diagenode

A Hologic Company

MagMeDIP Kit

Magnetic Methylated DNA Immunoprecipitation Kit

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

USER GUIDE

Version 5 05_2023

Contents

The MagMeDIP 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

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 Kit takes advantage of a specific antibody targeting this 5-mC to immunoprecipitated methylated DNA, which can be thereafter directly analyzed by qPCR or Next-Generation Sequencing (NGS). This methylation analysis is fast and highly specific as the immunoprecipitation (IP) assay 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 IP-Star Compact Automated System.

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

The kit includes:

- A XL GenDNA Extraction Module to prepare large amounts of DNA from cultured cells
- Protocol for DNA shearing using Diagenode's 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
- Reagents for fast DNA isolation after IP for qPCR application

We recommend Diagenode’s magnetic rack DiaMag 0.2ml (Cat. No. B04000001) together with our MagMeDIP 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.

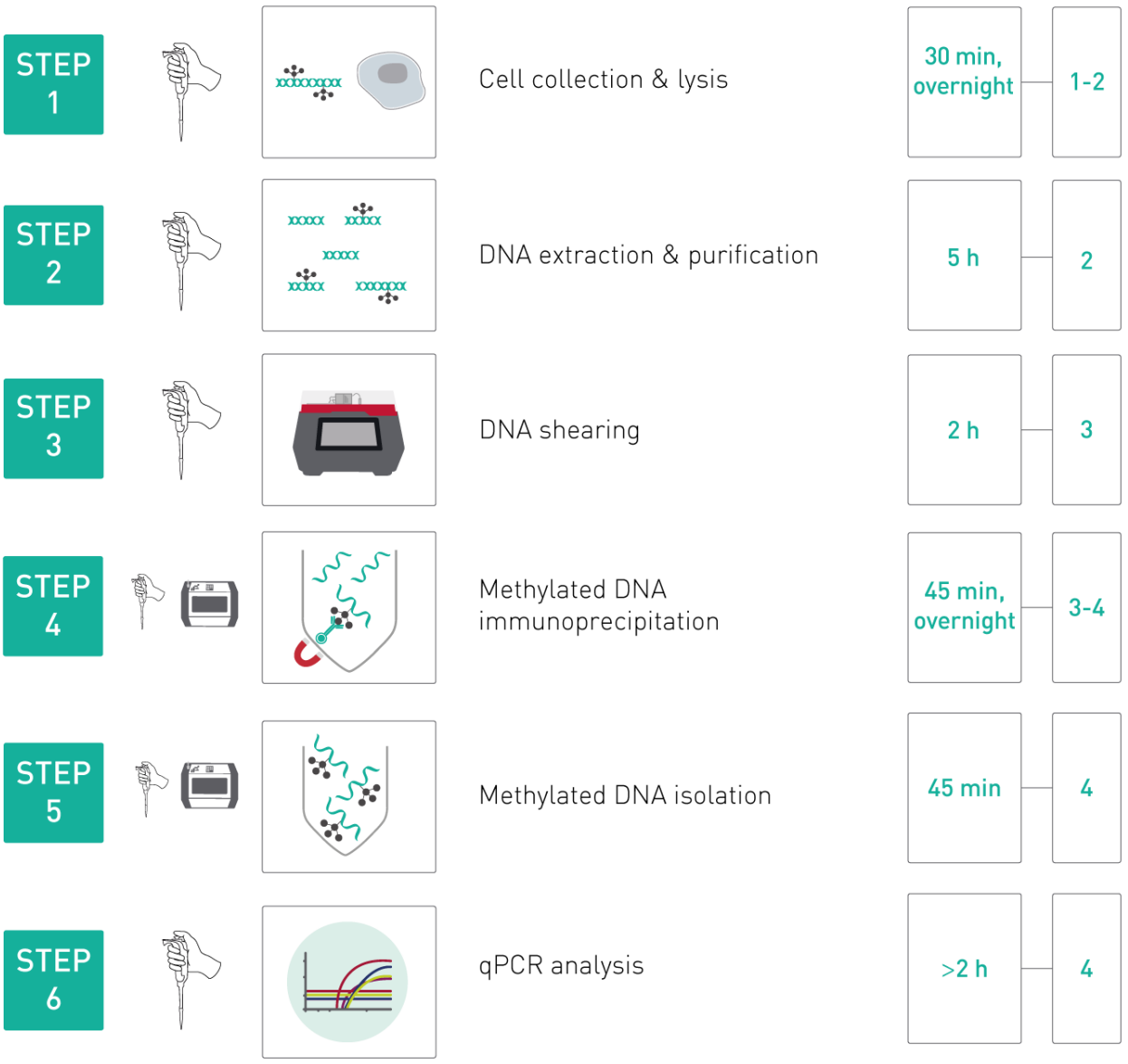
The MagMeDIP Kit can be used with DNA from every species.

Diagenode's 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 their corresponding qPCR primer pairs
- Easy to use with user-friendly magnetic beads and rack
- Highly validated protocols compatible with qPCR and NGS downstream analyses
- Automated protocol supplied

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 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 MagMeDIP Kit

Component	Qty (x10)	Qty (x48)	Storage
Magbeads (magnetic beads)	150 µl	750 µl	4°C Do not freeze
Nuclease-free water	4 ml	10 ml	4°C
5x MagBuffer A **	2 x 1 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 (5µM each)	50 µl	288 µl	-20°C
Primer pair for Unmethylated spike-in ctrl (5µM each)	50 µl	288 µl	-20°C
200 µl tube strips (8 tubes/strip)	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

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

** Avoid freeze-thawing cycles of this very sensitive antibody by aliquoting it before storage at -80°C.*

*** The composition of two buffers (5x MagBuffer A and MagWash buffer-1) has been slightly modified. The performance of the kit has been thoroughly tested and we confirm that this modification does not influence the kit performance. The change applies to all kits produced from December 2022 (lot 26 and the future lots).*

Required Materials Not Provided

Materials and Reagents

- Gloves to wear at all steps
- DNase-free 1.5 ml and 15 ml conical tubes
- Ice-cold PBS buffer
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol
- Ethanol

Equipment

- Diagenode magnetic rack: DiaMag 0.2ml (B04000001)
- Magnetic rack for 1.5 ml tubes
- Centrifuges for 1.5 ml tubes and 15 ml conical tubes (4°C)
- Microcentrifuge for 0.2 ml tubes
- Thermomixer (95°C)
- Thermocycler
- Cell counter
- Rotating wheel
- Diagenode Bioruptor® sonication device and the associated tube holders and tubes:
  - Bioruptor® Pico (Cat No. B01080010) or
  - Bioruptor® Plus (Cat No. B01020001)
- 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 automated protocol

Component	Cat. No.	Format
IP-Star Compact Automated System	B03000002	1 unit
200 µl tubes strips (8 tubes/strip) + cap strips	C30020002	120 pc
Tips (box)	C30040021	10 x 96 tips
Tips (bulk)	C30040020	1000 pc
2 ml microtubes	C30010014	100 pc
Medium reagent container	C30020003	10 pc
96 well microplates	C30080030	10 pc

Additional supplies (included and available separately)

Component	Cat. No.	Format
XL GenDNA Extraction Module	C03030020	6 rxns
Antibody anti-5-mC (33D3 clone)	C15200081	10/100/500 µg
DiaMag anti-mouse IgG coated magnetic beads	C03010022	1.5 ml
DNA methylation control package V2	C02040019	48 rxns

Optional supplies (not included and available separately)

Component	Cat. No.	Format
DNA Methylation control package	C02040012	40 rxns
Human TSH2B promoter primer pair (methylated region)	C17011041	50/500 µl
Human ChIP-seq grade GAPDH TSS primer pair (unmethylated region)	C17011047	50/500 µl
Mouse TSH2B coding region primer pair (methylated region)	C17021042	50/500 µl
Mouse GAPDH promoter primer pair (unmethylated region)	C17021045	50/500 µl
Rat TSH2B coding region primer pair (methylated region)	C17031043	50/500 µl
Rat GAPDH promoter +0.3 kb primer pair (unmethylated region)	C17031046	50/500 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

The MagMeDIP Kit can be used with DNA amounts ranging from 10 ng to 1 µg of sheared DNA 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
- At least 10 ng of sheared DNA is needed per IP. Please note that since low starting amount of DNA leads to higher background, we recommend using as much DNA as possible
- 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 for MeDIP-qPCR and 200 bp for MeDIP-sequencing. High quality shearing can be obtained with the Bioruptor Pico (Cat. No. B01080010) or the Bioruptor Plus (Cat. No. B01020001) following Diagenode's protocols. 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 number 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. We recommend including one input sample for each sheared DNA sample that undergoes MeDIP.

