diagenode

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 3 10_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

Contents

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.

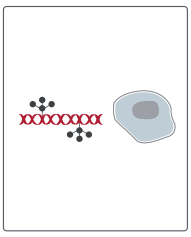
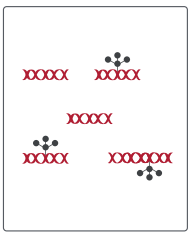
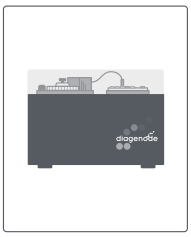
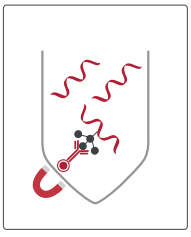
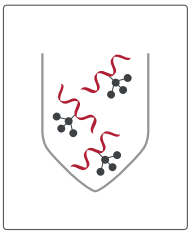
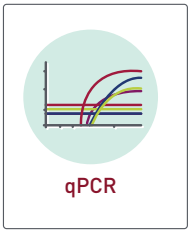
The kit ensures the use of a low amount of antibodies and buffers per reaction. The number of steps is reduced and handling is easier which makes this kit cost effective and simple to work with.

The MagMeDIP qPCR Kit can be used with DNA from every species.

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

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 µL	750 µL	4°C Do not freeze
Nuclease-free water	2x 2 mL	10 mL	4°C
MagBuffer A (5x concentrated)	2 mL	10 mL	4°C
MagBuffer B	100 µL	500 µL	4°C
MagBuffer C	40 µL	200 µL	-20°C
Antibody anti-5mC* (33D3 clone)	5 µL	25 µL	-80°C
Methylated spike-in control	6 µL	28 µL	-20°C
Unmethylated spike-in control	6 µL	28 µL	-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 µL	200 µL	-20°C
Primer pair for Methylated spike-in ctrl (5 µM each)	50 µL	50 µL	-20°C
Primer pair for Unmethylated spike-in ctrl (5 µM each)	50 µL	50 µL	-20°C
Human TSH2B primer pair (5 µM each)	50 µL	50 µL	-20°C
Human GAPDH primer pair (5 µM each)	50 µL	50 µL	-20°C
200 µL tube strips	2	8	RT
Cap strips	2	8	RT
GenDNA Digestion Buffer	3 mL	3 mL	4°C
GenDNA Proteinase K (200x)	300 µg/15 µL	300 µg/15 µL	-20°C
GenDNA precipitant	3 mL	3 mL	4°C
GenDNA TE	3 mL	3 mL	4°C
GenDNA RNAse (DNAse free)	5 µg/10 µL	5 µg/10 µL	-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

- Diagenode magnetic racks:
  - DiaMag1.5 (Cat. No. B04000003)
  - DiaMag02 (Cat. No. B04000001)
- 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
- 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)

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

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

Additionnal supplies (included and available separately)

Component	Cat. No.	Format
Human meDNA primer pair (TSH2B)	C17011041-500	500 µL
Human unDNA primer pair (GAPDH)	C17011047-500	500 µL
XL GenDNA Extraction Module	C03030020	500 µL
Antibody anti-5-mC (33D3 clone)	C15200081	10/100/500 µg

Optional supplies (not included and available separately)

Component	Cat. No.	Format
DNA Methylation control package	C02040012	40 rxns
Mouse meDNA primer pair (TSH2B)	C17021042-500	500 µL
Mouse unDNA primer pair (GAPDH)	C17021045-500	500 µL
Rat meDNA primer pair (TSH2B)	C17031043-500	500 µL
Rat unDNA primer pair (GAPDH)	C17031046-500	500 µL

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.

Methylated and unmethylated spike-in controls

The MagMeDIP qPCR Kit contains one methylated and one unmethylated spike-in control that can be added directly to DNA samples before the IP. Those two spike-in controls are not homologous to any model species and will not interfere with the sample of interest.

To check the efficiency of the MeDIP experiment, this kit also includes primer pairs (targeting methylated and unmethylated spike-in controls' regions) to calculate their recovery by qPCR.

