



Innovating Epigenetics Solutions

MagMeDIP qPCR Kit

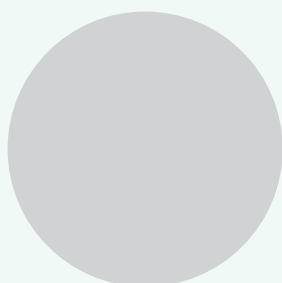
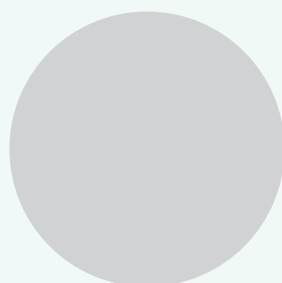
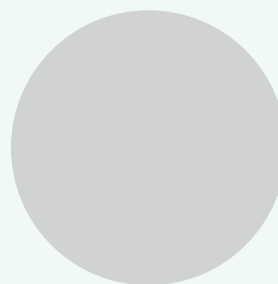
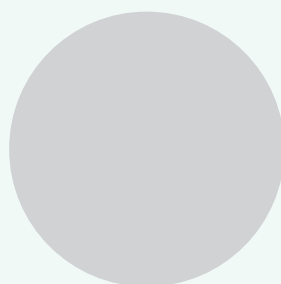
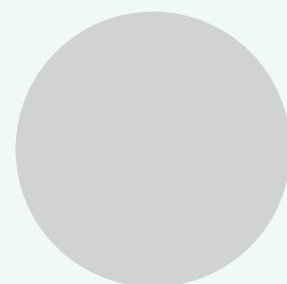
Magnetic Methylated DNA Immunoprecipitation Kit

MANUAL KIT

Cat. No. C02010020 (10 rxns)
C02010021 (48 rxns)

AUTOMATED KIT

Cat. No. C02010013 (10 rxns)
C02010014 (48 rxns)



USER GUIDE

Version 2 03_2019

The MagMeDIP qPCR/Auto MagMeDIP qPCR Kit has been validated on the IP-Star® Compact Automated System. Two versions of this protocol (manual and automated) are described in this document.



Please read this manual carefully
before starting your experiment

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Introduction

DNA methylation is a key epigenetic mechanism with important regulatory functions in biological processes such as genomic imprinting, control of transcription, embryonic development, X-chromosome inactivation, chromosome stability, and carcinogenesis.

DNA methylation occurs primarily as 5-methylcytosine (5-mC), and the Diagenode MagMeDIP qPCR/Auto MagMeDIP qPCR Kits take advantage of a specific antibody targeting this 5-mC to immunoprecipitate methylated DNA, which can be analyzed by qPCR or Next-Generation Sequencing (NGS). This methylation analysis is fast and highly specific as the IP has been optimized to specifically select and precipitate the methylated DNA by the use of our 5-mC monoclonal antibody 33D3 (Cat. No. C15200081), optimized buffers and protocol. This kit is provided with a manual protocol as well as an automated version on Diagenode SX-8G IP-Star Compact Robot (Cat. No. B03000002).

The MagMeDIP qPCR Kits are available in two formats for 10 and for 48 immunoprecipitation reaction (IPs).

The kit includes:

- A XL GenDNA Extraction Module to prepare large amounts of DNA from cultured cells
- Highly validated DNA shearing protocols on Diagenode Bioruptor®
- All the reagents for the IP, including 5-mC antibody 33D3 and magnetic beads
- Methylated and unmethylated DNA spike-in controls and their associated qPCR primers, allowing quality control of the IP
- qPCR primers targeting positive and negative regions on human genome to monitor the success of the IP
- DNA isolation module (after IP): 45 minutes for DNA Isolation Buffer method (DIB)

We recommend Diagenode's magnetic racks: DiaMag1.5 (Cat. No. B04000003) and DiaMag02 (Cat. No. B04000001) together with our MagMeDIP qPCR 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.


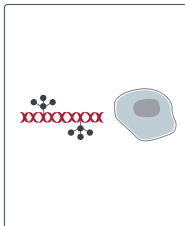

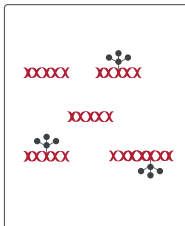

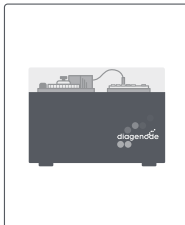


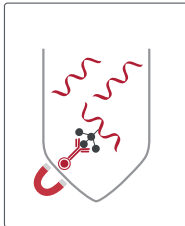


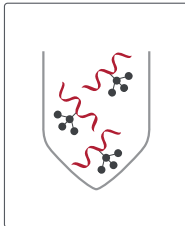


The kit ensures the use of a low amount of antibodies and buffers per reaction. The number of steps is reduced and handling is easier which makes this kit cost effective and simple to work with.

The MagMeDIP qPCR Kit can be used with DNA from every species. Please see section meDNA and unDNA spike-in controls for compatibility information.

Diagenode MagMeDIP Kits have been cited in many publications and chosen by leading laboratories worldwide thanks to their numerous benefits:

- Complete kits including DNA extraction module, antibody, spike-in controls and qPCR primer pairs for control regions
- Easy to use with user-friendly magnetic beads and racks
- Highly validated protocols compatible with qPCR (MagMeDIP qPCR Kit) and NGS downstream analyses.
- Available as an automated version

Kit Method Overview

| | | | Time needed | Day | |
|-------------------|---|---|--|----------------------------------|----------------|
| <div>STEP 1</div> |  |  | Cell collection & lysis | <div>30 min, overnight</div> | <div>1-2</div> |
| <div>STEP 2</div> |  |  | DNA extraction & purification | <div>5 h</div> | <div>2</div> |
| <div>STEP 3</div> |  |  | DNA shearing | <div>2 h</div> | <div>3</div> |
| <div>STEP 4</div> |   |  | Methylated, DNA immunoprecipitation | <div>45 min, overnight</div> | <div>3-4</div> |
| <div>STEP 5</div> |   |  | Methylated DNA isolation | <div>45 min</div> | <div>4</div> |
| <div>STEP 6</div> |  |  qPCR | qPCR analysis | <div>>2 h</div> | <div>4</div> |

LEGEND



Methylated DNA (5-mC)



Unmethylated DNA



Antibody



Magnetic bead



Magnet



Auto processing



Manual processing

Kit materials

The MagMeDIP qPCR/Auto MagMeDIP qPCR Kit is available in two formats so that the kit content (Table 1 below) is sufficient to perform either 10 or 48 methylated DNA immunoprecipitations and 6 DNA extractions.

Table 1. Components of the MagMeDIP qPCR Kit

| Component | Qty (x10) | Qty (x48) | Storage |
|--|------------------------|------------------------|----------------------|
| Magbeads (magnetic beads) | 150 μ l | 750 μ l | 4°C Do not freeze |
| Water | 2x 2 ml | 10 ml | 4°C |
| MagBuffer A (5x concentrated) | 2 ml | 10 ml | 4°C |
| MagBuffer B | 100 μ l | 500 μ l | 4°C |
| MagBuffer C | 40 μ l | 200 μ l | -20°C |
| Antibody anti-5mC* (33D3 clone) | 5 μ l | 25 μ l | -80°C |
| meDNA spike-in control | 20 μ l | 20 μ l | -20°C |
| unDNA spike-in control | 20 μ l | 20 μ l | -20°C |
| MagWash buffer-1 | 6 ml | 30 ml | 4°C |
| MagWash buffer-2 | 4 ml | 20 ml | 4°C |
| DNA Isolation Buffer (DIB) | 4 ml | 20 ml | 4°C |
| Proteinase K (100x) | 20 μ l | 200 μ l | -20°C |
| meDNA primer pair (for meDNA spike-in ctrl) (5 μ M each) | 50 μ l | 50 μ l | -20°C |
| unDNA primer pair (for unDNA spike-in ctrl) (5 μ M each) | 50 μ l | 50 μ l | -20°C |
| Human TSH2B primer pair (5 μ M each) | 50 μ l | 50 μ l | -20°C |
| Human GAPDH primer pair (5 μ M each) | 50 μ l | 50 μ l | -20°C |
| 200 μ l tube strips | 2 | 8 | RT |
| Cap strips | 2 | 8 | RT |
| GenDNA Digestion Buffer | 3 ml | 3 ml | 4°C |
| GenDNA Proteinase K (200x) | 300 μ g/15 μ l | 300 μ g/15 μ l | -20°C |
| GenDNA precipitant | 3 ml | 3 ml | 4°C |
| GenDNA TE | 3 ml | 3 ml | 4°C |
| GenDNA RNase (DNase free) | 5 μ g/10 μ l | 5 μ g/10 μ l | -20°C |

**Avoid freeze-thawing cycles of this very sensitive antibody! Make aliquots!*

NOTE: Upon receipt, store the components at the indicated temperatures.

