ab185904 – MeDIP Ultra Kit

Instructions for Use

For selective enrichment of DNA fragments containing 5-methylcytosine. It can use DNA isolated from various species

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

Version 4 Last Updated 14 April 2020

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1. BACKGROUND

Core mechanisms for epigenetic alteration of genomic DNA are hypermethylation of CpG islands in specific genes and global DNA hypomethylation. Region-specific DNA methylation plays an important role in the repression of gene transcription and is mainly found in 5'-CpG-3'dinucleotides within promoters or in the first exon of genes. Global DNA hypomethylation is likely caused by methyl-deficiency due to a variety of environmental influences. It has been demonstrated that alterations in DNA methylation are associated with many diseases, especially cancer.

Highly specific isolation of methylated DNA combined with next generation sequencing for genome-wide methylation analysis should provide an advantage for convenient and comprehensive identification of methylation status of normal and diseased cells, such as cancer cells. Such analysis requires the isolated methylated DNA to contain minimal background in order to achieve high specificity (>98%) for reliably identifying true methylated regions. The major method for enriching methylated DNA used for genome-wide methylation profiling is methylated DNA immunoprecipitation (MeDIP). However, currently used MeDIP methods, represented by most commercially available kits, have significant weaknesses including highly non-specific enrichment (amount of enriched DNA is >75% of the amount of input DNA), time consuming, labor intensive, and has low throughput. Thus, for effectively and specifically capturing methylated DNA used for next generation sequencing analysis, an ideal MeDIP method requires maximum sensitivity with minimal background levels. Abcam's MeDIP Ultra Kit is designed to achieve these goals by maximizing sensitivity and minimizing non-specific background signals.

The MeDIP Ultra Kit has the following advantages and features:

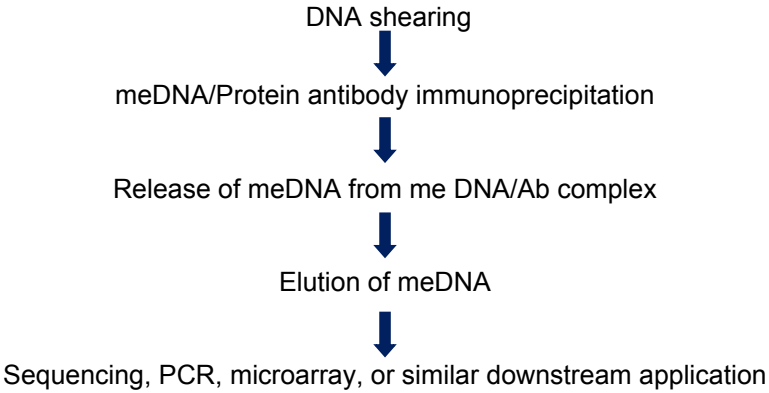
- Extremely fast and convenient protocol with a total procedure time (from input sample to ready-to-use methylated DNA) of less than 3 hours, which includes a minimal handling time of less than 20 minutes.
- Optimized buffers and protocol allow minimal background by overcoming the weaknesses that cause non-specific enrichment.
- A highly specific 5-mC monoclonal antibody included in the kit can strongly bind both single and double stranded DNA fragments containing 2 or more 5-mCs which enables highly sensitive enrichment of methylated DNA with >99% specificity.
- 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 24-48 reactions at a time.
- Spin columns and collection tubes conveniently included for a DNA purification step.
- Low DNA input requirement as low as 50 ng (10000 cells) per reaction.
- High reproducibility using pre-optimized MeDIP conditions.
- Compatible with various downstream analysis workflows including MeDIP-PCR and MeDIP-chip, and specifically for MeDIP-Seq.

The MeDIP Ultra Kit is suitable for selective enrichment of DNA fragments containing 5-methylcytosine. The highly sensitive and specific format can use DNA isolated from various species. The methylated DNA that is enriched with this kit can be used for various downstream applications including qualitative and quantitative PCR (MeDIP-PCR), microarray (MeDIP-chip) and especially sequencing (MeDIP-seq).

The MeDIP Ultra Kit contains all reagents required for carrying out a successful MeDIP procedure using DNA isolated from mammalian cells or tissues. This kit includes a methylated DNA (mDNA) control and an unmethylated DNA (unDNA) control, a negative control non-immune IgG, and control primers that can be used with the control DNA to demonstrate the enrichment efficacy and specificity for methylated DNA. The positive control DNA containing 5-mC can be immunoprecipitated by the 5-mC antibody but not by the non-immune IgG. In this MeDIP,

immunoprecipitation of 5-mC-enriched DNA fragments is processed in a microplate under optimized reaction conditions, which enables MeDIP to be completed within 3 hours with high efficiency. Immunoprecipitated methylated DNA is then cleaned, released, and eluted. Eluted DNA can be used for various downstream applications including PCR (MeDIP-PCR) and microarray (MeDIP-chip), and is especially suitable for MeDIP-seq.

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.