> ***CAUTION**: If you use the controls provided with the separate **DNA Methylation control package (C02040012)** as spike-in controls, please note that they can interfere with plant species. Please check FAQ section p.45*

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. Primers for control regions in mouse and rat can be purchased separately (see **Optional supplies**, page 9) 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 two solutions :

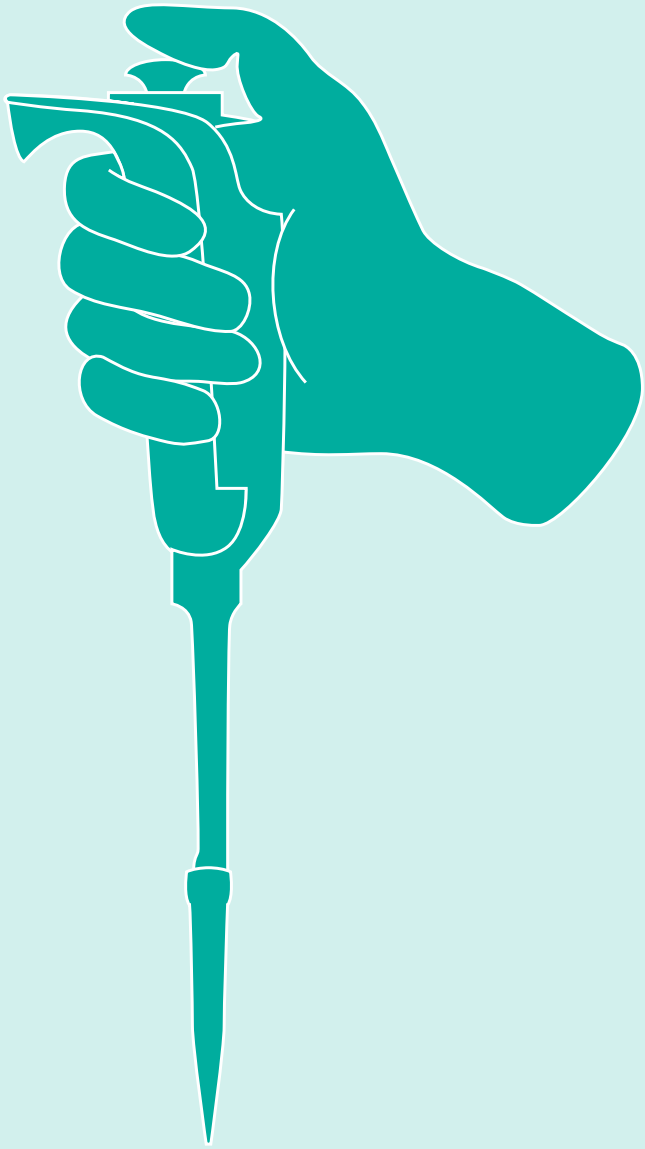
- Using our MagMeDIP-seq Package (Cat. No. C02010040) containing everything you need from gDNA extraction to library preparation.

OR

- Combine your actual MagMeDIP qPCR Kit (Cat. No. C02010020) in combination with Diagenode's iDeal Library Preparation Kit (Cat. No. C05010020) and IPure kit v2 (C03010014).

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

PROTOCOL



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

2 *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

3 *Day 3* *2 hours*

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
Methylated spike-in control	0.5 µL	1.2 µL	1.7 µL	2.3 µL	2.9 µL	3.5 µL	4.0 µL	4.6 µL
Unmethylated spike-in control	0.5 µL	1.2 µL	1.7 µL	2.3 µL	2.9 µL	3.5 µL	4.0 µL	4.6 µL
Nuclease-free water	2.0 µL	4.6 µL	6.9 µL	9.2 µL	11.5 µL	13.8 µL	16.1 µL	18.4 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
Methylated spike-in control	5.2 µL	5.8 µL	6.3 µL	6.9 µL	7.5 µL	8.1 µL	8.6 µL	9.2 µL
Unmethylated spike-in control	5.2 µL	5.8 µL	6.3 µL	6.9 µL	7.5 µL	8.1 µL	8.6 µL	9.2 µL
Nuclease-free water	20.7 µL	23.0 µL	25.3 µL	27.6 µL	29.9 µL	32.2 µL	34.5 µL	36.8 µL

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

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

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

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

4.10 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.

4.11 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.