Required materials not provided

Materials and Reagents

- Gloves to wear at all steps
- RNase/DNase-free 1.5 ml, 2 ml and 50 ml conical tubes
- Ice-cold PBS buffer
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol
- Ethanol

Equipment

- Centrifuges for 1.5 ml tubes and 50 ml conical tubes (4°C)
- Microcentrifuge for 0.2 ml tubes
- Thermomixer (95°C)
- Thermocycler
- Cell counter
- Diagenode magnetic racks:
 - DiaMag1.5 (Cat. No. B04000003)
 - DiaMag02 (Cat. No. B04000001)
- Rotating wheel such as DiaMag Rotator (Cat. No. B05000001)
- Bioruptor sonication device from Diagenode and the associated tubes:
 - Bioruptor Pico (Diagenode, Cat. No. B01060010), 0.5/0.65 ml tube holder (Cat. No. B01200043) or the holder (Cat. No. B01200051) with the 0.65 ml adaptor (Cat. No. B01200054), and 0.65 ml Bioruptor Microtubes (Cat. No. C30010011) or
 - Bioruptor Plus (Diagenode, Cat. No. B01020003), 0.5/0.65 ml tube holder (Cat. No. B01200043), and 0.5 ml TPX Microtubes (Cat. No. C30010013)

- Reagents and equipment for quantitative PCR
- Reagents and equipment for DNA analysis such as electrophoresis on agarose gel, BioAnalyzer (Agilent) or Fragment Analyzer (Agilent)
- Reagents and equipment for DNA quantification such as Qubit® Fluorometer (ThermoFisher Scientific)

Additional supplies if working with IP-Star® Compact (Auto MagMeDIP qPCR):

- IP-Star Compact Automated System (Cat. No. B03000002)
- 200 µl tubes strips (8 tubes/strip) + cap strips (Cat. No. C30020002)
- Tips (box) (Cat. No. C30040021)
- Tips (bulk) (Cat. No. C30040020)
- 2 ml microtubes (Cat. No. C30010014)
- Medium reagent container (Cat. No. C30020003)
- 96 well microplates (Cat. No. C30080030)

Optional supplies available separately

The MagMeDIP qPCR Kit contains all reagents you need for your MeDIP assay. However three of the products mentioned in the protocol can be purchased additionally if needed:

1. Additional **XL GenDNA Module (Cat. No. C03030020)** – recommended genomic DNA purification method.
2. Additional **DNA Methylation spike-in control package (Cat. No. C02040012)** includes unmethylated (unDNA) and in vitro methylated DNA (meDNA) together with specific primer sets for assessing the efficiency of your immunoprecipitation, carried out with Diagenode's MagMeDIP qPCR/Auto MagMeDIP qPCR Kit.
3. Additional **Antibody anti-5-mC* (33D3 clone) (Cat. No. C15200081)** Monoclonal antibody raised in mouse against 5-mC (5-methylcytosine) conjugated to ovalbumin (33D3 clone).

Remarks before starting

DNA extraction

The quality of the DNA to be used in MagMeDIP is important. Thus, we recommend using Diagenode XL GenDNA Extraction Module (included in this kit) 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 MagMeDIP qPCR Kit are optimized for a starting amount of 1 µg of sheared DNA per IP.

NOTE: *If starting material is limiting, it is possible to decrease the starting amount of sheared DNA down to 100 ng per IP.*

Guidelines regarding the required amount of cells:

- From 1.5 million cells, 8 to 12 µg of gDNA can be expected
- Some of the isolated gDNA will be used as a control for DNA preparation efficiency
- 1 µg of sheared DNA is needed per IP
- Some of the sheared DNA will be used as a control checking for: (a) shearing efficiency and (b) the IP experiment efficiency (see section Input below)

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).

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 amount of beads will decrease reproducibility. Do not freeze the Magbeads.

Input

“Input sample” corresponds to the sheared DNA that undergoes the full MeDIP procedure without incubation with the antibody and magnetic beads (Magbeads). The input sample is used as a reference to calculate the recovery by qPCR at the end of the MeDIP procedure. We recommend including one input sample for each sheared DNA sample that undergoes MeDIP.

meDNA and unDNA spike-in controls

The MagMeDIP qPCR Kit contains one methylated (meDNA) and one unmethylated (unDNA) spike-in control that can be added directly to DNA samples before the IP. To check the efficiency of the MeDIP experiment, this kit also includes primer pairs (targeting meDNA and unDNA spike-in controls’ regions) to calculate their recovery by qPCR.

NOTE: meDNA and unDNA spike-in controls are sheared DNA from *Arabidopsis thaliana*. They will not interfere with the sample of interest for Mammals; however, do not use them if working on *A. thaliana* and validate the controls before working on related plant species.

Technique for DNA isolation

The MagMeDIP qPCR Kit includes a DNA Isolation Buffer (DIB) for an easy and very fast DNA isolation after MeDIP 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) and the iDeal Library Preparation Kit (Cat. No. C05010020). The IPure kit v2 is the only DNA purification kit that is specifically optimized for extracting very low amounts of DNA after MeDIP.

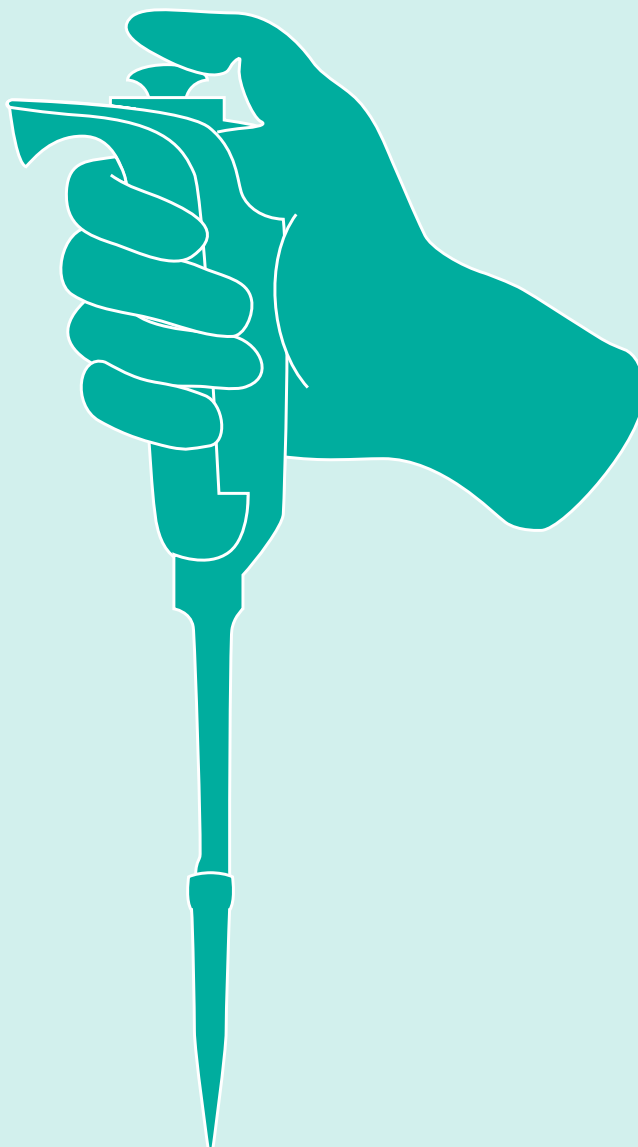
Quantitative PCR analysis

This kit contains two primer pairs targeting methylated (TSH2B) and unmethylated (GAPDH) human regions. For each primer pair, please run the input sample alongside the IP samples. As downstream analysis, qPCR reactions are recommended at least in duplicates (although triplicates are recommended as for potential outliers' identification).

MagMeDIP-sequencing (MagMeDIP-seq)

The MagMeDIP-qPCR kit has been designed for the analysis of a few loci by qPCR. To perform MeDIP-sequencing we recommend using our MagMeDIP qPCR Kit (Cat. No. C02010020) in combination with Diagenode's iDeal Library Preparation Kit (Cat. No. C05010020).

NOTE: in the MagMeDIP-seq protocol - ligation of the adaptors has to be performed BEFORE the IP, when the DNA is still double-stranded.



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MANUAL PROCESSING

STEP 1

Cell collection & lysis



Day 1



30 minutes, overnight

Starting material: cultured cells

NOTE: For recommendations about starting amounts of material, please refer to section “Remarks before starting” (page 10)

- 1.1 Pellet suspension culture out of its serum containing medium or trypsinize adherent cells and collect cells from the flask. Centrifuge at 300g for 5 minutes at 4°C.
 - 1.2 Discard the supernatant. Resuspend cells in 5 to 10 ml ice-cold **PBS**. Count cells. Centrifuge at 300g for 5 minutes. Discard the supernatant. Repeat this resuspension and centrifugation step once more. This step is to wash the cells.
 - Meanwhile, place the GenDNA Digestion Buffer at room temperature and the GenDNA Proteinase K on ice (to be used at Point 1.3 below).
- NOTE:** If needed, cell pellets can be fresh frozen at this step and stored at -80°C for several months.
- 1.3 Prepare the complete Digestion Buffer by adding 5 µl **GenDNA Proteinase K** to 1 ml **GenDNA Digestion Buffer**.
 - 1.4 Resuspend cells in complete Digestion Buffer.
 - For 1 to 1.5 million cells, use up to 500 µl complete Digestion Buffer
 - It might be necessary to use more buffer to avoid viscosity when performing the extractions
 - 1.5 Incubate the samples with shaking (500 rpm) at 50°C for 12 to 18 hours in tightly capped tubes.

STEP 2

Nucleic acid extraction & purification

 Day 2  5 hours

-
- 2.1** Thoroughly extract the samples with an equal volume of phenol/chloroform/isoamyl alcohol (work under a fume-hood).
- Add **1 volume** (500 µl according to step 1.4) of **phenol/chloroform/isoamyl alcohol (25:24:1)**.
 - Incubate the samples at room temperature for **10 minutes** on a rotating wheel. Use gentle rotation, do not vortex.
- 2.2** Centrifuge at 1700g for **10 minutes**.
- If the phases do not resolve properly, add another volume of GenDNA Digestion Buffer omitting Proteinase K, and repeat the centrifugation.
 - If there is a thick layer of white material at the interface between the phases, repeat the extraction.
- 2.3** Transfer the aqueous (top) layer to a new tube.
- Increase volume to avoid viscosity if necessary and pipette slowly.
- 2.4** Thoroughly extract the samples with **500 µl of chloroform/isoamyl alcohol (one volume)**.
- 2.5** Incubate for **10 minutes** at room temperature.
- 2.6** Centrifuge at 1700g for **10 minutes**.
- 2.7** Transfer aqueous layer to a new 2 ml tube.

