MagMeDIP-seq Package V2

Magnetic Methylated DNA Immunoprecipitation Package for Illumina® sequencing

Cat. No. C02010041 (10 rxns)

PACK CONTENT:
C02010020 MagMeDIP qPCR kit (10 rxns)
C05010024 iDeal DNA IP Library Preparation kit (24 rxns)
C05010025 iDeal Unique Dual Indexes module (Set A: 24 UDIs, 24 rxns)
C03010014 iPure kit V2 (24 rxns)

Version 3 01_2022

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Please read this manual carefully before starting your experiment

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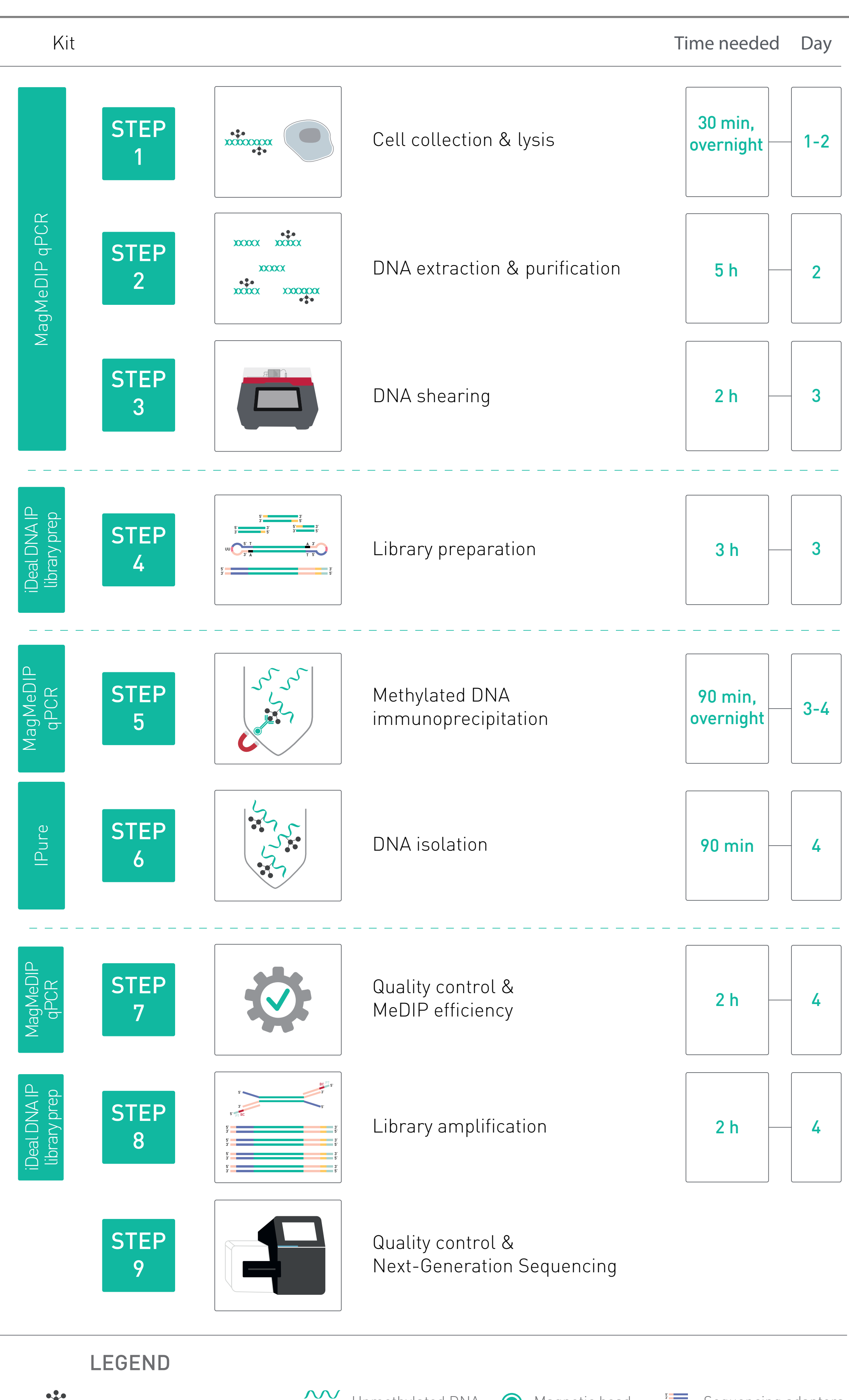
Kit method overview