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

Check if Wash Buffer contain 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	Storage Condition (Before Preparation)
Wash Buffer	15 mL	30 mL	4°C
MeDIP Buffer	4 mL	8mL	RT
DNA Release buffer	14 mL	28 mL	RT
DNA Binding Solution	7 mL	14 mL	RT
Blocker Solution	200 µL	400 µL	4°C
DNA Elution Buffer	1 mL	2 mL	RT
Non-Immunogen IgG	10 µL	20 µL	4°C
5-mC Antibody	25 µL	50 µL	4°C
Proteinase K (10 mg/mL)	28 µL	56 µL	4°C
Control unDNA (200 ng/mL)	5 mL	10 mL	–20°C
Control mDNA (200 ng/mL)	5 µL	10 µL	–20°C
Control Primer-F (20 µM)	5 µL	10 µL	4°C
Control Primer-R (20 µM)	5 µL	10 µL	4°C
Spin Column	30	50	RT
Collection Tube	30	50	RT
8-Well Assay Strip (With Frame)	3 strips	6 strips	4°C
8-Well Strip Caps	3 strips	6 strips	RT
Adhesive 8-Well Strip Film	3	6	RT

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- Variable temperature water bath or incubator oven
- Thermal cycler with 48- or 96-well block
- Sonication device
- Orbital shaker
- Adjustable pipette or multiple-channel pipette
- Aerosol resistant pipette tips
- Parafilm M
- 0.2 mL or 0.5 mL PCR tubes
- 1X TE buffer, pH 8.0
- 90% Ethanol

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

All reagents are supplied as ready to use.

10. SAMPLE PREPARATION

10.1 **Input DNA Amount:** DNA amount can range from 50 ng to 500 ng per reaction. An optimal amount is 100 ng per reaction.

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

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

10.4 **DNA Shearing**: Genomic DNA should be sheared by sonication before starting MeDIP. The sheared DNA fragments should range in size from 100-600 base pairs.

10.5 **Antibody:** The 5-methylcytosine antibody used in this kit is highly specific against methylated DNA fragments, both single and double stranded, and is not cross-reactive to hydroxymethylated and unmethylated DNA fragments. This antibody can capture >50% of DNA fragments containing as few as two 5-mCs and enriches all DNA fragments containing four or more 5-mCs.

10.6 **Methylated DNA yield:** The yielded methylated DNA is about 4 ng for 100 ng input DNA (4%), which is consistent with the expected percentage (4-5%) at which the highest sensitivity and specificity for enriched methylated DNA has been demonstrated by bisulfite sequencing.

11. ASSAY PROCEDURE

Internal Control: Negative control (non-immune IgG) and specificity are provided in this kit. The specificity controls are 200 base pair DNA fragments containing 44 cytosine residues which are methylated (mDNA) or unmethylated (unDNA). The kit also includes control PCR primers that can be used for verifying the enrichment efficiency and specificity of control DNA.

11.1 **Shearing of Genomic DNA**

11.1.1 **Water bath Sonication:** Follow the supplier's instruction.

11.1.2 Probe-based Sonication: Use 300 µL of DNA solution per 1.5 mL microcentrifuge tube. As an example, sonication can be carried out with a microtip set to 25% power output. Sonicate 3-4 pulses of 10-15 seconds each, followed by 30-40 seconds rest on ice between each pulse. The conditions of DNA shearing should be optimized based on the sonication instrument.

Note: *When probe-based sonication is carried out, shearing effect may be reduced if foam is formed in the DNA sample solution. Under this condition, discontinue sonication and centrifuge the sample at 4°C at 12000 rpm for 3 minutes to remove the air bubbles.*

The isolated DNA can also be sheared with various enzyme-based methods. Optimization of the shearing conditions, such as enzyme concentration and incubation time, is needed in order to use enzyme-based methods. The DNA solution can now be used immediately or stored at -80°C after aliquoting appropriately until further use. Avoid multiple freeze/thaw cycles. Note: The size of sonicated DNA can be verified before starting the immunoprecipitation step. Use 10 µL (>500 ng) for DNA fragment size analysis along with a DNA marker on a 1-2% agarose gel; Stain with ethidium bromide or a suitable fluorescent dye for DNA and visualize it under ultraviolet light. The length of sheared DNA should be between 100-600 bp with peak size of 250 bp.

11.2 Preparation of MeDIP Reaction

11.2.1 Dilute the Control mDNA and unDNA to 50 ng/mL (50 pg/µL) by adding 1 µL of Control mDNA or Control unDNA to 9 µL of MeDIP Buffer. Dilute your sample DNA with MeDIP Buffer to 10 µg/mL (10 ng/µL). Setup the MeDIP reactions by adding the appropriate reagents to each corresponding 0.5 mL vial according to the following chart:

Reagents	Sample Vial (µL)	Negative control (µL)	Control mDNA vial (µL)	Control unDNA vial (µL)
MeDIP Buffer	84	84	93	93
Sample DNA (10 ng/µL)	10	10	0	0
Control DNA (50 pg/µL)	0	0	1	1
Blocker Solution	5	5	5	5
5-mC antibody	1	0	1	1
Non-immunogen IgG	0	1	0	0

11.2.2 Cap vials and incubate at room temperature for 60 minutes on an end-to-end rotator or a rolling shaker for 1 hour.