2.8 Add **250 µl of GenDNA precipitant** (which is ½ volume) and **1 ml of 100% ethanol** (2 volumes).

2.9 Recover gDNA by centrifugation at 1700g for **5 minutes**.

- Do not use higher speed to avoid genomic DNA fragmentation
- This brief precipitation in the presence of an optimized high salt precipitant (GenDNA precipitant) reduces the amount of RNA in the DNA sample. For long-term storage, it is convenient to leave the DNA in the presence of ethanol.

2.10 Rinse the pellet with **70% ethanol**. Decant ethanol and air-dry the pellet.

- It is important to rinse extensively to remove any residual salt or phenol

2.11 Resuspend the pellet of gDNA at **~1 mg/ml in GenDNA TE** until dissolved. Shake gently at room temperature or at 65°C for several hours to facilitate solubilization. Store at 4°C.

- From 1-1.5 million cells, ~8 to 12 µg of gDNA can be expected (in a volume of 8 to 12 µl)
- From 3 million cells, ~20 to 30 µg of gDNA can be expected (in a volume of 20 to 30 µl)

NOTE: At this step the DNA quality can be analyzed by electrophoresis on 0.8% agarose gel or with a Fragment Analyzer (Agilent) and Standard Sensitivity Genomic DNA Analysis Kit (DNF-487, Agilent) (please refer to manufacturer's instructions).

2.12 If present, residual RNA has to be removed at this step by adding **2 µl of GenDNA RNase (DNase-free)** per ml of DNA sample and incubating 1 hour at 37°C, followed by phenol/chloroform extraction and ethanol precipitation (same protocol as above, starting from step 2.1).

2.13 Store the gDNA at 4°C until the shearing.

NOTE: For long term storage the gDNA can be placed at -20°C.

STEP 3

DNA shearing



Genomic DNA must be randomly sheared by sonication on Diagenode's Bioruptor to generate fragments around 400 bp (see Figure 1 in "Example of results" section). To perform the MagMeDIP, 1.2 µg of sheared DNA is needed in a volume smaller than 55 µl.

- 3.1 Dilute the required amount of DNA sample in GenDNA TE and transfer DNA sample into appropriate sonication tubes (see below).
- 3.2 Shear DNA by sonication using the Bioruptor. Choose the protocol and consumables which are adapted to your device.

CAUTION: Only use the recommended tubes for high quality results.

- When using the **Bioruptor Pico** (Cat. No. B01060010) use 0.65 ml Bioruptor Microtubes (Cat. No. C30010011) and the holder (Cat. No. B01200051) with the 0.65 ml adaptor (Cat. No. B01200054) and shear your 100 µl samples for 8 cycles (sonication parameters: 15" ON and 90" OFF).

NOTE: For a volume of sample below 100 µl, use 0.2 ml Microtubes for Bioruptor Pico (Cat. No. C30010020) and the holder (Cat. No. B01200051) with the 0.2 ml adaptor (Cat. No. B01200053) for 16 samples, then follow the protocol <https://www.diagenode.com/files/protocols/protocol-dna-shearing-tubes-02ml.pdf>

- When using the **Bioruptor Plus** (Cat. No. B01020001) use Diagenode 0.5 ml Microtubes (Cat. No. C30010013) with the 0.5 ml tube holder (Cat. No. B01200043) and shear your 100 µl samples for 6 cycles or 12 minutes (sonication parameters: 30" ON and 90" OFF).

STEP 4

Methylated DNA immunoprecipitation



3-4

Day 3-4



45 minutes, overnight

BEADS PREPARATION

- 4.1** Determine the number of IP reactions to be run. It is recommended to perform two IPs per DNA sample. Input will not undergo immunoprecipitation and is therefore not considered as an IP.
- 4.2** Prepare **MagBuffer A 1x** (bead wash buffer) as described in the table below. The volumes contain a sufficient excess.

| | 1 IP | 2 IPs | 3 IPs | 4 IPs | 5 IPs | 6 IPs | 7 IPs | 8 IPs |
|-------------------------|-------|--------|--------|--------|--------|--------|--------|--------|
| MagBuffer A (5x) | 20 µl | 40 µl | 60 µl | 80 µl | 100 µl | 120 µl | 140 µl | 160 µl |
| Water | 80 µl | 160 µl | 240 µl | 320 µl | 400 µl | 480 µl | 560 µl | 640 µl |

| | 9 IPs | 10 IPs | 11 IPs | 12 IPs | 13 IPs | 14 IPs | 15 IPs | 16 IPs |
|-------------------------|--------|--------|--------|--------|---------|---------|---------|---------|
| MagBuffer A (5x) | 180 µl | 200 µl | 220 µl | 240 µl | 260 µl | 280 µl | 300 µl | 320 µl |
| Water | 720 µl | 800 µl | 880 µl | 960 µl | 1040 µl | 1120 µl | 1200 µl | 1280 µl |

- 4.3** Take the required amount of **Magnetic beads (Magbeads)** and transfer it to a clean 1.5 ml tube. **11 µl of beads** are needed **per IP**.

NOTE: Keep beads in liquid suspension at all handling steps, as drying will result in reduced performance.

- 4.4** Place the tube on the DiaMag1.5 to discard the supernatant. Keep the beads.
- 4.5** Wash the magnetic beads (Magbeads) twice with ice-cold MagBuffer

A 1x (bead wash buffer). To wash the beads, add the required volume of MagBuffer A 1x directly to the beads: **27.5 µL of MagBuffer A 1x** are needed **per IP**. Resuspend the beads by pipetting up and down several times, spin the tubes and place them in the DiaMag1.5. Wait for **1 minute** to allow the beads to be captured by the magnet and remove the supernatant.

- 4.6** After washing, resuspend the beads in **22 µl of MagBuffer A 1x** per IP. Keep on ice.

CAUTION: Do not freeze the beads.

DNA IMMUNOPRECIPITATION

- 4.7** In a new 1.5 ml tube, prepare the Mag master mix as described in the following table. The volumes also include all inputs needed (1 input/IP) and contain a sufficient excess.

| | 1 IP | 2 IPs | 3 IPs | 4 IPs | 5 IPs | 6 IPs | 7 IPs | 8 IPs |
|-------------------------------|--------|---------|---------|---------|---------|---------|---------|---------|
| MagBuffer A 5x* | 24 µl | 55 µl | 83 µl | 110 µl | 138 µl | 166 µl | 193 µl | 221 µl |
| MagBuffer B | 6,0 µl | 13,8 µl | 20,7 µl | 27,6 µl | 34,5 µl | 41,4 µl | 48,3 µl | 55,2 µl |
| meDNA spike-in control | 1,5 µl | 3,5 µl | 5,2 µl | 6,9 µl | 8,6 µl | 10,4 µl | 12,1 µl | 13,8 µl |
| unDNA spike-in control | 1,5 µl | 3,5 µl | 5,2 µl | 6,9 µl | 8,6 µl | 10,4 µl | 12,1 µl | 13,8 µl |

| | 9 IPs | 10 IPs | 11 IPs | 12 IPs | 13 IPs | 14 IPs | 15 IPs | 16 IPs |
|-------------------------------|---------|---------|---------|---------|---------|---------|----------|----------|
| MagBuffer A 5x* | 248 µl | 276 µl | 304 µl | 331 µl | 359 µl | 386 µl | 414 µl | 442 µl |
| MagBuffer B | 62,1 µl | 69 µl | 75,9 µl | 82,8 µl | 89,7 µl | 96,6 µl | 103,5 µl | 110,4 µl |
| meDNA spike-in control | 15,5 µl | 17,3 µl | 19 µl | 20,7 µl | 22,4 µl | 24,2 µl | 25,9 µl | 27,6 µl |
| unDNA spike-in control | 15,5 µl | 17,3 µl | 19 µl | 20,7 µl | 22,4 µl | 24,2 µl | 25,9 µl | 27,6 µl |

* Contains detergent; if its appearance is cloudy and crystallized please warm gently prior to use.

4.8 In 1.5 ml tubes, prepare the incubation mix as described in the table below.

| | Volume per 1 IP + 1 input | Volume per 2 IPs + 1 input |
|--------------------------|---------------------------|----------------------------|
| Mag master mix | 33 µl | 66 µl |
| Sheared DNA (100 ng/µl)* | 12 µl | 24 µl |
| Water | 45 µl | 90 µl |
| TOTAL volume | 90 µl | 180 µl |

* If the DNA sample is not at a concentration of 100 ng/µl, adjust the volume of water and DNA.

- For 1 IP + 1 input 1.2 µg of DNA is required and the total volume of incubation mix must remain 90 µl
- For 2 IPs + 1 input 2.4 µg of DNA is required and the total volume of incubation mix must remain 180 µl

4.9 Incubate the incubation mix at 95°C for 3 minutes.

4.10 Quickly chill the incubation mix on ice. Perform a pulse spin to consolidate your sample.

4.11 Take out **7.5 µl (that will be your 10% input)** from each tube and transfer to a new labeled 0.2 ml tube (or 8tube-strip). Keep the input sample at 4°C: it is to be used as a control of starting material and it is therefore not to be used in IP.

4.12 Then, transfer **75 µl of incubation mix** for each IP into one 200 µl tube (using the provided 200 µl tube strips or individual 200 µl tubes that fit in the DiaMag02). Keep at 4°C.

4.13 In a new tube, dilute the antibody 1:2 with water (e.g. mix 1 µl of antibody with 1 µl of water).

NOTES:

- Discard remaining master mixes not used on the day.
- Do not omit the dilution step as the amount of antibody to be used is critical.