Methylated and unmethylated spike-in controls

The MagMeDIP Kit contains one methylated and one unmethylated spike-in controls 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 the primer pairs - targeting the methylated and unmethylated spike-in control regions - to calculate their recovery by qPCR.

> ***NOTE:*** *The spike-in controls and the primer pairs included in the MagMeDIP Kit are available separately within the* ***DNA methylation control package V2*** *(Cat. No. C02040019).*
>
> ***CAUTION:*** *The spike-in controls and the primer pairs provided in the separate* ***DNA methylation control package*** *(Cat. No. C02040012) are different from the ones included in the MagMeDIP Kit. Those spike-in controls are made from A. thaliana. Therefore, they can interfere with DNA samples derived from plant species.*

Technique for DNA isolation

The MagMeDIP Kit includes a DNA Isolation Buffer (DIB) for an easy and very fast DNA isolation after MeDIP providing DNA suitable for qPCR analysis. If DNA of higher purity is needed (e.g. for Next-Generation Sequencing), we recommend using the IPure kit v2 (Cat. No. C03010014). 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 – Control regions

This kit contains two primer pairs targeting the methylated and unmethylated spike-in controls to be used in qPCR to check the success of the immunoprecipitation in each experiment.
Primers for methylated (TSH2B, also known as H2BC1) and unmethylated (GAPDH) control regions in human, mouse and rat can be purchased separately (see Optional supplies, page 9). Endogenous regions likeTSH2B and GAPDH allow to check the IP success on the DNA sample rather than on the exogenous spike-in controls. This is therefore a useful tool but it requires a minimum amount of immunoprecipitated DNA to work properly. Consequently, depending on the sample type, it may be difficult to obtain accurate results when immunoprecipitation is performed on less than 100 ng of sheared genomic DNA. As cell-free

DNA is fragmented at a size close to the amplicon size, it is also not appropriate to run those qPCR on endogenous regions while using such template.
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 advised as for potential outliers' identification).

Quantitative PCR analysis – Regions of interest

To study DNA methylation level of a few loci of interest, MagMeDIP can be coupled with qPCR. The primer design is thus an important step of the procedure. Here are some guidelines and tools to design good primers:

- The length should be around 20 nucleotides.
- Self-complementarity and secondary structure of the primers can be tested during primer design (https://bioinfo.ut.ee/primer3/).
- Annealing temperature of 60°C (+/- 3.0ºC) is recommended.
- Short length of amplified DNA fragment (50 – 100 bp) facilitates the PCR efficiency and reduces potential problems in amplification of G/C-rich regions.
- Difference in melting temperature between forward and reverse primers should not exceed 2 to 3°C.
- G/C stretches at the 3' end of the primers should be avoided.
- A % GC of 50% (+/- 4%) is recommended.

After reception of candidate primer pairs a validation step is needed:

- Test primer sets by in silico PCR (http://genome.cse.ucsc.edu/cgi-bin/hgPcr). Primers should amplify unique DNA products from the genome.
- Test every primer set in qPCR using 10-fold serial dilutions of input DNA. Calculate amplification efficiency (AE) of primer set using the following formula: AE= 10^(-1 / slope). The ideal amplification factor is 2. If it is not the case the qPCR reagents from different brand or new primers should be tested.

MagMeDIP-sequencing (MagMeDIP-seq)

For DNA methylation analysis on the whole genome, MagMeDIP can be coupled with Next-Generation Sequencing. To perform MeDIP-sequencing we recommend the following strategy:

- Choose a library preparation solution which is compatible with the starting amount of DNA you are planning to use (from 10 ng to 1 µg). It can be a home-made solution or a commercial one.
- Choose the indexing system that fits your needs considering the following features:
  - Single-indexing, combinatorial dual-indexing or unique dual-indexing
  - Number of barcodes
  - Full-length adaptors containing the barcodes or barcoding at the final amplification step

- Presence / absence of Unique Molecular Identifiers (for PCR duplicates removal)

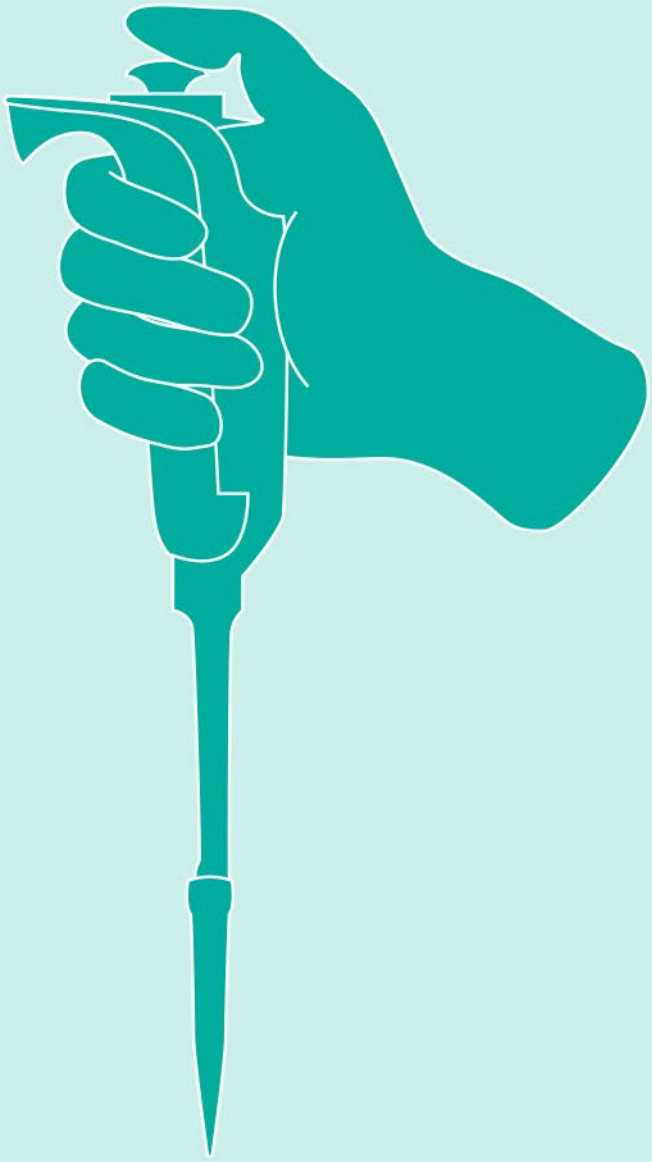
- Standard library preparation protocols are compatible with double-stranded DNA only, therefore the first steps of the library preparation (end repair, A-tailing, adaptor ligation and clean-up) will have to be performed on sheared DNA, before the IP.

  CAUTION: As the immunoprecipitation step occurs at the middle of the library preparation workflow, single-tube solutions for library preparation are usually not compatible with MeDIP-sequencing.

- For DNA isolation after the IP, we recommend using the IPure kit v2 (available separately, Cat. No. C03010014) instead of DNA isolation Buffer.

- Perform library amplification after the DNA isolation following the standard protocol of the chosen library preparation solution.

PROTOCOL



MANUAL PROCESSING

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".

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 300 x g 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 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

1.1 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.

1.2 Centrifuge at 1700 x g 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.

1.3 Transfer the aqueous (top) layer to a new tube.

- Increase volume to avoid viscosity if necessary and pipette slowly.

1.4 Thoroughly extract the samples with **500 µl of chloroform/isoamyl alcohol (one volume)**.

1.5 Incubate for 10 minutes at room temperature.

1.6 Centrifuge at 1700 x g for 10 minutes.

1.7 Transfer the aqueous layer to a new 2 ml tube.

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

1.9 Recover gDNA by centrifugation at 1700 x g 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.

1.10 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.

1.11 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.
- From 3 million cells, ~20 to 30 µg of gDNA can be expected.

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).*

1.12 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).

1.13 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 for qPCR application or 200bp for NGS application (see Figure 1 in "Example of results" section).

2.1 Switch on the Bioruptor and set the temperature of the cooler at 4°C.

2.2 Prepare the gDNA solution at concentration 2-100 ng/µl using GenDNA TE.

NOTE: The smear of a sample with a concentration below 2 ng/µl will not be detected by most instruments for quality control.