4.12 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.*

4.13 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

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

4.15 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.*

4.16 Place on a rotating wheel at 4ºC overnight.

DNA WASHES

4.17 Place the MagWash buffers and the DiaMag02 on ice.

4.18 Spin down and place the IP tubes in the ice-cold the DiaMag02, wait 1 minute and discard the buffer.

4.19 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.*

4.20 Wash the beads once with **100 µL ice-cold MagWash Buffer-2**.

4.21 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

Day 4  *› 2 hours*

The MagMeDIP qPCR Kit includes four validated primer pairs:

1. Primer pair for Methylated spike-in ctrl (5µM each)
2. Primer pair for Unmethylated spike-in ctrl (5µM each)
3. Human TSH2B (also known as HIST1H2BA) primer pair (5µM each) (methylated region)
4. Human GAPDH primer pair (5µM 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 cycler 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 µM each)	1 µL
2x SYBR Green PCR master mix	12.5 µL
IP'ed DNA	5 µL
Nuclease-free water	6.5 µL
TOTAL volume	25 µL

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:

% recovery= 2^[Ct(10% input) - 3.32 - Ct(IP sample)] x 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.*

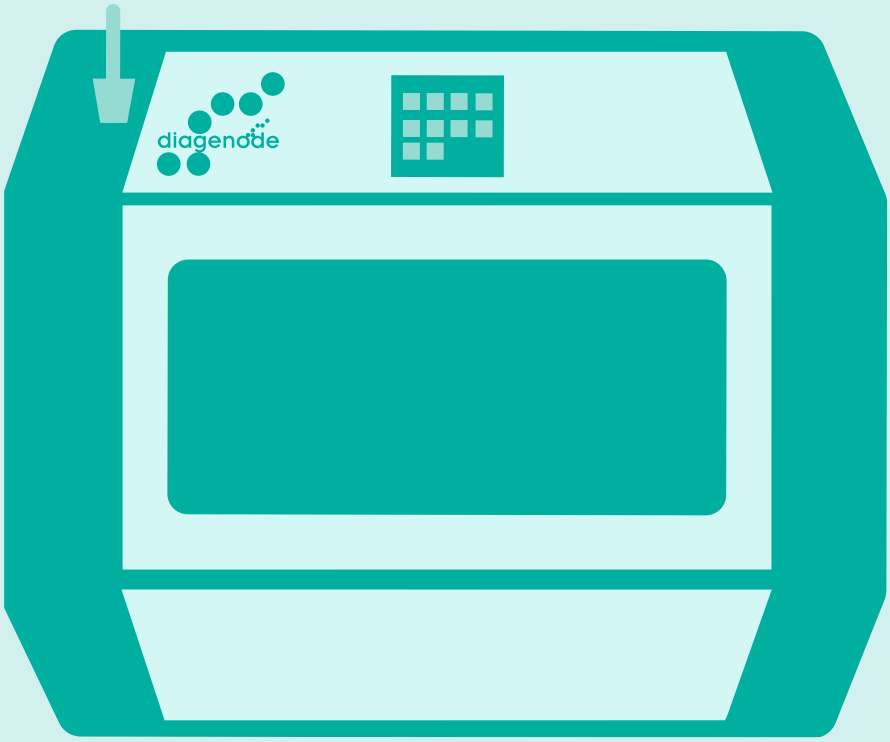
Background determination

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.

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

AUTO PROTOCOL



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

1 *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

2 *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

3 *Day 3* *2 hours*

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).