4.14 Prepare the diluted antibody mix as described in the following table.

The volumes contain a sufficient excess. **Mix the diluted antibody 1:2, MagBuffer A 5x and water first and add MagBuffer C at the end.**

| | 1 IP | 2 IPs | 3 IPs | 4 IPs | 5 IPs | 6 IPs | 7 IPs | 8 IPs |
|-----------------------|---------|----------|----------|----------|----------|----------|----------|----------|
| Antibody 1:2 | 0.30 µl | 0.75 µl | 1.05 µl | 1.50 µl | 1.80 µl | 2.10 µl | 2.40 µl | 3.00 µl |
| MagBuffer A 5x | 0.60 µl | 1.50 µl | 2.10 µl | 3.00 µl | 3.60 µl | 4.20 µl | 4.80 µl | 6.00 µl |
| Water | 2.10 µl | 5.25 µl | 7.35 µl | 10.50 µl | 12.60 µl | 14.70 µl | 16.80 µl | 21.00 µl |
| MagBuffer C | 2.00 µl | 5.00 µl | 7.00 µl | 10.00 µl | 12.00 µl | 14.00 µl | 16.00 µl | 20.00 µl |
| TOTAL volume | 5.00 µl | 12.50 µl | 17.50 µl | 25.00 µl | 30.00 µl | 35.00 µl | 40.00 µl | 50.00 µl |

| | 9 IPs | 10 IPs | 11 IPs | 12 IPs | 13 IPs | 14 IPs | 15 IPs | 16 IPs |
|-----------------------|----------|----------|----------|----------|----------|----------|----------|-----------|
| Antibody 1:2 | 3.30 µl | 3.60 µl | 3.90 µl | 4.20 µl | 4.50 µl | 4.80 µl | 5.40 µl | 6.00 µl |
| MagBuffer A 5x | 6.60 µl | 7.20 µl | 7.80 µl | 8.40 µl | 9.00 µl | 9.60 µl | 10.80 µl | 12.00 µl |
| Water | 23.10 µl | 25.20 µl | 27.30 µl | 29.40 µl | 31.50 µl | 33.60 µl | 37.80 µl | 42.00 µl |
| MagBuffer C | 22.00 µl | 24.00 µl | 26.00 µl | 28.00 µl | 30.00 µl | 32.00 µl | 36.00 µl | 40.00 µl |
| TOTAL volume | 55.00 µl | 60.00 µl | 65.00 µl | 70.00 µl | 75.00 µl | 80.00 µl | 90.00 µl | 100.00 µl |

4.15 Add **5 µl of diluted antibody mix** to each 200 µl IP tube containing the incubation mix (from point 4.12)

4.16 Mix and add **20 µl of washed Magbeads** to each 200 µl IP tube (from point 4.15). The final volume is 100 µl.

NOTE: Keep the beads homogenously in suspension at all times when pipetting. Variation in the amount of beads will lead to lower reproducibility.

4.17 Place on a rotating wheel at 4°C overnight.

DNA WASHES

- 4.18** Place the MagWash buffers and the DiaMag02 on ice.
- 4.19** Spin down and place the IP tubes in the ice-cold the DiaMag02, wait **1 minute** and discard the buffer.
- 4.20** Wash the DNA IP Samples 3 times as follows. Add per tube, **100 µl ice-cold MagWash Buffer-1**, 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 and place in the DiaMag02. Wait **1 minute** and discard the buffer. Keep the captured beads.
- NOTE:** 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 DiaMag02.*
- 4.21** Wash the beads once with **100 µl ice-cold MagWash Buffer-2**.
- 4.22** After the last wash, discard the last traces of Wash Buffer and keep the bead pellets on ice. The bound DNA can be now purified from Magbeads.

STEP 5

Methylated DNA isolation

 Day 4  45 minutes

-
- 5.1 Take the input samples, centrifuge briefly and from now onwards treat the input samples and IP samples in parallel.
 - 5.2 Prepare **100 µl DNA Isolation Buffer (DIB)** per sample. Add **1 µl of Proteinase K** per **100 µl of DIB**. Scale accordingly knowing that 100 µl are needed per IP'ed sample and 92.5 µl per input sample.
 - 5.3 Remove the tubes from the DiaMag02 and add **100 µl of DIB** per IP'ed sample. Resuspend the beads.
 - 5.4 Add **92.5 µl** of DIB to each « 7.5 µL input sample » from step 4.11.
 - 5.5 Incubate at 55°C for **15 minutes** both IP'ed samples and input samples.
 - 5.6 Incubate all the samples at 100°C for **15 minutes**.
 - 5.7 Spin down and place the tubes into the DiaMag02.
 - 5.8 Transfer the supernatants containing DNA in new labeled tubes, discard Magbeads. This isolated DNA is ready for qPCR analysis. Make aliquots of this isolated DNA, store at -20°C.

STEP 6

qPCR analysis



The MagMeDIP qPCR Kit includes four validated primer pairs:

1. meDNA primer pair (for meDNA spike-in ctrl) (5 μ M each)
2. unDNA primer pair (for unDNA spike-in ctrl) (5 μ M each)
3. Human TSH2B (also known as HIST1H2BA) primer pair (5 μ M each) (methylated region)
4. Human GAPDH primer pair (5 μ M each) (unmethylated region)

NOTE: Primer pairs for mouse and rat are available! For more information please visit www.diagenode.com

- 6.1** Prepare your qPCR mix using a SYBR Green PCR master mix and start qPCR.

NOTES:

- We recommend performing qPCR in duplicates or triplicates.
- Depending on the sensitivity of the Master Mix and qPCR cyclers used, the samples dilution before PCR and the volume per PCR may vary. The IP'ed and input samples can be diluted up to 10x in water when needed.

- 6.2** Example of qPCR mix:

| Reagents | Volume for 1 reaction |
|-------------------------------------|-----------------------|
| Primer pair (Stock: 5 μ M each) | 1 μ l |
| 2x SYBR Green PCR master mix | 12.5 μ l |
| IP'ed DNA | 5 μ l |
| Nuclease-free water | 6.5 μ l |
| TOTAL volume | 25 μ l |

6.3 Use the following qPCR program:

| Step | Temperature | Time & Cycles | |
|----------------------|---|---------------|-------------|
| 1. Denaturation* | 95°C | 7 minutes | |
| 2. PCR Amplification | 95°C | 15 seconds | X 40 cycles |
| | 60°C | 60 seconds | |
| | 95°C | 1 minute | |
| 3. Melting curve** | Follow qPCR instrument manufacturer recommendations | | |

*Please check carefully supplier's recommendations about Taq polymerase activation time and temperature.

**Include and inspect the melting curves to ensure that primer pairs amplify only a single specific product.

6.4 Data analysis. Some major advices how to analyse qPCR results are given below.

Data interpretation

The efficiency of methylated DNA immunoprecipitation of a particular genomic locus can be calculated from qPCR data and reported as a recovery of starting material using the following formula:

$$\% \text{ recovery} = 2^{[Ct(10\% \text{ input}) - 3.32 - Ct(IP \text{ sample})]} \times 100$$

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

NOTE: This equation assumes that the qPCR is 100% efficient (amplification efficiency = 2). The real amplification efficiency, if known, should be used.

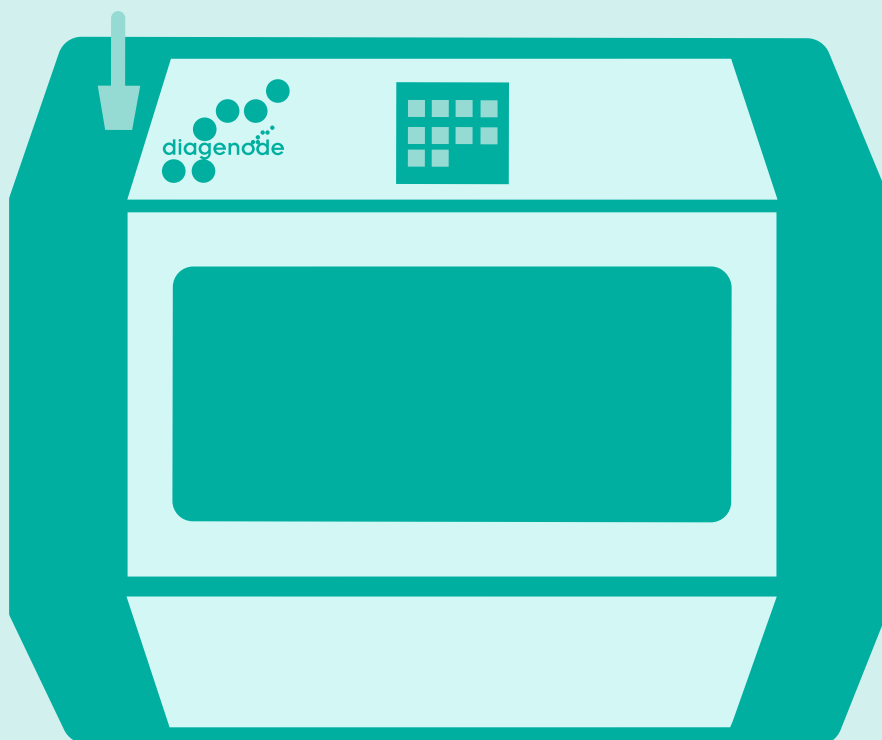
Background determination

The final goal of IP is to calculate the enrichment in the same IP sample of:

- The specific DNA fragments (corresponding to the methylated DNA) in comparison with background (corresponding to unmethylated DNA).
- This enrichment can be calculated as a ratio of specific signal over background.

$$\text{enrichment} = \% \text{ recovery (specific locus)} / \% \text{ recovery (background locus)}$$

AUTO PROTOCOL



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AUTOMATED PROCESSING

Protocol for Auto MagMeDIP qPCR using the IP-Star Compact Automated System

The Auto MagMeDIP qPCR Kit has been optimized on the IP-Star Compact Automated System (Cat. No. B03000002) for higher reproducibility.