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. Its study in diseased cells is of growing interest for new diagnostic and therapeutic methods development.

DNA methylation primarily takes place by the addition of a methyl group to the C5 position of a cytosine nucleotide, which occurs mainly in the context of CG dinucleotides, forming 5-methylcytosine (5-mC). The most common technique used in DNA methylation studies remains bisulfite conversion prior to analysis. However, use of bisulfite treatment can be challenging. Indeed, bisulfite conversion leads to DNA fragmentation and reduces genome complexity to three nucleotides (by converting the majority of C, which are unmethylated, into U), making NGS data analysis difficult.

Performing MeDIP-seq experiments, i.e. methylated DNA immunoprecipitation followed by next generation sequencing, provides a useful alternative for convenient and comprehensive identification of differentially methylated regions, where single-base resolution is not necessary. This technique enables to obtain region-resolution assessment of DNA methylation by enrichment of methylated DNA using a 5mC-specific antibody.

Diagenode **MagMeDIP kits** take advantage of Diagenode **highly specific monoclonal antibody targeting 5-mC** (33D3 clone, C15200081) to immunoprecipitate and enrich samples in methylated DNA. Recovered DNA is suitable for many downstream applications to analyze genome-wide DNA methylation including qPCR or Next-Generation Sequencing (NGS). The high affinity of the 5-mC antibody enables detection of methylated cytosines regardless of their genomic context. Indeed, MeDIP assays cover 5mC in dense, less dense, and repeat CpG regions.

Diagenode **MagMeDIP-seq package V2** has been specifically developed and optimized to generate DNA libraries during MeDIP assay from the **lowest DNA amounts** and secure **high-quality NGS data** for DNA methylation analysis. **Only 10 ng** of fragmented double-stranded DNA is required for library generation, while preserving the efficiency of the enrichment. In addition, the protocol can be applied on DNA from **any species.**

MagMeDIP-seq package V2 contains:

- **MagMeDIP qPCR Kit (C02010020)** including:
  - A XL GenDNA Extraction Module to extract genomic DNA from cultured cells
  - Highly validated DNA shearing protocols on Diagenode Bioruptor®
  - All the reagents for the immunoprecipitation (IP), including 5-mC monoclonal antibody (clone 33D3) and magnetic beads
  - Methylated and unmethylated DNA spike-in controls and their associated qPCR primers to verify the efficiency of the IP
  - qPCR primers targeting positive and negative regions on human genome to monitor the success of the IP on your sample

- **iPure Kit V2 (C03010014)** for highest quality purification before sequencing

- **iDeal DNA IP Library Preparation Kit (C05010024)*** to obtain ready-to-sequence libraries for Illumina sequencing

- **iDeal Unique Dual Indexes Module – Set A (C05010025)*** to mitigate index hopping during sequencing

  * Product is available for ordering only in combination with, and cannot be ordered without, MagMeDIP qPCR Kits.

The immunoprecipitation and purification steps of the MagMeDIP-seq package V2 protocol use magnetic beads. We recommend using Diagenode magnetic racks - **Magnetic rack - DiaMag 1.5ml (B04000003)** and **Magnetic rack – DiaMag 0.2ml (B04000001)** - together with our MagMeDIP kits.