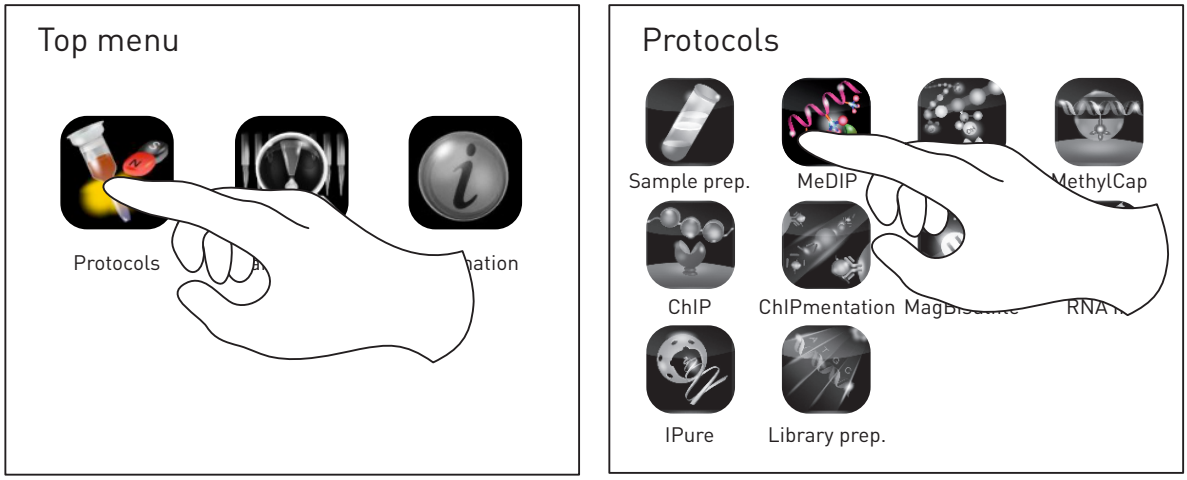
STEP 4

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.

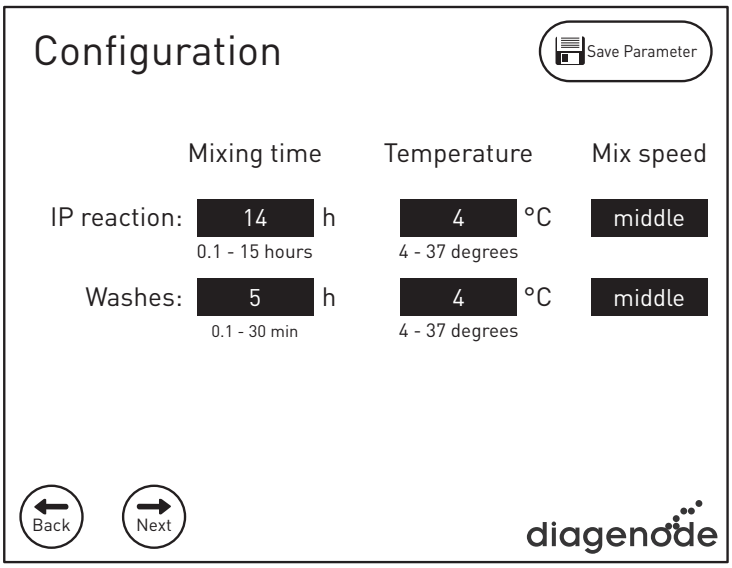
4.3 Select "**MeDIP_8_DIB**" if you plan to run between 1 and 8 samples or "**MeDIP_16_DIB**" if you plan to run between 9 and 16 samples.

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.

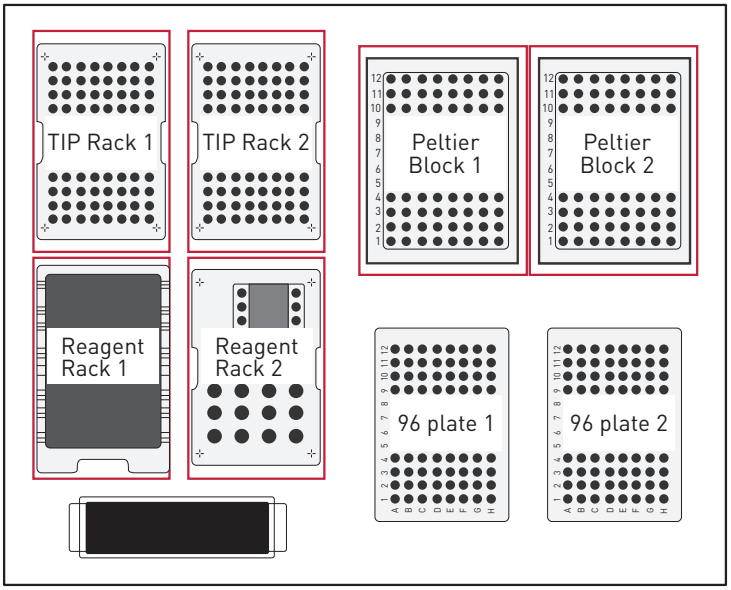
***NOTE**: The Peltier block is now cooling down to 4°C to keep your samples cold.*

4.5 Setup the parameters for your MagMeDIP experiment and press "Next".

Recommended parameters:



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
Methylated spike-in control	0.5 µL	1.2 µL	1.7 µL	2.3 µL	2.9 µL	3.5 µL	4.0 µL	4.6 µL
Unmethylated spike-in control	0.5 µL	1.2 µL	1.7 µL	2.3 µL	2.9 µL	3.5 µL	4.0 µL	4.6 µL
Nuclease-free water	2.0 µL	4.6 µL	6.9 µL	9.2 µL	11.5 µL	13.8 µL	16.1 µL	18.4 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
Methylated spike-in control	5.2 µL	5.8 µL	6.3 µL	6.9 µL	7.5 µL	8.1 µL	8.6 µL	9.2 µL
Unmethylated spike-in control	5.2 µL	5.8 µL	6.3 µL	6.9 µL	7.5 µL	8.1 µL	8.6 µL	9.2 µL
Nuclease-free water	20.7 µL	23.0 µL	25.3 µL	27.6 µL	29.9 µL	32.2 µL	34.5 µL	36.8 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

Day 4 *› 2 hours*

The MagMeDIP qPCR Kit includes four validated primer pairs:

1. Primer pair for Methylated spike-in ctrl (5µM each)
2. Primer pair for Unmethylated spike-in ctrl (5µM each)
3. Human TSH2B (also known as HIST1H2BA) primer pair (5µM each) (methylated region)
4. Human GAPDH primer pair (5µM 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 cycler 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 µM each)	1 µL
2x SYBR Green PCR master mix	12.5 µL
IP'ed DNA	5 µL
Nuclease-free water	6.5 µL
TOTAL volume	25 µL

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:

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

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

Example of results

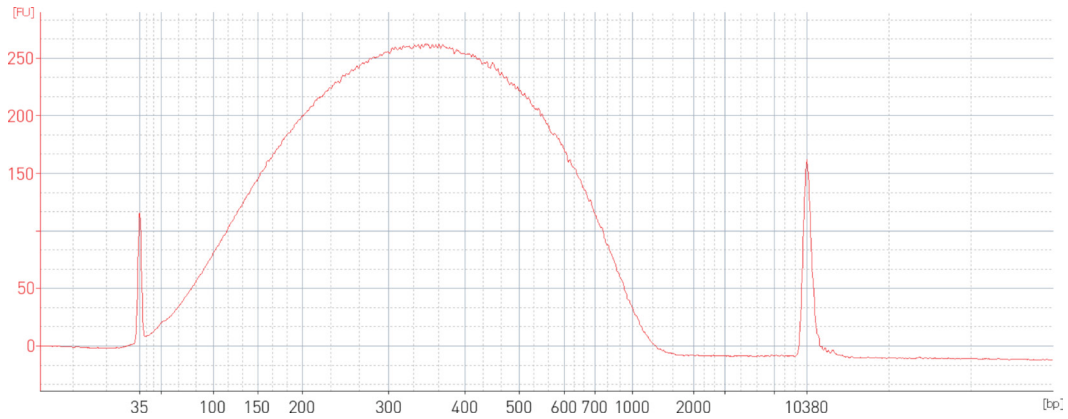


Figure 1. *Agilent High Sensitivity DNA chip profile of sheared genomic DNA: smear around 400 bp. The genomic DNA was diluted in GenDNA TE to reach a concentration of 100 ng/µL and 100 µL were sheared in a 0.65 mL Bioruptor Microtube (Cat. No. C30010011). The following program was used: 8 Cycles: [15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