If you have an older version of the IP-Star, please, contact us at customer.support@diagenode.com for the corresponding Auto MagMeDIP qPCR manual.

STEP 1

Cell collection & lysis



Day 1



30 minutes, overnight

Starting material: cultured cells

NOTE: For recommendations about starting amounts of material, please refer to section “Remarks before starting” (page 10)

- 1.6 Pellet suspension culture out of its serum containing medium or trypsinize adherent cells and collect cells from the flask. Centrifuge at 300g for 5 minutes at 4°C.
 - 1.7 Discard the supernatant. Resuspend cells in 5 to 10 ml ice-cold **PBS**. Count cells. Centrifuge at 300g for 5 minutes. Discard the supernatant. Repeat this resuspension and centrifugation step once more. This step is to wash the cells.
 - Meanwhile, place the GenDNA Digestion Buffer at room temperature and the GenDNA Proteinase K on ice (to be used at Point 1.3 below).
- NOTE:** If needed, cell pellets can be fresh frozen at this step and stored at -80°C for several months.
- 1.8 Prepare the complete Digestion buffer by adding 5 µl **GenDNA Proteinase K** to 1 ml **GenDNA Digestion Buffer**.
 - 1.9 Resuspend cells in complete Digestion Buffer.
 - For 1 to 1.5 million cells, use up to 500 µl **complete Digestion Buffer**
 - It might be necessary to use more buffer to avoid viscosity when performing the extractions
 - 1.10 Incubate the samples with shaking (500 rpm) at 50°C for 12 to 18 hours in tightly capped tubes.

STEP 2

Nucleic acid extraction & purification

 Day 2  5 hours

-
- 2.1** Thoroughly extract the samples with an equal volume of phenol/chloroform/isoamyl alcohol (work under a fume-hood).
- Add **1 volume** (500 µl according to step 1.4) of **phenol/chloroform/isoamyl alcohol (25:24:1)**.
 - Incubate the samples at room temperature for **10 minutes** on a rotating wheel. Use gentle rotation, do not vortex.
- 2.2** Centrifuge at 1700g for **10 minutes**.
- If the phases do not resolve properly, add another volume of GenDNA Digestion Buffer omitting Proteinase K, and repeat the centrifugation.
 - If there is a thick layer of white material at the interface between the phases, repeat the extraction.
- 2.3** Transfer the aqueous (top) layer to a new tube.
- Increase volume to avoid viscosity if necessary and pipette slowly.
- 2.4** Thoroughly extract the samples with **500 µl of chloroform/isoamyl alcohol (one volume)**.
- 2.5** Incubate for **10 minutes** at room temperature.
- 2.6** Centrifuge at 1700g for **10 minutes**.
- 2.7** Transfer aqueous layer to a new 2 ml tube.

2.8 Add **250 µl of GenDNA precipitant** (which is ½ volume) and **1 ml of 100% ethanol** (2 volumes).

2.9 Recover gDNA by centrifugation at 1700g for **5 minutes**.

- Do not use higher speed to avoid genomic DNA fragmentation
- This brief precipitation in the presence of an optimized high salt precipitant (GenDNA precipitant) reduces the amount of RNA in the DNA sample. For long-term storage, it is convenient to leave the DNA in the presence of ethanol.

2.10 Rinse the pellet with **70% ethanol**. Decant ethanol and air-dry the pellet.

- It is important to rinse extensively to remove any residual salt or phenol

2.11 Resuspend the pellet of gDNA at **~1 mg/ml in GenDNA TE** until dissolved. Shake gently at room temperature or at 65°C for several hours to facilitate solubilization. Store at 4°C.

- From 1-1.5 million cells, ~8 to 12 µg of gDNA can be expected (in a volume of 8 to 12 µl)
- From 3 million cells, ~20 to 30 µg of gDNA can be expected (in a volume of 20 to 30 µl)

NOTE: At this step the DNA quality can be analyzed by electrophoresis on 0.8% agarose gel or with a Fragment Analyzer (Agilent) and Standard Sensitivity Genomic DNA Analysis Kit (DNF-487, Agilent) (please refer to manufacturer's instructions).

2.12 If present, residual RNA has to be removed at this step by adding **2 µl of GenDNA RNase (DNase-free)** per ml of DNA sample and incubating 1 hour at 37°C, followed by phenol/chloroform extraction and ethanol precipitation (same protocol as above, starting from step 2.1).

2.13 Store the gDNA at 4°C until the shearing.

NOTE: For long term storage the gDNA can be placed at -20°C.

STEP 3

DNA shearing



Genomic DNA must be randomly sheared by sonication on Diagenode's Bioruptor to generate fragments around 400 bp (see Figure 1 in "Example of results" section). To perform the MagMeDIP, 1.2 µg of sheared DNA is needed in a volume smaller than 55 µl.

- 3.1 Dilute the required amount of DNA sample in GenDNA TE and transfer DNA sample into appropriate sonication tubes (see below).
- 3.2 Shear DNA by sonication using the Bioruptor. Choose the protocol and consumables which are adapted to your device.

CAUTION: Only use the recommended tubes for high quality results.

- When using the **Bioruptor Pico** (Cat. No. B01060010) use 0.65 ml Bioruptor Microtubes (Cat. No. C30010011) and the holder (Cat. No. B01200051) with the 0.65 ml adaptor (Cat. No. B01200054) and shear your 100 µl samples for 8 cycles (sonication parameters: 15'' ON and 90'' OFF).

NOTE: For a volume of sample below 100 µl, use 0.2 ml Microtubes for Bioruptor Pico (Cat. No. C30010020) and the holder (Cat. No. B01200051) with the 0.2 ml adaptor (Cat. No. B01200053) for 16 samples, then follow the protocol <https://www.diagenode.com/files/protocols/protocol-dna-shearing-tubes-02ml.pdf>

- When using the **Bioruptor Plus** (Cat. No. B01020001) use Diagenode 0.5 ml Microtubes (Cat. No. C30010013) with the 0.5 ml tube holder (Cat. No. B01200043) and shear your 100 µl samples for 6 cycles or 12 minutes (sonication parameters: 30'' ON and 90'' OFF).



STEP 4

Methylated DNA immunoprecipitation



3-4

Day 3-4

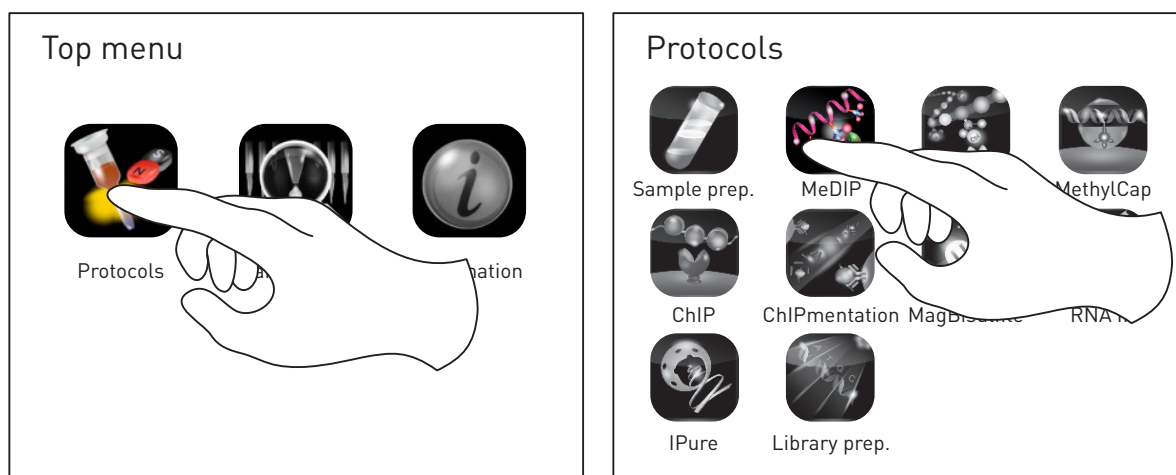


1 hour hands on time and overnight run

A- Protocol Set-up

4.1 Switch ON the IP-Star Compact.

4.2 Select “**Protocols**” icon and then “**MeDIP**” category.



4.3 Select “**MeDIP_8_DIB**” if you plan to run between 1 and 8 samples or “**MeDIP_16_DIB**” if you plan to run between 9 and 16 samples.

4.4 Set up the exact number of samples for your experiment by pressing the black box. This number includes each IP and it is recommended to perform the IP in duplicates. Input will not undergo immunoprecipitation in the IP-Star and is therefore not considered as a sample.

NOTE: The Peltier block is now cooling down to 4°C to keep your samples cold.

- 4.5** Setup the parameters for your MagMeDIP experiment and press “Next”.