2.3 Transfer a required volume of DNA solution to the appropriate sonication microtubes:

Type	Sample volume	Microtubes	Tube holder
Bioruptor Pico	100 µl	0.65 ml Microtubes for Bioruptor Pico Cat. No. C30010011	Tube holder for 0.65 ml tubes - Bioruptor® Pico Cat. No. B01201143
	<100 µl	0.2 ml Microtubes for Bioruptor Pico Cat. No. C30010020	Tube holder for 0.2 ml tubes - Bioruptor® Pico Cat. No. B01201144
Bioruptor Plus	100 µl	0.5 ml Bioruptor Plus Microtubes (Cat. No. C30010013)	0.5/0.65 ml tube holder for Bioruptor® Standard & Plus Cat. No. B01200043

2.4 Briefly vortex (5-10 sec) and spin samples.

2.5 Incubate on ice for 10 minutes.

2.6 Shear gDNA by sonication using the Bioruptor. Choose the protocol which is adapted to your sonicator model, sample volume and target size as described in the below table:

Application	Target size	Bioruptor model	Sample volume	Tubes	Shearing protocol
MagMeDIP-qPCR	300-500 bp	Bioruptor Pico	100 µl	0.65 ml Microtubes for Bioruptor Pico	8 cycles 15 sec ON 90 sec OFF
	300-500 bp	Bioruptor Pico	50 µl	0.2 ml Microtubes for Bioruptor Pico	5 cycles 30 sec ON 30 sec OFF
	300-500 bp	Bioruptor Plus	100 µl	0.5 ml Bioruptor Plus Microtubes	6 cycles 30 sec ON 90 sec OFF
MagMeDIP-seq	200 bp	Bioruptor Pico	100 µl	0.65 ml Microtubes for Bioruptor Pico	13 cycles 30 sec ON 30 sec OFF
	200 bp	Bioruptor Pico	50 µl	0.65 ml Microtubes for Bioruptor Pico	13 cycles 30 sec ON 30 sec OFF
	200 bp	Bioruptor Plus	100 µl	0.5 ml Bioruptor Plus Microtubes	30 cycles 30 sec ON 90 sec OFF

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

NOTE: To guarantee homogeneity of shearing, always completely fill the tube holder with tubes. Never leave empty spaces in the tube holder. Fill the empty spaces with tubes containing the same volume of distilled water.

STEP 4

Methylated DNA immunoprecipitation

3-4 Day 3-4 45 minutes, overnight

BEADS PREPARATION

Note: *In order to save time, the beads preparation can also be performed during the 15 minutes incubation time of step 4.11.*

3.1 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.

3.2 Prepare **1x MagBuffer A** (bead wash buffer) as described in the table below and keep it on ice. The volumes contain a sufficient excess.

	1 IP	2 IPs	3 IPs	4 IPs	5 IPs	6 IPs	7 IPs	8 IPs
5x MagBuffer A	20 µl	40 µl	60 µl	80 µl	100 µl	120 µl	140 µl	160 µl
Nuclease-free 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
5x MagBuffer A	180 µl	200 µl	220 µl	240 µl	260 µl	280 µl	300 µl	320 µl
Nuclease-free Water	720 µl	800 µl	880 µl	960 µl	1040 µl	1120 µl	1200 µl	1280 µl

3.3 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. Do not vortex.*

3.4 Place the tube on a magnetic rack for 1.5 ml tubes to discard the supernatant. Keep the beads.

3.5 Wash the magnetic beads (Magbeads) twice with ice-cold 1x **MagBuffer A** (bead wash buffer) as follows:

- Take the required amount of ice-cold 1x **MagBuffer A** and transfer it to the tube containing the beads. **27.5 µl of 1x MagBuffer A** are needed **per IP**
- Resuspend the beads by pipetting up and down several times.
- Briefly spin the tube, place it on the magnetic rack and wait until the beads are completely bound to the magnet (~1 minute).
- Carefully remove and discard the supernatant without disturbing the beads.

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

CAUTION: Do not freeze the beads.

DNA IMMUNOPRECIPITATION

3.7 Prepare your DNA samples in a total volume of 57 µl in a 1.5 ml nuclease-free tube as described in the following table:

Component	Quantity/Volume
DNA*	10 ng to 1 µg + 10% surplus for inputs
Nuclease-free water	Up to 57 µl
Total Volume	**57 µl**

** It can be sheared DNA or adapters-ligated DNA in case of NGS application*

3.8 In a new 1.5 ml tube, prepare the **MagMaster 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
5x MagBuffer A	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
5x MagBuffer A	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

3.9 Add **33 µl of MagMaster Mix** to each DNA sample to form the Incubation Mix.

3.10 Incubate the Incubation Mix at 95°C for 3 minutes.

3.11 Quickly chill the Incubation Mix on ice. Perform a pulse spin to consolidate your sample and let it cool on ice for 15 minutes.

CAUTION: Be careful to maintain the denatured DNA sample on ice until the DNA immunoprecipitation in order to keep the DNA single-stranded and avoid re-annealing to double-stranded DNA.

3.12 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 8 tube-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.

3.13 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 DiaMag 0.2ml). Keep at 4°C.

3.14 In a new tube, dilute the antibody 1:1 with nuclease-free water as described in the following table and keep on ice:

Reagent (µl)	1 IP	2 IPs	3 IPs	4 IPs	5 IPs	6 IPs	7 IPs	8 IPs
Antibody anti-5mC (33D3 clone)*	0.5 µl	0.5 µl	1.0 µl	1.0 µl	1.0 µl	1.2 µl	1.4 µl	1.6 µl
Nuclease-free water	0.5 µl	0.5 µl	1.0 µl	1.0 µl	1.0 µl	1.2 µl	1.4 µl	1.6 µl

Reagent (µl)	9 IPs	10 IPs	11 IPs	12 IPs	13 IPs	14 IPs	15 IPs	16 IPs
Antibody anti-5mC (33D3 clone)*	1.8 µl	2.0 µl	2.2 µl	2.4 µl	2.6 µl	2.8 µl	3.0 µl	3.2 µl
Nuclease-free water	1.8 µl	2.0 µl	2.2 µl	2.4 µl	2.6 µl	2.8 µl	3.0 µl	3.2 µl

**Avoid freeze-thawing cycles of this very sensitive antibody. For optimal storage conditions freeze at -20°C or -80°C in small aliquots.*

3.15 Prepare the Diluted Antibody Mix as described in the following table. The volumes contain a sufficient excess. **Mix the Diluted Antibody, 5x MagBuffer A 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
Diluted Antibody	0.30 µl	0.75 µl	1.05 µl	1.50 µl	1.80 µl	2.10 µl	2.40 µl	3.00 µl
5x MagBuffer A	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
Diluted Antibody	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**	**100.0 µl**

3.16 Add **5 µl of Diluted Antibody Mix** to each 200 µl IP tube containing the Incubation Mix (from point 4.13)

3.17 Mix and add **20 µl of washed Magbeads** from point 4.6 to each 200 µl IP tube (from point 4.16). The final volume is 100 µl.

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

3.18 Place the IP tube on a rotating wheel (40 rpm) at 4°C overnight.

DNA WASHES

3.19 Place the **MagWash Buffer-1**, **MagWash Buffer-2** and the **Magnetic rack – DiaMag 0.2ml** on ice.

3.20 Briefly spin down the IP tube, place it on the ice-cold magnetic rack and wait until the beads are completely bound to the magnet (~1 minute).

3.21 Carefully remove and discard the supernatant without disturbing the beads.

3.22 Wash the beads 3 times with ice-cold **MagWash Buffer-1** as follows:

- Add **100 µl of** ice-cold **MagWash Buffer-1**, close the tube cap, and invert the tube to resuspend the beads.
- Incubate at 4°C for 5 minutes on a rotating wheel (40 rpm).
- Briefly spin down the IP tube, place it on the magnetic rack and wait until the beads are completely bound to the magnet (~1 minute).
- Carefully remove and discard the supernatant without disturbing the beads.

3.23 Wash the beads once with **100 µl ice-cold MagWash Buffer-2** as follows:

- Add **100 µl of** ice-cold **MagWash Buffer-2**, close the tube cap, and invert the tube to resuspend the beads.
- Incubate at 4°C for 5 minutes on a rotating wheel (40 rpm).

