with ab117134. 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.



Sensitive detection of gene-specific hydroxymethylation by hMeDIP-QPCR. Human brain DNA (500 ng) was fragmented to 200-600 bps with a sonicator. The fragmented DNA was used for hydroxymethylated DNA enrichment with the 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.

Suggested PCR running program

These cycle times are only a guideline, we strongly recommend running optimization testing.

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

13. TROUBLESHOOTING

Problem	Cause	Solution
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
Little or no PCR products generated from samples.	Improper PCR program settings	Ensure PCR program settings are properly programmed

Problem	Cause	Solution
	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
Little or no PCR products generated from samples.	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)

Problem	Cause	Solution
No difference in signal intensity between negative control and positive control	Insufficient washing of wells	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: 1. Increase wash time at each wash step: after adding 1X Wash Buffer, 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 1X Wash Buffer is sufficient for at least two extra washes for each sample

Problem	Cause	Solution
No difference in signal intensity between negative control and positive control	Too many PCR cycles	Plateau 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

14. NOTES

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