Recommended parameters:

Configuration

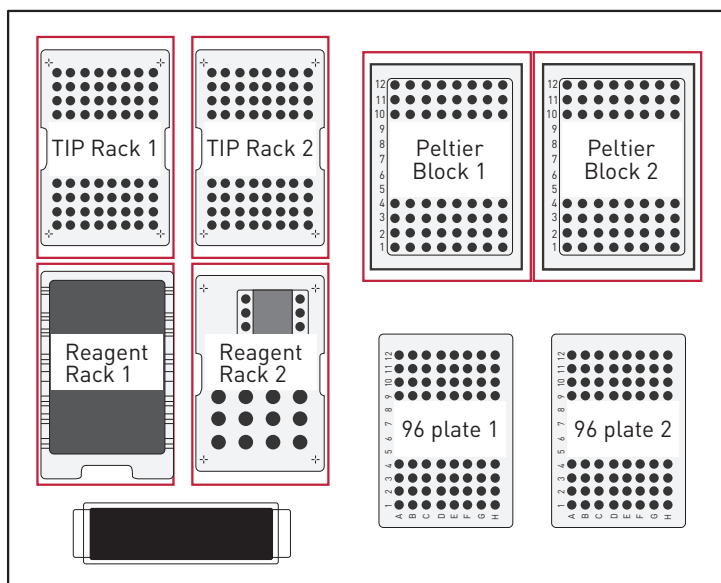
Save Parameter

| | Mixing time | Temperature | Mix speed |
|--------------|--|--|---|
| IP reaction: | <div style="border: 1px solid black; padding: 2px 10px;">14</div> h <small>0.1 - 15 hours</small> | <div style="border: 1px solid black; padding: 2px 10px;">4</div> °C <small>4 - 37 degrees</small> | <div style="border: 1px solid black; padding: 2px 10px;">middle</div> |
| Washes: | <div style="border: 1px solid black; padding: 2px 10px;">5</div> h <small>0.1 - 30 min</small> | <div style="border: 1px solid black; padding: 2px 10px;">4</div> °C <small>4 - 37 degrees</small> | <div style="border: 1px solid black; padding: 2px 10px;">middle</div> |

Back

Next

- 4.6** Setup all the plastic consumables on the platform according to the screen layout



- 4.7** Fill TIP Rack 1 (and 2 if processing more than 8 samples) with tips according to the screen.
- 4.8** Fill **Reagent Racks 1 & 2** with Reagent containers according to the screen.
- 4.9** Fill **Peltier Block 1** (and Block 2 if processing more than 8 samples) with strips according to the screen.

NOTE: All the rows of the Peltier(s) Block(s) must be filled with a strip.

- 4.10** Prepare the mixes and fill the strips with your samples and the reagents from the kit as described below. Make sure that the liquid is at the bottom of each well.

B- Prepare Reagent and Mixes

- 4.11** In a new 1.5 ml tube, prepare MagBuffer A 1x (bead wash buffer) as described in the table below. The volumes contain a sufficient excess. Keep the reagent on ice.

| | 1 IP | 2 IPs | 3 IPs | 4 IPs | 5 IPs | 6 IPs | 7 IPs | 8 IPs |
|-------------------------|--------|--------|--------|--------|--------|--------|--------|--------|
| MagBuffer A (5x) | 50 µl | 80 µl | 110 µl | 130 µl | 160 µl | 190 µl | 210 µl | 240 µl |
| Water | 200 µl | 320 µl | 440 µl | 520 µl | 640 µl | 760 µl | 840 µl | 960 µl |

| | 9 IPs | 10 IPs | 11 IPs | 12 IPs | 13 IPs | 14 IPs | 15 IPs | 16 IPs |
|-------------------------|---------|---------|---------|---------|---------|---------|---------|---------|
| MagBuffer A (5x) | 260 µl | 290 µl | 320 µl | 340 µl | 370 µl | 400 µl | 420 µl | 450 µl |
| Water | 1040 µl | 1160 µl | 1280 µl | 1360 µl | 1480 µl | 1600 µl | 1680 µl | 1800 µl |

- 4.12** In a new 1.5 ml tube, prepare the Mag master mix as described in the following table. The volumes also include all inputs needed (1 input/IP) and contain a sufficient excess. Keep the mix on ice.

| | 1 IP | 2 IPs | 3 IPs | 4 IPs | 5 IPs | 6 IPs | 7 IPs | 8 IPs |
|-------------------------------|--------|---------|---------|---------|---------|---------|---------|---------|
| MagBuffer A 5x* | 24 µl | 55 µl | 83 µl | 110 µl | 138 µl | 166 µl | 193 µl | 221 µl |
| MagBuffer B | 6,0 µl | 13,8 µl | 20,7 µl | 27,6 µl | 34,5 µl | 41,4 µl | 48,3 µl | 55,2 µl |
| meDNA spike-in control | 1,5 µl | 3,5 µl | 5,2 µl | 6,9 µl | 8,6 µl | 10,4 µl | 12,1 µl | 13,8 µl |
| unDNA spike-in control | 1,5 µl | 3,5 µl | 5,2 µl | 6,9 µl | 8,6 µl | 10,4 µl | 12,1 µl | 13,8 µl |

| | 9 IPs | 10 IPs | 11 IPs | 12 IPs | 13 IPs | 14 IPs | 15 IPs | 16 IPs |
|-------------------------------|---------|---------|---------|---------|---------|---------|----------|----------|
| MagBuffer A 5x* | 248 µl | 276 µl | 304 µl | 331 µl | 359 µl | 386 µl | 414 µl | 442 µl |
| MagBuffer B | 62,1 µl | 69 µl | 75,9 µl | 82,8 µl | 89,7 µl | 96,6 µl | 103,5 µl | 110,4 µl |
| meDNA spike-in control | 15,5 µl | 17,3 µl | 19 µl | 20,7 µl | 22,4 µl | 24,2 µl | 25,9 µl | 27,6 µl |
| unDNA spike-in control | 15,5 µl | 17,3 µl | 19 µl | 20,7 µl | 22,4 µl | 24,2 µl | 25,9 µl | 27,6 µl |

* Contains detergent; if its appearance is cloudy and crystallized please warm gently prior to use.

4.13 In 1.5 ml tubes, prepare the incubation mix as described in the table below. The incubation mix is prepared in excess. 75 µl will be needed for the IP. Keep the mix on ice

| | Volume per 1 IP + 1 input | Volume per 2 IPs + 1 input |
|---------------------------------|---------------------------|----------------------------|
| Mag master mix | 33 µl | 66 µl |
| Sheared DNA (100 ng/µl)* | 12 µl | 24 µl |
| Water | 45 µl | 90 µl |
| TOTAL volume | 90 µl | 180 µl |

** If the DNA sample is not at a concentration of 100 ng/µl, adjust the volume of water and DNA.*

- For 1 IP + 1 input 1.2 µg of DNA is required and the total volume of incubation mix must remain 90 µl.
- For 2 IPs + 1 input 2.4 µg of DNA is required and the total volume of incubation mix must remain 180 µl.

4.14 Incubate the incubation mix at 95°C for 3 minutes.

4.15 Quickly chill the incubation mix on ice. Perform a pulse spin to consolidate your sample.

4.16 Take out **7.5 µl (that will be your 10% input)** from each tube and transfer to a new labeled 0.2 ml tube (or 8tube-strip). Keep the input sample at 4°C until step 5: it is to be used as a control of starting material and it is therefore not to be used in IP.

4.17 In a new tube, dilute the **antibody 1:2 with water (e.g. mix 1 µl of antibody with 1 µl of water)**. Keep on ice.

NOTES:

- Discard remaining master mixes not used on the day.
- Do not omit the dilution step as the amount of antibody to be used is critical.

4.18 Prepare the Diluted Antibody mix as described in the following table. The volumes contain a sufficient excess. **Mix the diluted antibody 1:2, MagBuffer A 5x and water first and add MagBuffer C at the end.** Keep the mix on ice.

| | 1 IP | 2 IPs | 3 IPs | 4 IPs | 5 IPs | 6 IPs | 7 IPs | 8 IPs |
|-----------------------|---------|----------|----------|----------|----------|----------|----------|----------|
| Antibody 1:2 | 0.30 µl | 0.75 µl | 1.05 µl | 1.50 µl | 1.80 µl | 2.10 µl | 2.40 µl | 3.00 µl |
| MagBuffer A 5x | 0.60 µl | 1.50 µl | 2.10 µl | 3.00 µl | 3.60 µl | 4.20 µl | 4.80 µl | 6.00 µl |
| Water | 2.10 µl | 5.25 µl | 7.35 µl | 10.50 µl | 12.60 µl | 14.70 µl | 16.80 µl | 21.00 µl |
| MagBuffer C | 2.00 µl | 5.00 µl | 7.00 µl | 10.00 µl | 12.00 µl | 14.00 µl | 16.00 µl | 20.00 µl |
| TOTAL volume | 5.00 µl | 12.50 µl | 17.50 µl | 25.00 µl | 30.00 µl | 35.00 µl | 40.00 µl | 50.00 µl |

| | 9 IPs | 10 IPs | 11 IPs | 12 IPs | 13 IPs | 14 IPs | 15 IPs | 16 IPs |
|-----------------------|----------|----------|----------|----------|----------|----------|----------|-----------|
| Antibody 1:2 | 3.30 µl | 3.60 µl | 3.90 µl | 4.20 µl | 4.50 µl | 4.80 µl | 5.40 µl | 6.00 µl |
| MagBuffer A 5x | 6.60 µl | 7.20 µl | 7.80 µl | 8.40 µl | 9.00 µl | 9.60 µl | 10.80 µl | 12.00 µl |
| Water | 23.10 µl | 25.20 µl | 27.30 µl | 29.40 µl | 31.50 µl | 33.60 µl | 37.80 µl | 42.00 µl |
| MagBuffer C | 22.00 µl | 24.00 µl | 26.00 µl | 28.00 µl | 30.00 µl | 32.00 µl | 36.00 µl | 40.00 µl |
| TOTAL volume | 55.00 µl | 60.00 µl | 65.00 µl | 70.00 µl | 75.00 µl | 80.00 µl | 90.00 µl | 100.00 µl |

C- Fill the strips with the reagents and the mixes

4.19 Distribute **10 µl of Magnetic beads (Magbeads)** in each well of row 3.

NOTE: Keep the beads homogenously in suspension at all times when pipetting. Variation in the amount of beads will lead to lower reproducibility.

4.20 Prepare the Immunoprecipitation Mix in a 200 µl IP-Star tube strip as described in the table below. Insert the strip in row 7.

| | 1IP (100 µl) |
|-------------------------|--------------|
| MagBuffer A (1x) | 20 µl |
| Incubation Mix | 75 µl |
| Antibody Mix | 5 µl |

NOTE: Before dispensing, mix the content in the tube by pipetting up and down.