- Briefly spin down the IP tube, place it on the magnetic rack and wait until the beads are completely bound to the magnet (~1 minute).
- Carefully remove and discard the supernatant without disturbing the beads.
- Be sure to remove all traces of MagWash Buffer-2. If necessary, briefly spin down the tube, place it back on the magnetic rack and remove traces of liquids with a pipette.

3.24 Place the IP tube with bead pellet on ice. The bound DNA can be now purified from Magbeads.

STEP 5

Methylated DNA isolation

📅 Day 4 ⏳ 45 minutes

4.1 Take the input samples, centrifuge briefly and from now onwards treat the input samples and IP samples in parallel.

CAUTION: The following protocol describes the use of the DIB method to purify DNA. This is suitable for qPCR application. For NGS application, we recommend using the IPure Kit V2 (Cat. No. C03010014) instead of DIB.

4.2 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.

4.3 Remove the tubes from the DiaMag 0.2ml and add **100 µl** of DIB per IP'ed sample. Resuspend the beads.

4.4 Add **92.5 µl** of DIB to each « 7.5 µl input sample » from step 4.11.

4.5 Incubate at 55°C for 15 minutes both IP'ed samples and input samples.

4.6 Incubate all the samples at 100°C for 15 minutes.

4.7 Spin down and place the tubes into the DiaMag02.

4.8 Transfer the supernatants containing DNA in new labeled tubes, discard Magbeads. This isolated DNA can be stored at -20°C until further processing.

NOTES:

- *For MagMeDIP-qPCR use the isolated DNA for qPCR analyses.*
- *For MagMeDIP-seq use the isolated DNA for library amplification. We recommend performing qPCR on a small aliquot of each library for quality control before the sequencing.*

STEP 6

qPCR analysis

4 Day 4 > 2 hours

The MagMeDIP Kit includes two validated primer pairs:

1. Primer pair for Methylated spike-in ctrl (5µM each)
2. Primer pair for Unmethylated spike-in ctrl (5µM each)

NOTE: Primer pairs for endogenous control regions in human, mouse and rat are available - for more information, please visit www.diagenode.com

For large starting amounts of DNA, we advise to control the 4 DNA sequences.
For experiments starting with less than 100 ng DNA, or for MagMeDIP-seq experiments the analysis of the two spike-in controls is enough for quality control.

5.1 Prepare the qPCR Mix for each targeted DNA sequence as described in the following table for the number of desired reactions. Mix by pipetting and keep on ice until further use.

Reagents	Volume for 1 reaction
Primer pair (Stock: 5 µM each)	1 µl
2x SYBR Green PCR master mix	12.5 µl
Nuclease-free water	6.5 µl
TOTAL volume	20 µl

NOTES:

- *qPCR reagents are not provided for this step so that every researcher can use their regular SYBRGreen Master Mix that fits best their qPCR device.*
- *We recommend performing qPCR in duplicates or triplicates.*

5.2 Transfer 20 µl of the qPCR Mix per qPCR reaction into appropriate strip or well plate.

5.3 Add 5 µl of DNA sample.

NOTE: *The IP'ed and input samples can be diluted up to 10x in nuclease-free water before the qPCR when needed (e.g. if libraries have to be saved for library amplification and sequencing or if many regions have to be analyzed by qPCR).*

5.4 Run 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.*

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

IP efficiency

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=log2(10))) is used to take into account the 10% quantity of the input compared to the IP quantity.

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

IP enrichment and specificity

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)

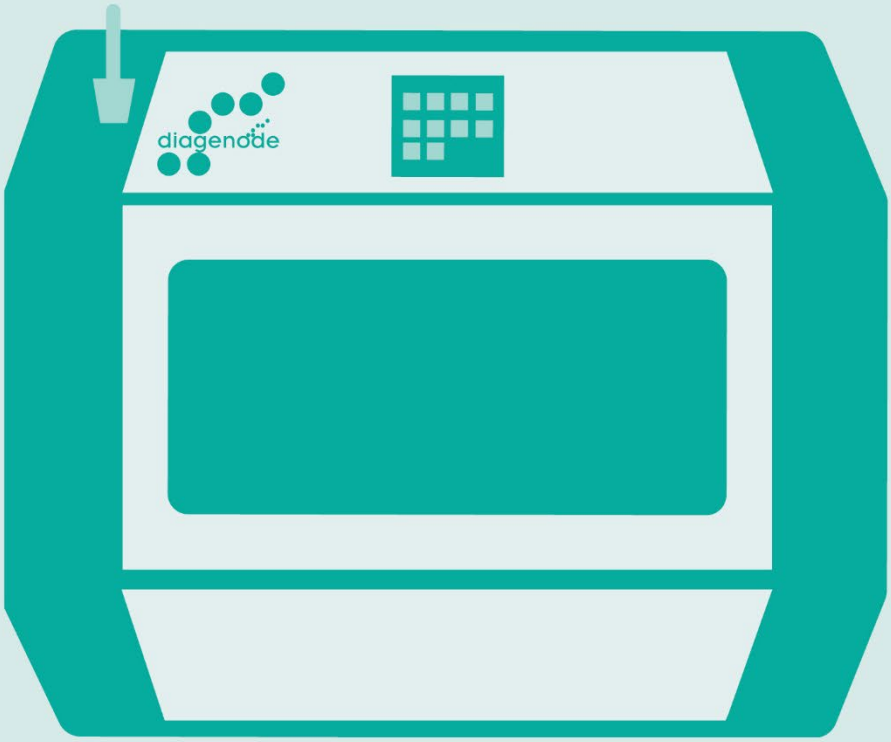
IP specificity can be calculated:

Specificity = 1- (% recovery background locus/%recovery specific locus) = 1- (1/enrichment)

Characteristics of a good quality IP:

- Methylated spike-in recovery **>** 20%
- Unmethylated spike-in recovery **<** 1%
- Spike-in IP specificity **>** 99%

AUTO PROTOCOL



AUTOMATED PROCESSING

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

The below chapter describes the protocol for MagMeDIP assay using the IP-Star Compact Automated System (Cat. No. B03000002).

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

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 300 x g 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 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 1700 x g 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 1700 x g for 10 minutes.

2.7 Transfer the 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 1700 x g 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.
- From 3 million cells, ~20 to 30 µg of gDNA can be expected.

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 for qPCR application or 200bp for NGS application (see Figure 1 in "Example of results" section).

3.1 Switch on the Bioruptor and set the temperature of the cooler at 4°C.

3.2 Prepare the gDNA solution at concentration 2-100 ng/µl using GenDNA TE.

NOTE: The smear of a sample with a concentration below 2 ng/µl will not be detected by most instruments for quality control.

3.3 Transfer a required volume of DNA solution to the appropriate sonication microtubes:

Type	Sample volume	Microtubes	Tube holder
Bioruptor Pico	100 µl	0.65 ml Microtubes for Bioruptor Pico Cat. No. C30010011	Tube holder for 0.65 ml tubes - Bioruptor® Pico Cat. No. B01201143
	<100 µl	0.2 ml Microtubes for Bioruptor Pico Cat. No. C30010020	Tube holder for 0.2 ml tubes - Bioruptor® Pico Cat. No. B01201144
Bioruptor Plus	100 µl	0.5 ml Bioruptor Plus Microtubes (Cat. No. C30010013)	0.5/0.65 ml tube holder for Bioruptor® Standard & Plus Cat. No. B01200043

3.4 Briefly vortex (5-10 sec) and spin samples.

3.5 Incubate on ice for 10 minutes.

3.6 Shear gDNA by sonication using the Bioruptor. Choose the protocol which is adapted to your sonicator model, sample volume and target size as described in the below table:

Application	Target size	Bioruptor model	Sample volume	Tubes	Shearing protocol
MagMeDIP-qPCR	300-500 bp	Bioruptor Pico	100 µl	0.65 ml Microtubes for Bioruptor Pico	8 cycles 15 sec ON 90 sec OFF
	300-500 bp	Bioruptor Pico	50 µl	0.2 ml Microtubes for Bioruptor Pico	5 cycles 30 sec ON 30 sec OFF
	300-500 bp	Bioruptor Plus	100 µl	0.5 ml Bioruptor Plus Microtubes	6 cycles 30 sec ON 90 sec OFF
MagMeDIP-seq	200 bp	Bioruptor Pico	100 µl	0.65 ml Microtubes for Bioruptor Pico	13 cycles 30 sec ON 30 sec OFF
	200 bp	Bioruptor Pico	50 µl	0.65 ml Microtubes for Bioruptor Pico	13 cycles 30 sec ON 30 sec OFF
	200 bp	Bioruptor Plus	100 µl	0.5 ml Bioruptor Plus Microtubes	30 cycles 30 sec ON 90 sec OFF

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

NOTE: To guarantee homogeneity of shearing, always completely fill the tube holder with tubes. Never leave empty spaces in the tube holder. Fill the empty spaces with tubes containing the same volume of distilled water.