11.2.3 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).

11.2.4 Transfer the reaction solution from each vial to the strip-wells. Carefully seal the wells with Adhesive Covering Film. Incubate at room temperature for 60 minutes on an orbital shaker (100 rpm).

11.2.5 Peel away the Adhesive Covering Film carefully to avoid contamination between each well.

11.3 **Washing of the Reaction Wells**

11.3.1 Carefully remove and discard the solution by pipetting it out of each well.

11.3.2 Wash each well three times with 200 µL of Wash Buffer each time. This can be done by simply pipetting WB into the wells and then removing it from the wells.

11.3.3 Wash each well once with 200 1 µL of 1X TE Buffer.

11.3.4 **Release and Purification of DNA:** For each sample, prepare DRB-PK Solution by adding 1 µL of Proteinase K to 39 µL of DRB and mix. Then add 40 µL of the DRB-PK Solution to each well. Cover with Strip Caps and incubate at 60°C for 20 minutes.

11.3.5 Quickly transfer the DNA solution from each well to a 0.2 mL strip PCR tube. Cap the PCR tubes.

11.3.6 Incubate the PCR tubes containing DNA solution at 95°C for 5 minutes in a thermal cycler.

11.3.7 Place the PCR tubes at room temperature. If liquid is collected on the inside of the caps, briefly spin the liquid down to the bottom. The eluted DNA can now be directly used for PCR. Otherwise, continue to Step 11.3.8 for further purification for use in MeDIP sequencing.

11.3.8 Place a Spin Column into a 2 mL Collection Tube. Add 250 µL of DNA Binding Solution to each sample and transfer the solution to a column. Centrifuge at 12000 rpm for 30 seconds.

11.3.9 Add 200 µL of 90% ethanol to the Spin Column; centrifuge for 30 seconds at 12000 rpm. Remove the Spin Column from the Collection Tube and discard the flow-through.

11.3.10 Place Spin Column back into the Collection Tube. Add 200 µL of 90% Ethanol to the column. Centrifuge at 12000 rpm for 30 seconds.

11.3.11 Remove the Spin Column and discard the flow-through. Place the Spin Column back into the Collection Tube and wash the

Spin Column again with 200 µL of 90% Ethanol at 12000 rpm for 1 minute.

11.3.12 Place Spin Column in a 1.5 mL tube. Elute DNA by adding 20 µL DNA Elution Buffer directly to the filter of the Spin Column and centrifuge at 12000 rpm for 30 seconds.

Purified DNA is now ready for PCR, MeDIP-chip, and MeDIP-seq use, or for storage at –20°C.

Note: *For real time PCR analysis, we recommend the use of 1 µL of eluted DNA in a 20 µL PCR reaction. For end point PCR, the number of PCR cycles may need to be optimized for better PCR results.*

In general, the amplification difference between "normal IgG control" and "5-mC enriched DNA" may vary from 3 to 8 cycles, depending on experimental conditions. The amplification difference between "mDNA control" and "unDNA control" is generally >7 cycles (Specificity >99%).

In general, at least 10 ng of MeDIPed DNA are required for methylated DNA library construction. We recommend pooling the DNA solution from several wells with the MeDIP reaction from the same sample to gain 10 ng or higher DNA concentrations. We also recommend performing qPCR to verify the quality of the MeDIPed DNA.

12. ANALYSIS

Fold enrichment (FE) can be calculated by simply using a ratio of amplification efficiency of the MeDIP sample over that of non-immune IgG.

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

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

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

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 MeDIP should be between 50-500 ng with 260/280 ratio >1.6
	Inappropriate DNA fragmenting conditions.	DNA fragment size should be between 100-600 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 is 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 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
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 WB, leave it in the wells for 2-3 minutes before removing it. 2. Add an additional one or two wash steps: The volume of WB is sufficient for at least two extra washes for each sample
	Too many PCR cycles.	Plateau phase of amplification caused by excessive 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
No difference in signal intensity between Control mDNA and Control unDNA	Amount of the controls added into the PCR reaction is too high.	Make sure that the final concentration of both Control mDNA and Control unDNA is < 0.01 pg/20 µL of PCR reaction solution
	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 WB, leave it in the wells for 2-3 minutes before removing it. 2. Add an additional one or two wash steps: The volume of WB is sufficient for at least two extra washes for each sample

14. APPENDIX

Real Time PCR

Primer Design: The 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.

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	Volume (µL)	Final concentration
Master Mix (2X)	10	1X
Forward Primer	1	0.4-0.5 µM
Reverse Primer	1	0.4-0.5 µM
DNA Template	1-2	50 pg – 0.1 µg
DNA/RNA free H2O	6-7	
Total volume	20	

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 follows:

Cycle Step	Temperature(°C)	Time (seconds)	Cycle Number
Activation	95	120	1
Denature	95	600	1
Cycling	95	10	40
	55	10	
	72	10	
Final Extension	72	60	1

15. NOTES

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