4.21 Fill in reagent racks 1 & 2 with reagents according to the screen instructions.

4.22 Check the proper insertion of the racks and the consumables, and press “Next”.

- 4.23** Check the selected parameters, close the door and press “Run” to start.
- 4.24** MeDIP protocol is running. The “Remaining time” calculation will give you an estimation of the processing time of your experiment.

STEP 5

Methylated DNA isolation



Day 4



10 minutes hands on time and 40 minute run

5.1 The next morning, recover the sample tubes in row 12 (at 4°C).

NOTE: Remove all the plastics from the IP-Star.

5.2 Prepare the INPUT as described in the table below.

| 1 INPUT | |
|----------------------------|---------|
| Sheared DNA | 7.5 µl |
| DNA Isolation Buffer (DIB) | 92.5 µl |

From now onwards treat the input DNA samples and IP samples in parallel.

5.3 Add **1 µl of Proteinase K** in all IP samples and input samples.

5.4 Close the tubes with caps and insert them on the Peltier Block 1.

NOTE: The Peltier Block 1 heats evenly, so the strips can be placed in every row of this block.

5.5 Close the door and press “OK” to start the incubation.

5.6 The incubation is now starting. The “Remaining time” calculation will give you an estimation of the processing time.

- 5.7 When the protocol is completed, a screen appears telling you the run is over. Recover the sample tubes, spin down the tubes to bring down the liquid caught in the lid.
- 5.8 Place the tubes in the DiaMag02 (Cat. No. B04000001) and wait for 1 minute.
- 5.9 Transfer the supernatants containing DNA in new labeled tubes and discard Magbeads. This isolated DNA is ready for qPCR analysis. Make aliquots of this isolated DNA, store at -20°C.
- 5.10 Now back to the IP-Star screen, press “OK” and then “Yes” to start a new run or press “Back” until the homepage appears on the screen. Press “Shutdown” and wait until the screen is black before switching off the IP-Star.
- 5.11 Remove all the plastics from the IP-Star platform, empty the waste shuttle, and clean the inner side of the IP-Star with **70% ethanol**.

STEP 6

qPCR analysis



The MagMeDIP qPCR Kit includes four validated primer pairs:

1. meDNA primer pair (for meDNA spike-in ctrl) (5 μ M each)
2. unDNA primer pair (for unDNA spike-in ctrl) (5 μ M each)
3. Human TSH2B (also known as HIST1H2BA) primer pair (5 μ M each) (methylated region)
4. Human GAPDH primer pair (5 μ M each) (unmethylated region)

NOTE: Primer pairs for mouse and rat are available! For more information please visit www.diagenode.com

- 6.1** Prepare your qPCR mix using a SYBR Green PCR master mix and start qPCR.

NOTES:

- We recommend performing qPCR in duplicates or triplicates.
- Depending on the sensitivity of the Master Mix and qPCR cyclyer used, the samples dilution before PCR and the volume per PCR may vary. The IP'ed and input samples can be diluted up to 10x in water when needed

Example of qPCR mix:

| Reagents | Volume for 1 reaction |
|-------------------------------------|-----------------------|
| Primer pair (Stock: 5 μ M each) | 1 μ l |
| 2x SYBR Green PCR master mix | 12.5 μ l |
| IP'ed DNA | 5 μ l |
| Nuclease-free water | 6.5 μ l |
| TOTAL volume | 25 μ l |

6.2 Use the following qPCR program:

| Step | Temperature | Time & Cycles | |
|----------------------|---|---------------|-------------|
| 1. Denaturation* | 95°C | 7 minutes | |
| 2. PCR Amplification | 95°C | 15 seconds | X 40 cycles |
| | 60°C | 60 seconds | |
| | 95°C | 1 minute | |
| 3. Melting curve** | Follow qPCR instrument manufacturer recommendations | | |

*Please check carefully supplier's recommendations about Taq polymerase activation time and temperature.

**Include and inspect the melting curves to ensure that primer pairs amplify only a single specific product.

6.3 Data analysis. Some major advices how to analyse qPCR results are given below.

Data interpretation

The efficiency of methylated DNA immunoprecipitation of particular genomic locus can be calculated from qPCR data and reported as a recovery of starting material using the following formula:

$$\% \text{ recovery} = 2^{[(Ct(10\% \text{ input}) - 3.32) - Ct(IP \text{ sample})]} \times 100$$

In this example, 2 is the amplification efficiency, Ct (IP sample) and Ct (10% input) are threshold values obtained from exponential phase of qPCR for the IP sample and input sample respectively; the compensatory factor (3.32) is used to take into account the dilution 1:10 of the input.

NOTE: This equation assumes that the qPCR is 100% efficient (amplification efficiency = 2). For accurate results, the amplification efficiency of the primer pairs has to be close to 100% meaning that for each cycle the amount of product doubles ($E=2$). The real amplification efficiency, if known, should be used.

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 methylated DNA) in comparison with background (corresponding to unmethylated DNA).
- This enrichment can be calculated as a ratio of specific signal over background.

$$\text{enrichment} = \% \text{ recovery (specific locus)} / \% \text{ recovery (background locus)}$$

Example of results

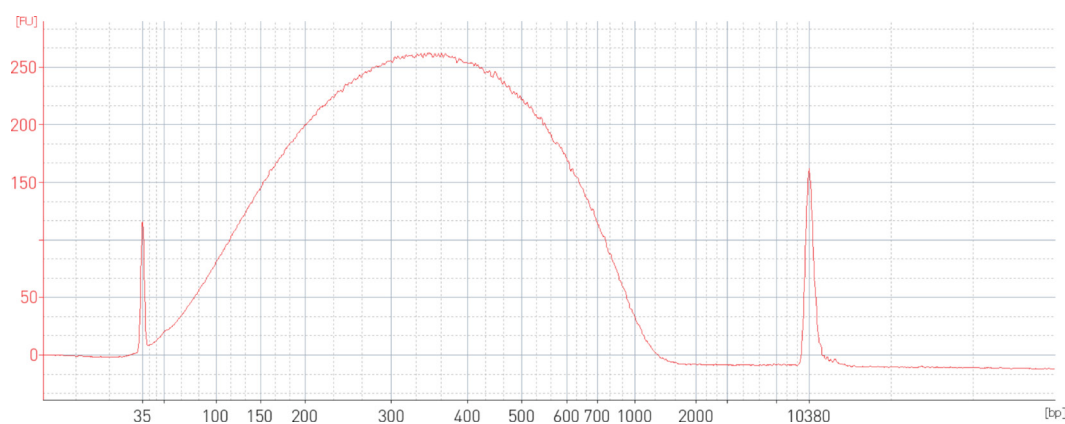


Figure 1. Agilent High Sensitivity DNA chip profile of sheared genomic DNA: smear around 400 bp. The genomic DNA was diluted in GenDNA TE to reach a concentration of 100 ng/μl and 100 μl were sheared in a 0.65 ml Bioruptor Microtube (Cat. No. C30010011). The following program was used: 8 Cycles: [15 seconds “ON” & 90 seconds “OFF”].

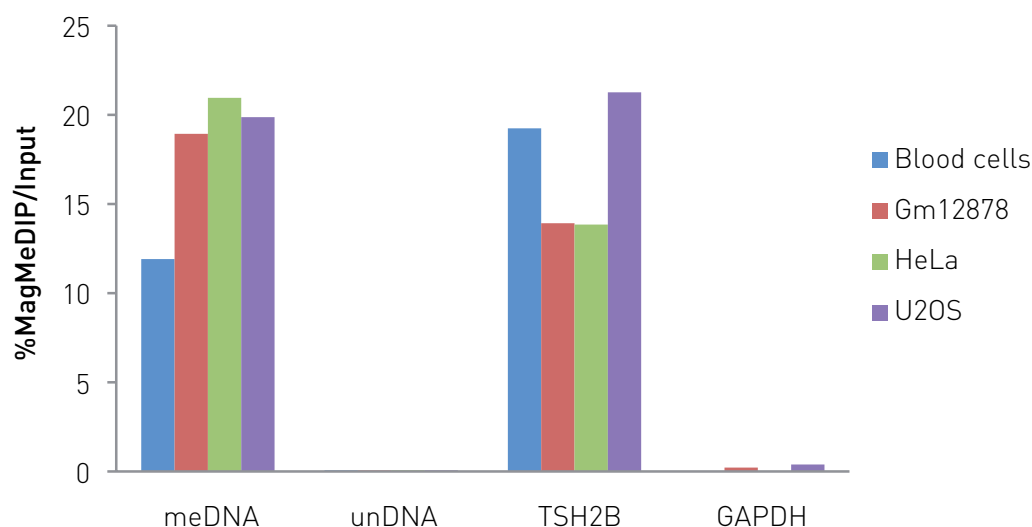


Figure 2. IP results obtained with Diagenode MagMeDIP qPCR Kit

MeDIP assays were performed manually using DNA from blood, Gm12878, HeLa and U20S cells and the MagMeDIP qPCR kit (Diagenode). The DNA was prepared with the XL GenDNA Extraction Module. The IP was performed including the kit meDNA and unDNA spike-in controls, together with the human DNA sample controls. The DNA was isolated/purified using DIB. Afterwards, qPCR was performed using the primer pairs included in this kit.