STEP 4

Methylated DNA immunoprecipitation

3-4 Day 3-4 1 hour hands on time and overnight run

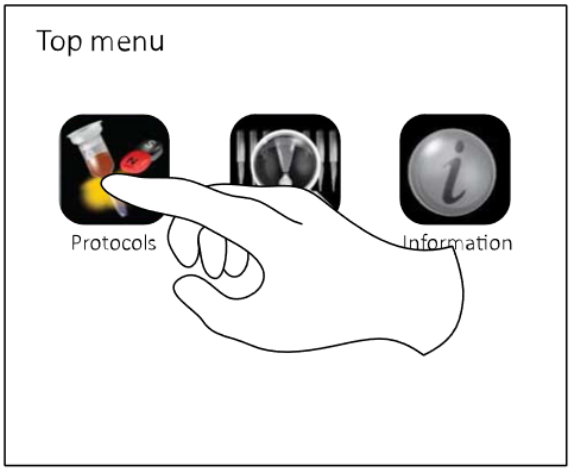
A – Protocol Set-up

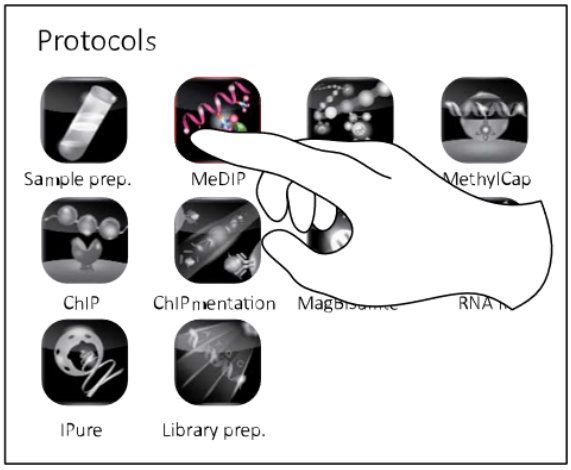
4.1 Switch ON the IP-Star Compact.

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.

CAUTION: The following protocol describes the use of the DIB method to purify DNA. This is suitable for qPCR application. For NGS application we recommend using the IPure Kit V2 (Cat. No. C03010014) instead of DIB and the MeDIP_8_IPure or MeDIP_16_IPure programs. For more information refer to the manual of the kit IPure v2.



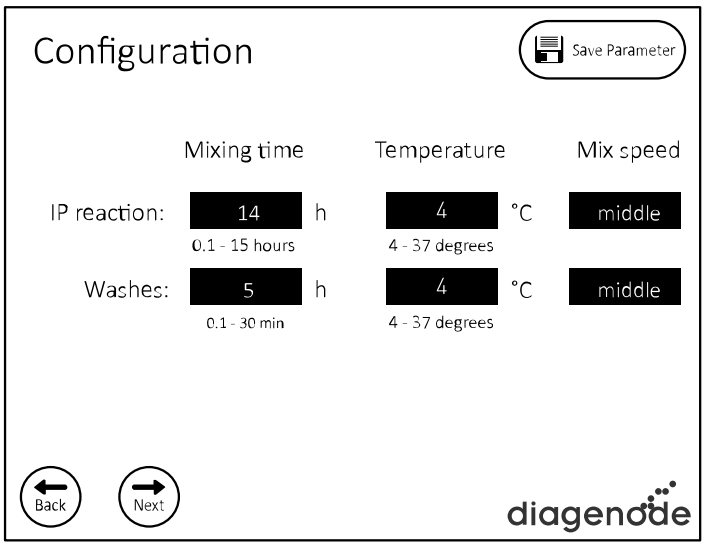


4.4 Set up the exact number of samples for your experiment by pressing the black box. This number includes each IP and it is recommended to perform the IP in duplicates. Input will not undergo immunoprecipitation in the IP-Star and is therefore not considered as a sample.

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:

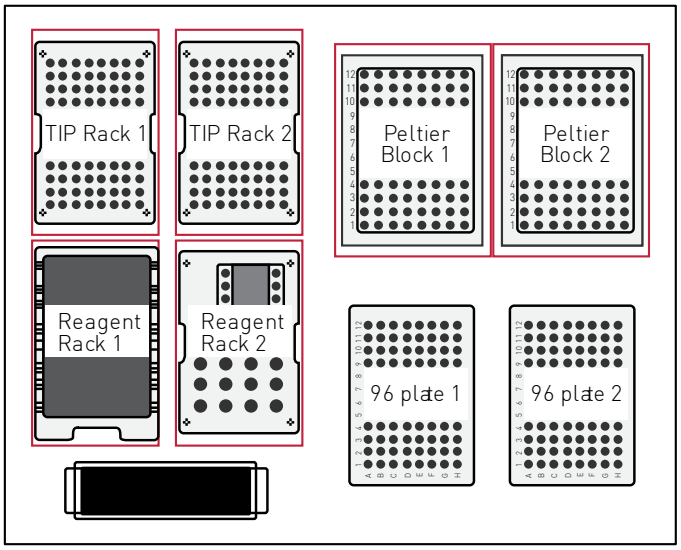


4.6 Setup all the plastic consumables on the platform according to the screen layout

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

  NOTE: *For qPCR applications – DIB (included in the kit MagMeDIP) should be used; for NGS applications – Elution Buffer (from the kit IPure v2) should be used.*
- 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.*



4.7 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

4.8 In a new 1.5 ml tube, prepare 1x MagBuffer A (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
5x MagBuffer A	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
5x MagBuffer A	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

4.9 Prepare your DNA samples in a total volume of 57 µl in a 1.5 ml nuclease-free tube as described in the following table:

Component	Quantity/Volume
DNA*	10 ng to 1µg + 10% surplus for inputs
Nuclease-free water	Up to 57 µl
Total Volume	**57 µl**

** It can be sheared DNA or adapters-ligated DNA for NGS application*

4.10 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
5x MagBuffer A	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
5x MagBuffer A	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

4.11 Add **33 µl of MagMaster Mix** to each DNA sample to form the Incubation Mix.

4.12 Incubate the Incubation Mix at 95ºC for 3 minutes.

4.13 Quickly chill the Incubation Mix on ice. Perform a pulse spin to consolidate your sample and let it cool on ice for 15 minutes.

CAUTION: Be careful to maintain the denatured DNA sample on ice until the DNA immunoprecipitation in order to keep the DNA single-stranded and avoid re-annealing to double-stranded DNA.

4.14 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.

4.15 Then, transfer **75 µl of incubation mix** for each IP into one 200 µl IP-Star tube strip. Keep at 4°C.

4.16 In a new tube, dilute the antibody 1:1 with nuclease-free water as described in the following table and keep on ice:

Reagent (µl)	1 IP	2 IPs	3 IPs	4 IPs	5 IPs	6 IPs	7 IPs	8 IPs
Antibody anti-5mC (33D3 clone)*	0.5 µl	0.5 µl	1.0 µl	1.0 µl	1.0 µl	1.2 µl	1.4 µl	1.6 µl
Nuclease-free water	0.5 µl	0.5 µl	1.0 µl	1.0 µl	1.0 µl	1.2 µl	1.4 µl	1.6 µl

Reagent (µl)	9 IPs	10 IPs	11 IPs	12 IPs	13 IPs	14 IPs	15 IPs	16 IPs
Antibody anti-5mC (33D3 clone)*	1.8 µl	2.0 µl	2.2 µl	2.4 µl	2.6 µl	2.8 µl	3.0 µl	3.2 µl
Nuclease-free water	1.8 µl	2.0 µl	2.2 µl	2.4 µl	2.6 µl	2.8 µl	3.0 µl	3.2 µl

**Avoid freeze-thawing cycles of this very sensitive antibody. For optimal storage conditions freeze at -20°C or -80°C in small aliquots.*

4.17 Prepare the Diluted Antibody Mix as described in the following table. The volumes contain a sufficient excess. **Mix the Diluted Antibody, 5x MagBuffer A 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
Diluted Antibody	0.30 µl	0.75 µl	1.05 µl	1.50 µl	1.80 µl	2.10 µl	2.40 µl	3.00 µl
5x MagBuffer A	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
Diluted Antibody	3.30 µl	3.60 µl	3.90 µl	4.20 µl	4.50 µl	4.80 µl	5.40 µl	6.00 µl
5x MagBuffer A	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**	**101.0 µl**

C – Fill the strips with the reagents and the mixes

4.18 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 number of beads will lead to lower reproducibility.