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

- **Start-to-finish**: Complete kits including gDNA extraction module, antibody, spike-in controls and qPCR primer pairs for control regions, validated library preparation module
- **Easy to use:** Streamlined, magnetic protocols with user-friendly magnetic beads and racks
- **High enrichment yield:** Targeted capture of 5-mC DNA fragments with Diagenode's highly specific antibody
- **Robust and reproducible data:** Extensively validated for qPCR and NGS downstream analysis

Kit Materials

The MagMeDIP-seq package V2 is available in one format so that the content is sufficient to perform 6 DNA extractions of 10 µg each and 10 methylated DNA immunoprecipitations with 20 purifications and 20 library preparations.

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

Table 1. Components of MagMeDIP qPCR kit (C02010020)

Component	Quantity	Storage
MagBeads (magnetic beads)	150 µl	4°C/39°F Do not freeze
Nuclease-free water	4 ml	4°C/39°F
MagBuffer A (5x concentrated)	2 ml	4°C/39°F
MagBuffer B	100 µl	4°C/39°F
MagBuffer C	40 µl	-20°C/-4°F
Antibody anti-5mC* (33D3 clone)	5 µl	-20°C / -4°F Long term storage: -80°C/-112°F
Methylated spike-in control	6 µl	-20°C/-4°F
Unmethylated spike-in control	6 µl	-20°C/-4°F
MagWash Buffer-1	6 ml	4°C/39°F
MagWash Buffer-2	4 ml	4°C/39°F
DNA Isolation Buffer (DIB)	4 ml	4°C/39°F
Proteinase K (100x)	20 µl	-20°C/-4°F
Primer pair for Methylated spike-in ctrl (5µM each)	50 µl	-20°C/-4°F
Primer pair for Unmethylated spike-in ctrl (5µM each)	50 µl	-20°C/-4°F
Human TSH2B primer pair (5µM each)	50 µl	-20°C/-4°F
Human GAPDH primer pair (5µM each)	50 µl	-20°C/-4°F
200 µl tube strips (8 tubes/strip)	2	RT
Cap strips	2	RT
GenDNA Digestion Buffer	3 ml	4°C/39°F
GenDNA Proteinase K (200X)	300 µg / 15 µl	-20°C/-4°F
GenDNA Precipitant	3 ml	4°C/39°F
GenDNA TE	3 ml	4°C/39°F
GenDNA RNase (DNAse free)	5 µg / 10 µl	-20°C/-4°F

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

Table 2. Components of iPure kit V2 (C03010014)

Component	Quantity	Storage
200 µl tube strips (8 tubes/strip)	4	RT
Cap strips	4	RT
Buffer A	2.8 ml	4°C/39°F
Buffer B	115 µl	4°C/39°F
Buffer C	1.2 ml	4°C/39°F
Wash Buffer 1 w/o isopropanol	1.5 ml	4°C/39°F
Wash Buffer 2 w/o isopropanol	1.5 ml	4°C/39°F
iPure Beads V2	240 µl	4°C/39°F
Carrier**	48 µl	-20°C/-4°F

***This product is shipped at 4°C. Store it at -20°C upon arrival. This is an optimized buffer (NOT CARRIER DNA).*

Table 3. Components of iDeal DNA IP Library Preparation kit (C05010024)

Component	Quantity	Storage
iDeal DNA IP End Prep Enzyme Mix ●	72 µl	-20°C/-4°F
iDeal DNA IP End Prep Reaction Buffer ●	168 µl	-20°C/-4°F
iDeal DNA IP Ligation Master Mix ●	720 µl	-20°C/-4°F
iDeal DNA IP Ligation Enhancer ●	24 µl	-20°C/-4°F
iDeal UDI Adapter ●	60 µl	-20°C/-4°F
iDeal UDI Uracil Excision Reagent ●	72 µl	-20°C/-4°F
iDeal Adapter Dilution Buffer ●	54 µl	-20°C/-4°F
Resuspension Buffer ●	1080 µl	-20°C/-4°F

Table 4. Components of iDeal Unique Dual Indexes module – Set A (C05010025)