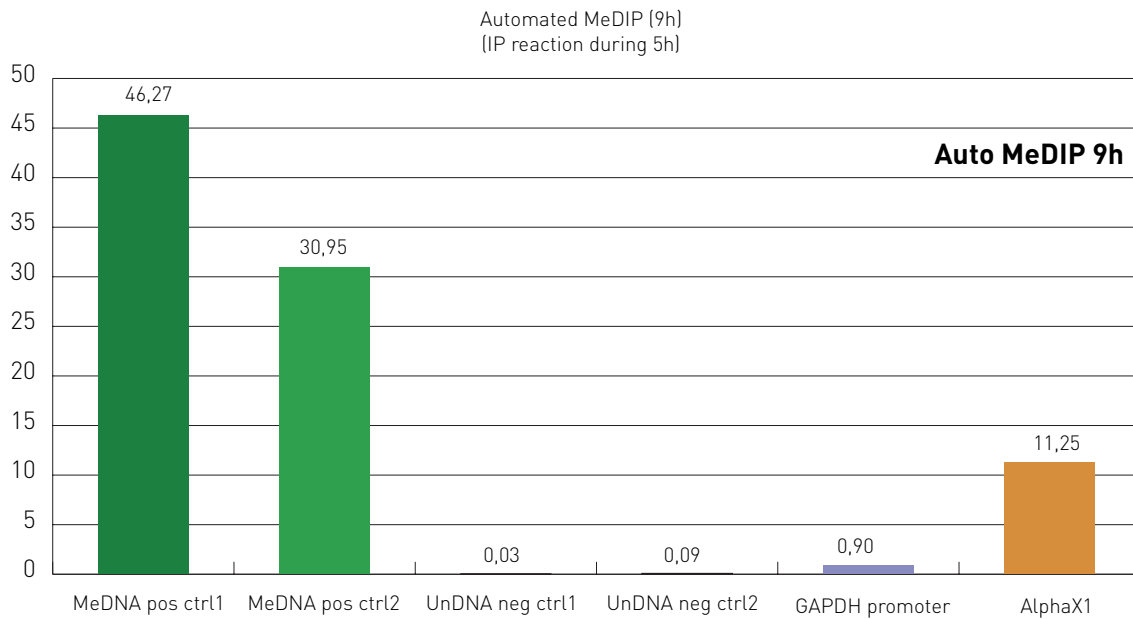


Figure 3. Automated MeDIP (9h)

IP reaction was performed with the anti-5-meC antibody. Methylated and unmethylated DNA were used as internal controls. Unmethylated DNA region of GAPDH and a methylated DNA region of AlphaX1 were used to test DNA sample-IP efficiency. DNA has been isolated by using DNA Isolation Buffer.

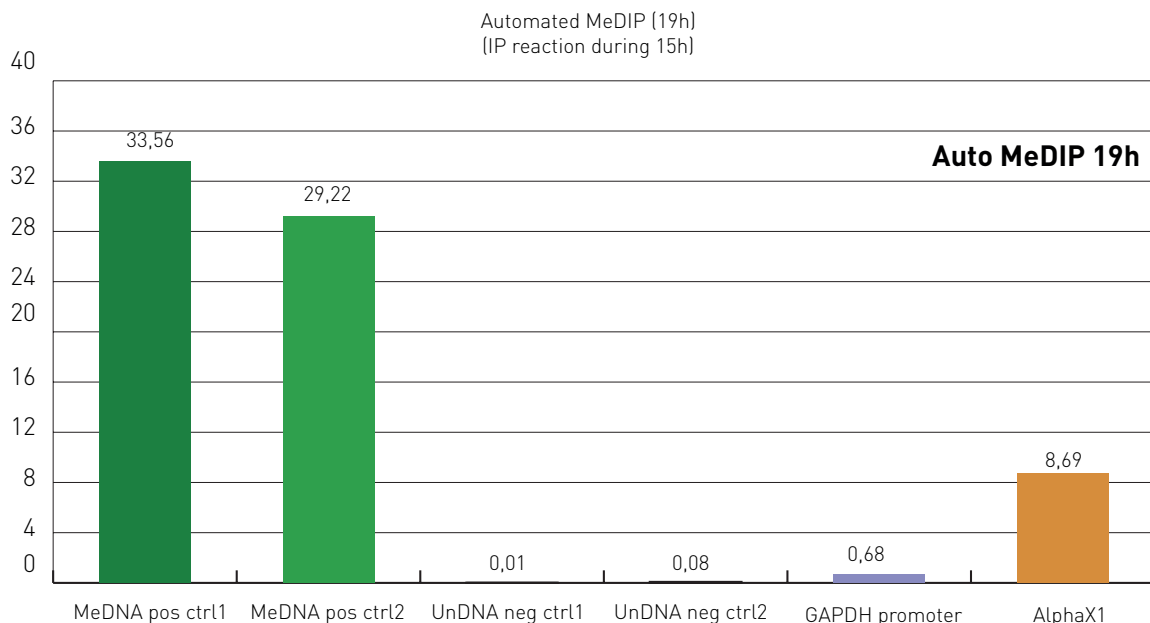


Figure 4. Automated MeDIP (19h)

The IP reaction was performed with the anti-5-mC antibody. Methylated and unmethylated DNA were used as internal controls. Unmethylated DNA region of GAPDH and a methylated DNA region of AlphaX1 were used to test DNA sample-IP efficiency. DNA has been isolated by using DNA Isolation Buffer.

FAQs

What is the resolution for MagMeDIP?

The resolution is related to the size of the fragments. Usually the sheared DNA is a smear of 100-600 bp which means that the resolution is also 100-600 bp.

Would it be possible to obtain genomic DNA by other means and start using the kit at the DNA shearing step? Can we clean up the DNA by column, to minimize the contact with phenol?

The XL GenDNA extraction module allows the extraction of high molecular weight DNA, but you can definitely use columns instead of the double phenol/chloroform extraction, as long as you are confident with the quality of the genomic DNA you will get. The most important is to obtain pure and high molecular weight DNA. You can choose which DNA extraction protocol you want to use to reach this goal and then start using the MagMeDIP qPCR kit from the immunoprecipitation step.

I will shear the DNA using the Bioruptor Pico. How do I choose the right tubes?

For the shearing, the size of the tubes is not important as long as you use the recommended volume according to each format and the recommended tubes. To help you with DNA shearing we have developed a tool available at <https://www.diagenode.com/en/dna-shearing-guide> that contains all the information needed to choose the best shearing protocol for DNA.

Can I shear DNA using an enzyme for MagMeDIP DNA preparation?

As long as you obtain DNA fragments from 100-600 bp, you can use the fragmentation technique of your choice. We usually do not recommend using restriction enzymes because the fragmentation is less random compared to sonication.

Can I use more than 1 µg DNA for the MeDIP? Will it increase the overall yield?

It is possible to use more DNA, but the amount of antibody may need to be adjusted, requiring further optimization without a guarantee of improved output. Therefore, we recommend following the standard and optimized protocol. To increase the output we suggest to process several samples with 1 µg and to pool them at the end.

What is the lowest DNA amount that we can investigate with your MagMeDIP qPCR kit?

We recommend using at least 100 ng of sheared DNA per IP.

Can I use another antibody with the kit?

This kit has been validated with the provided antibody only - Cat. No. C15200081, therefore we do not recommend changing the antibody. Moreover not all antibodies are compatible with the beads which are included in the kit.

What is the reference of the antibody used in the MagMeDIP qPCR kit? Which amount of antibody per IP should I use?

The antibody used is the Cat. No. C15200081. To ensure efficient IP it is important to use the diluted antibody as described in the protocol. A lack of antibody can result in low IP efficiency whereas a large excess of antibody might lead to lower specificity.

What are the internal controls that can be added to the incubation mix during MeDIP? Is this a required step?

The internal spike-in controls allow you to check the immunoprecipitation efficiency of a positive and a negative template in the same tube as your DNA of interest. The purpose of these controls is to confirm antibody specificity. It is better to add them to every sample, but you can also choose to add a spike-in control to only one sample per experimental condition.

You can find the full description of the spike-in controls for MagMeDIP

qPCR in the datasheet of the DNA Methylation Control Package here: https://www.diagenode.com/files/products/kits/Datasheet_DNA_methylation_control_package.pdf

Is it possible to buy other spike-in controls tubes or primers? If possible, are they the same as in the initial MagMeDIP qPCR kit?

Yes, the spike-in controls and their associated primers are available in the DNA Methylation Control Package: <https://www.diagenode.com/en/p/dna-methylation-control-package-40-rxns>. The controls and primers from the DNA Methylation Control Package are exactly the same as in the MagMeDIP qPCR kit.

What are the lengths of the PCR products?

Here are the lengths of the PCR products obtained with the different primer pairs:

- TSH2B: 170 pb
- unDNA: 92 pb
- GAPDH: 64 pb
- meDNA1 : 81 pb

How do I design my own qPCR primers?

We recommend the following parameters for qPCR primers:

- Length: 18 to 24 nucleotides
- T_m: 60°C (+/- 3.0°C)
- % GC: 50% (+/- 4%)

I did not obtain a Ct value for the negative control GAPDH. Which value should I use for the calculation?

The absence of a qPCR signal for GAPDH after the MeDIP, means that the IP was highly specific since GAPDH signal is a measurement of the background. Therefore it already shows that the background is very low. If you want to put a Ct value you can apply the maximal number of qPCR cycles performed (usually 40).

At which step can I freeze my samples and stop safely?

Samples can be frozen at several steps of the protocol:

- After DNA extraction (step 2)
- After DNA shearing (step 3)
- After isolation of IP'ed DNA (step 6)

How can I troubleshoot errors on the IP-Star Compact?

Regarding the questions related to the IP-Star Compact, please refer to the troubleshooting guide and the list of error codes pages 29-31 of the [SX-8G IP-Star Compact manual](#).

Related products

| Product | Cat. No. |
|--|-----------|
| 5-methylcytosine (5-mC) monoclonal antibody 33D3 | C15200081 |
| XL GenDNA Extraction Module | C03030020 |
| IPure kit v2 | C03010015 |
| DNA Methylation control package | C02040012 |
| iDeal Library Preparation Kit x24 | C05010020 |
| DiaMag 0.2ml - magnetic rack | B04000001 |
| DiaMag 1.5ml - magnetic rack | B04000003 |
| DiaMag rotator | B05000001 |
| Bioruptor® Pico sonication device | B01060010 |
| IP-Star® Compact Automated System | B03000002 |

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