4.19 Prepare the Immunoprecipitation Mix in the 200 µl IP-Star tube strip from step 4.18 as described in the table below. Insert the strip in row 7.

	1IP (100 µl)
MagBuffer A (1x)	20 µl
Incubation Mix (already in the strip)	75 µl
Antibody Mix	5 µl

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

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

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

4.22 Check the selected parameters, close the door and press “Run” to start.

4.23 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

4 Day 4 ⏳ 10 minutes hands on time and 40 minutes 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

**For NGS application follow the protocol for IPure kit v2 (Cat. No. C03010014).*

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 DiaMag 0.2ml 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

📅 4 Day 4 ⏳ > 2 hours

The MagMeDIP 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)

NOTE: Primer pairs for endogenous control regions in human, mouse and rat are available! For more information please visit www.diagenode.com

For large starting amounts of DNA we advise to control the 4 DNA sequences.
For experiments starting with less than 100 ng DNA, or for MagMeDIP-seq experiments the analysis of the two spike-in controls is enough for quality control.

6.1 Prepare the qPCR Mix for each targeted DNA sequence as described in the following table for the number of desired reactions. Mix by pipetting and keep on ice until further use.

Reagents	Volume for 1 reaction
Primer pair (Stock: 5 µM each)	1 µl
2x SYBR Green PCR master mix	12.5 µl
Nuclease-free water	6.5 µl
TOTAL volume	20 µl

NOTES:

- *qPCR reagents are not provided for this step so that every researcher can use their regular SYBRGreen Master Mix that fits best their qPCR device.*
- *We recommend performing qPCR in duplicates or triplicates.*

6.2 Transfer 20 µl of the qPCR Mix per qPCR reaction into appropriate strip or well plate.

6.3 Add 5 µl of DNA sample.

NOTE: *The IP'ed and input samples can be diluted up to 10x in nuclease-free water before the qPCR when needed (e.g. if libraries have to be saved for library amplification and sequencing or if many regions have to be analyzed by qPCR).*

6.4 Run 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.*

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

IP efficiency

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=log2(10))) is used to take into account the 10% quantity of the input compared to the IP quantity.

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

IP enrichment and specificity

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)

IP specificity can be calculated:

Specificity = 1- (% recovery background locus/%recovery specific locus) = 1- (1/enrichment)

To consider an IP to be of good quality, find below some key criteria:

- Methylated spike-in recovery **>** 20%
- Unmethylated spike-in recovery **<** 1%
- Spike-in IP specificity **>** 99%

Example of Results

MAGMEDIP-QPCR

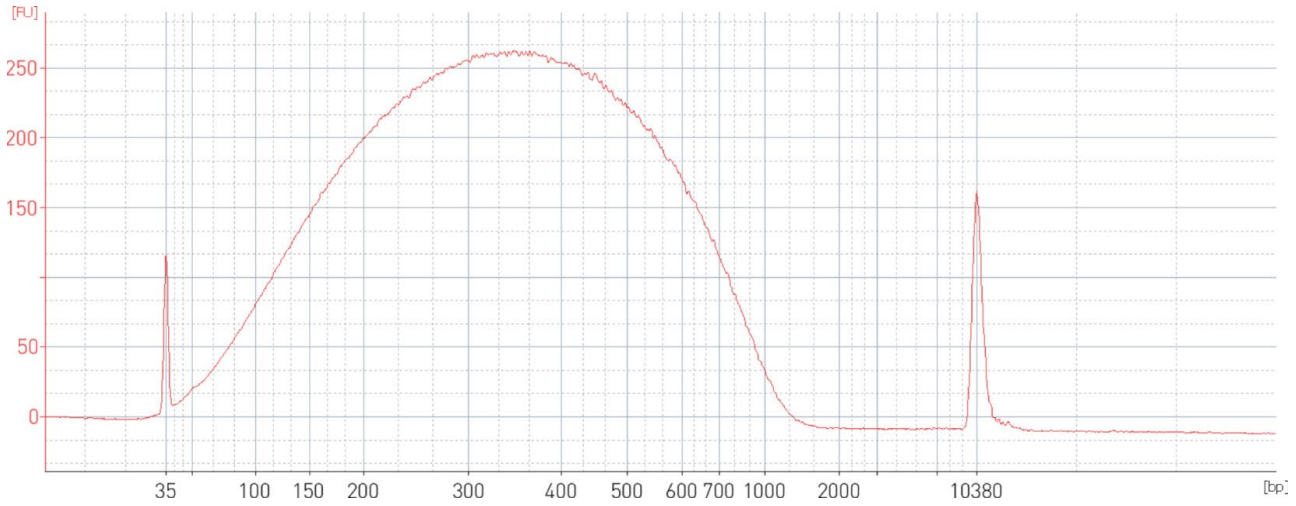


Figure 1. BioAnalyzer (Agilent) High Sensitivity DNA chip profile of sheared genomic DNA: smear around 400 bp.

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"].

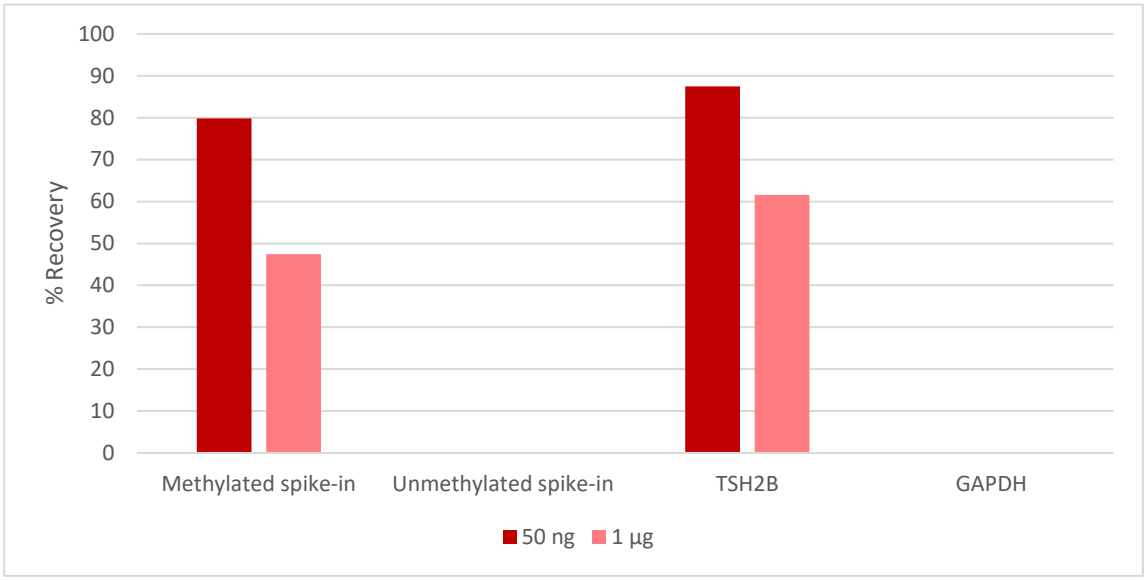


Figure 2. Immunoprecipitation results obtained with Diagenode MagMeDIP Kit

MeDIP assays were performed manually using 1 µg or 50 ng gDNA from blood cells with the MagMeDIP kit (Diagenode). The IP was performed with the Methylated and Unmethylated spike-in controls included in the kit, together with the human DNA samples. The DNA was isolated/purified using DIB. Afterwards, qPCR was performed using the Human TSH2B promoter and GADPH TSS primer pairs (Cat. No. C17011041 and C17011047, respectively).