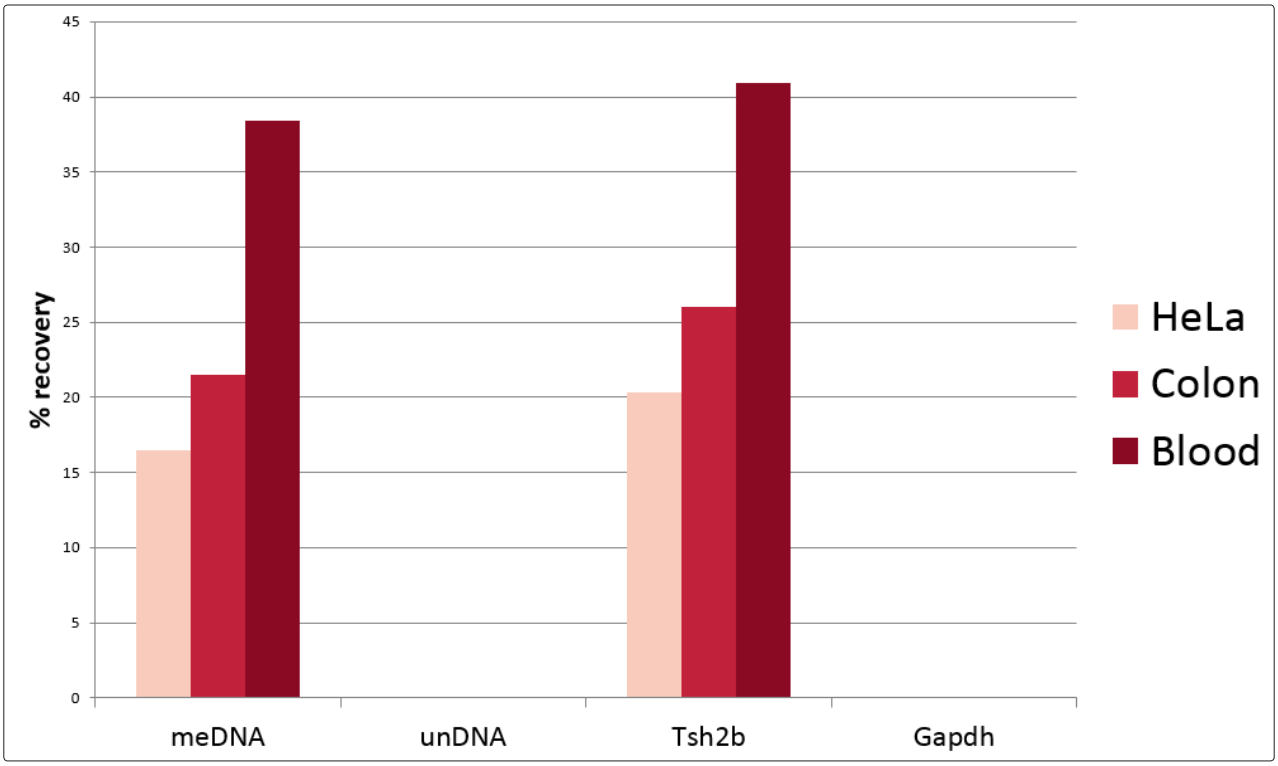


Figure 2. *Immunoprecipitation results obtained with Diagenode MagMeDIP qPCR Kit*

MeDIP assays were performed manually using DNA from HeLa, colon and blood cells with the MagMeDIP qPCR kit (Diagenode). The IP was performed with Methylated and Unmethylated spike-in controls included in the kit, 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.

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.

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?

If you need more spike-in controls, you can buy the **DNA Methylation Control Package (Cat. No. C02040012)** which provides methylated and unmethylated controls and their associated primers. They are produced from the genome of *Arabidopsis thaliana* and may therefore interfere with plant samples. They were validated with MagMeDIP qPCR Kit and are compatible with every other sample species.

Please check the product datasheet on the website for more information: https://www.diagenode.com/en/p/dna-methylation-control-package-40-rxns.

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
- GAPDH: 64 pb
- unmethylated spike-in control: 110 pb
- methylated spike-in control: 103 pb

How do I design my own qPCR primers?

We recommend the following parameters for qPCR primers:

- Length: 18 to 24 nucleotides
- Tm: 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

Equipment	Cat. No.
DiaMag 1.5mL - magnetic rack	B04000003
DiaMag 0.2mL - magnetic rack	B04000001
DiaMag rotator	B05000001
Bioruptor® Pico sonication device	B01060010
IP-Star® Compact Automated System	B03000002
Product	**Cat. No.**
5-methylcytosine (5-mC) monoclonal antibody (33D3)	C15200081
XL GenDNA Extraction Module	C03030020
DNA Methylation control package	C02040012
Human meDNA primer pair (TSH2B)	C17011041-500
Human meDNA primer pair (GAPDH)	C17011047-500
Mouse meDNA primer pair (TSH2B)	C17021042-500
Mouse meDNA primer pair (GAPDH)	C17021045-500
Rat meDNA primer pair (TSH2B)	C17031043-500
Rat meDNA primer pair (GAPDH)	C17031046-500
IPure kit v2	C03010015
iDeal Library Preparation Kit x24	C05010020

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