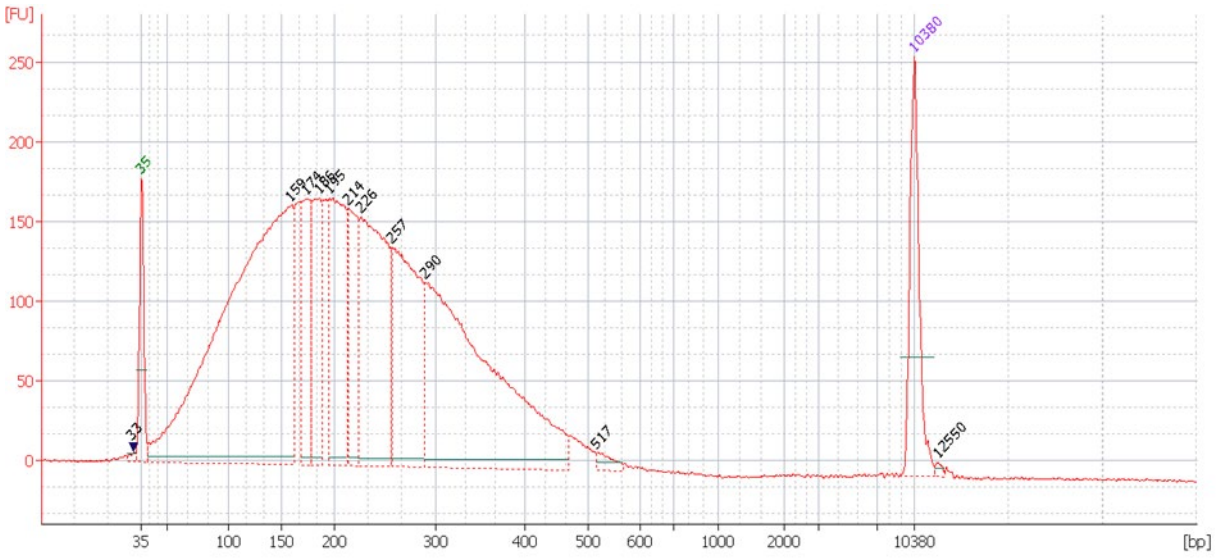


Figure 3. *BioAnalyzer (Agilent) High Sensitivity DNA chip profile of sheared human gDNA: smear around 200 bp.*

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 (C30010011). The following program was used: 13 Cycles [30 seconds "ON" & 30 seconds "OFF"], on Bioruptor® Pico sonication device (B01080010).

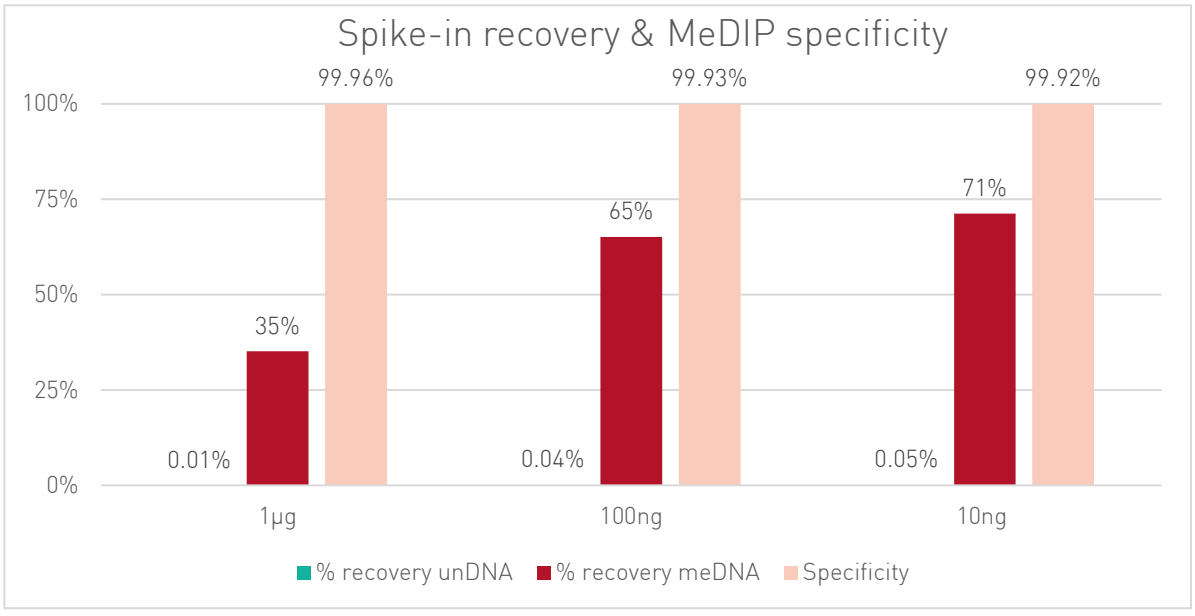


Figure 4. *qPCR analysis of external spike-in DNA controls (methylated and unmethylated) after IP.* Samples were prepared using 1µg – 100ng -10ng sheared human gDNA with the MagMeDIP kit (Diagenode) and a commercially available library prep kit. DNA isolation after IP has been performed with IPure kit V2 (Diagenode).

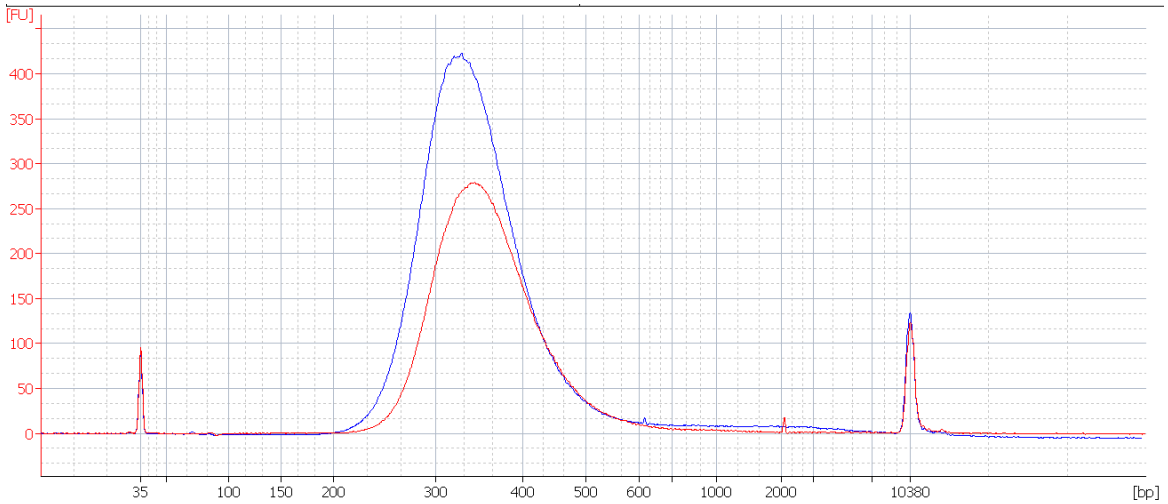
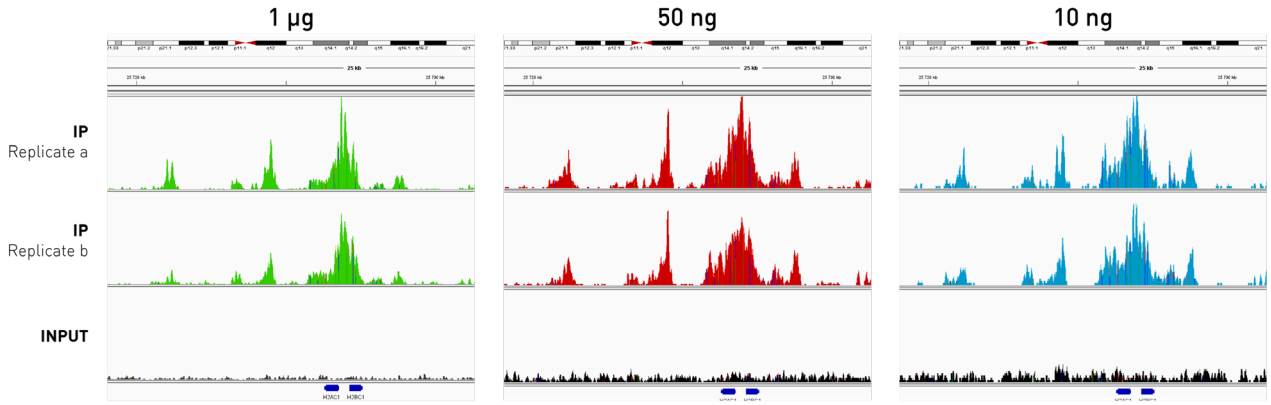


Figure 5. *BioAnalyzer (Agilent) High Sensitivity DNA chip profile of IP (red) and corresponding INPUT (blue) libraries*.

Samples were prepared using 100 ng sheared human gDNA with the MagMeDIP kit (Diagenode) and a commercially available library preparation kit. DNA isolation after IP has been performed with IPure kit V2 (Diagenode).

TSH2B



GADPH

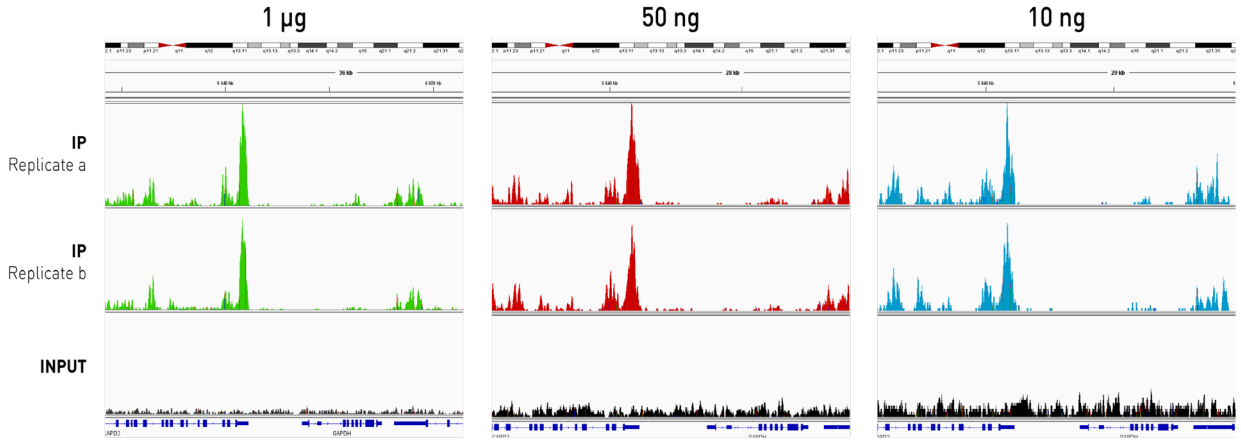


Figure 6. *Sequencing profiles of MeDIP-seq libraries prepared from different starting amounts of sheared gDNA on the positive and negative methylated control regions.*
MeDIP-seq libraries were prepared from decreasing starting amounts of gDNA (1 µg (green), 50 ng (red), and 10ng (blue)) originating from human blood with the MagMeDIP kit (Diagenode) and a commercially available library prep kit. DNA isolation after IP has been performed with IPure kit V2 (Diagenode). IP and corresponding INPUT samples were sequenced on Illumina NovaSeq SP with 2x50 PE reads. The reads were mapped to the human genome (hg19) with bwa and the alignments were loaded into IGV (the tracks use an identical scale). The top IGV figure shows the TSH2B (also known as H2BC1) gene (marked by blue boxes in the bottom track) and its surroundings. The TSH2B gene is coding for a histone variant that does not occur in blood cells, and it is known to be silenced by methylation. Accordingly, we see a high coverage in the vicinity of this gene. The bottom IGV figure shows the GADPH locus (marked by blue boxes in the bottom track) and its surroundings. The GADPH gene is a highly active transcription region and should not be methylated, resulting in no reads accumulation following MeDIP-seq experiment.

FAQs

What is the resolution for MagMeDIP?

The resolution is related to the size of the fragments analyzed.

- For MagMeDIP-qPCR the sheared DNA is a smear of 50-1000 bp which means that the resolution is also 50-1000 bp.
- For MagMeDIP-seq the resolution is related to the size of the fragments incorporated into the library and then effectively sequenced. While the sheared DNA is a smear between 50-500 bp, after library preparation and IP we observed fragment size between 50 bp and maximum 450 bp (thus libraries including adaptors around 200-600 bp). On Illumina sequencers, clustering of libraries above 500 bp is not very efficient, thus you can expect a resolution between 50 bp and 350 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 kit from the immunoprecipitation step.

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

For shearing it is paramount to follow recommendations on appropriate volumes and tubes according to your sample quantity. 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.

Can I use another antibody with the kit?

This kit has been validated with the provided antibody only: 5-methylcytosine (5-mC) Antibody - clone 33D3, 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 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 kit?

If you need more spike-in controls, you can buy the **DNA Methylation Control Package V2** (Cat. No. C02040019) which provides the methylated and unmethylated controls and the primer pairs included in the MagMeDIP Kit. Those spike-in controls have been produced using synthetic oligonucleotides and are not homologous to any model species. Therefore, they will not interfere with your DNA sample.

Another kind of methylated and unmethylated controls and primer pairs are available separately in the **DNA Methylation Control Package** (Cat. No. C02040012). Those spike-in controls are produced from the genome of A. thaliana and may therefore interfere with plant samples.

Please check the products datasheet on the website for more information:

DNA Methylation Control Package	Link towards datasheet
V1 (C02040012)	https://www.diagenode.com/files/products/kits/Datasheet_DNA_methylation_control_package.pdf
V2 (C02040019)	https://www.diagenode.com/files/products/kits/Datasheet_DNA-methylation-control-package-V2.pdf

What are the lengths of the PCR products?

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

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

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 sections Troubleshooting and list of error codes of the IP-Star Compact manual.

Related Products

Equipment	Cat. No.
DiaMag 0.2ml - magnetic rack	B04000001
Bioruptor® Pico sonication device	B01080010
Bioruptor® Plus sonication device	B01020001
Product	**Cat. No.**
5-methylcytosine (5-mC) monoclonal antibody (33D3)	C15200081
DiaMag anti-mouse IgG coated magnetic beads	C03010022
XL GenDNA Extraction Module	C03030020
DNA methylation control package V2	C02040019
DNA Methylation control package	C02040012
Human meDNA primer pair (TSH2B)	C17011041
Human meDNA primer pair (GAPDH)	C17011047
Mouse meDNA primer pair (TSH2B)	C17021042
Mouse meDNA primer pair (GAPDH)	C17021045
Rat meDNA primer pair (TSH2B)	C17031043
Rat meDNA primer pair (GAPDH)	C17031046
IPure kit v2	C03010014

Revision history

Version	Date of modification	Description of modifications
Version 5 05_2023	May 2023	Page 8 - Adjustment of reagents quantity
Version 5 03_2023	March 2023	Correction of small errors (point 4.15, 4.16, 4.17) Removal of TSHB2 and GAPDH primer pairs from the list of kit components and adjustment of the qPCR protocol Adjustment of the auto protocol – the use of the IPure kit instead of DIB
Version 5 12_2022	December 2022	Page 8 - Addition of the information about the modification of two buffers
Version 5 11_2022	November 2022	- Modification of the kit name - Removal of discontinued products - Adjustment of the recommendations and protocol for starting amount - Addition of the recommendations for primer design - Updating of the protocol for DNA shearing - Addition of the recommendations for MeDIP-seq - Addition of the table for antibody dilution

FOR RESEARCH USE ONLY.

Not intended for any animal or human therapeutic or diagnostic use.

NOTICE TO PURCHASERLIMITED LICENSE

The information provided herein is owned by Diagenode and/or its affiliates. Subject to the terms and conditions that govern your use of such products and information, Diagenode and/or its affiliates grant you a nonexclusive, nontransferable, non-sublicensable license to use such products and information only in accordance with the manuals and written instructions provided by Diagenode and/or its affiliates. You understand and agree that except as expressly set forth in the terms and conditions governing your use of such products, that no right or license to any patent or other intellectual property owned or licensable by Diagenode and/or its affiliates is conveyed or implied by providing these products. In particular, no right or license is conveyed or implied to use these products in combination with any product not provided or licensed to you by Diagenode and/or its affiliates for such use. Limited Use Label License: Research Use Only The purchase of this product conveys to the purchaser the limited, non-transferable right to use the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact info@diagenode.com.

TRADEMARKS

The trademarks mentioned herein are the property of Diagenode or their respective owners. Bioanalyzer is a trademark of Agilent Technologies, Inc. Illumina® is a registered trademark of Illumina®Inc; Qubit is a registered trademark of Life Technologies Corporation.

www.diagenode.com