Component	Quantity	Storage
iDeal Primer UDI (x24) ●	5 µl each	-20°C/-4°F
iDeal DNA IP PCR Master Mix ●	600 µl	-20°C/-4°F
100X SYBR ●	5 µl	-20°C/-4°F

Table 5. Sequences of iDeal Unique Dual Indexes – Set A (C05010025)

iDeal Primer UDI #	i7 sequence	i5 sequence for forward strand reading (NovaSeq 6000 v1.0, MiniSeq Rapid Reagent, MiSeq, HiSeq 2500, HiSeq 2000).	i5 sequence for reverse complement reading (iSeq 100, MiniSeq Standard reagents, NextSeq, NovaSeq 6000 v1.5, HiSeq X, HiSeq 4000, and HiSeq 3000).
1	CACTGTAG	AAGCGACT	AGTCGCTT
2	GTGCACGA	TGATAGGC	GCCTATCA
3	AAGCGACT	ACGAATCC	GGATTCGT
4	TGATAGGC	GTCTGAGT	AACTCAGAC
5	ACGAATCC	ATTACCCA	TGGGTAAT
6	GTCTGAGT	GACTTGTG	CACAAGTC
7	ATTACCCA	CACTGTAG	CTACAGTG
8	GACTTGTG	GTGCACGA	TCGTGCAC
9	TTCAATAG	TCCCACGA	TCGTGGGA
10	GTTTGCTC	ACCAACAG	CTGTTGGT
11	ACCGGAGT	AAGGAAGG	CCTTCCTT
12	CTTGACGA	GCACACAA	TTGTGTGC
13	TGTTCGCC	AGGTAGGA	TCCTACCT
14	ACAAGGCA	TCGCGCAA	TTGCGCGA
15	CCTGTCAA	ATGGCTGT	ACAGCCAT
16	CCATCCGC	AAGGCGTA	TACGCCTT
17	ATGGCTGT	GTGAATAT	TTGACAGG
18	AAGGCGTA	CCATCCGC	GCGGATGG
19	AGGTAGGA	TGTTCGCC	GGCGAACA
20	TCGCGCAA	ACAAGGCA	TGCCTTGT
21	AAGGAAGG	ACCGGAGT	ACTCCGGT
22	GCACACAA	CTTGACGA	TCGTCAAG
23	TCCCACGA	TTCAATAG	CTATTGAA
24	ACCAACAG	GTTTGCTC	GAGCAAAC

Required Materials Not Provided

Materials and Reagents

- Gloves to wear at all steps
- RNAse/DNase-free 15 or 50 ml tubes, 1.5 ml and 0.2 ml conical tubes
- Ice-cold PBS buffer
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol
- Ethanol
- Nuclease-free water
- AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- Isopropanol

Equipment

- Centrifuges for 1.5 ml tubes and 15-50 ml conical tubes (4°C)
- Microcentrifuge for 0.2 ml tubes
- Thermomixer (95°C)
- Thermocycler
- Cell counter
- Rotating wheel such as DiaMag Rotator (B05000001)
- Diagenode magnetic racks:
  - Magnetic rack – DiaMag 1.5ml (B04000003)
  - Magnetic rack – DiaMag 0.2ml (B04000001)
- Diagenode Bioruptor® sonication device and the associated tubes:
  - Bioruptor® Pico (B01080010) and tube holder for 0.2 ml tubes (B01201144) + 0.2 ml Pico Microtubes (C30010020) AND/OR tube holder for 0.65 ml tubes (B01201143) + 0.65 ml Pico Microtubes (C30010011)
  - Bioruptor® Plus (B01020001) and tube holder for 0.5/0.65ml tubes (B01200043) + 0.5 ml Bioruptor Plus Microtubes (C30010013)
- Reagents and equipment for quantitative PCR

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

Additional supplies (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
DNA methylation control package V2	C02040019	48 rxns
XL GenDNA Extraction Module	C03030020	6 rxns
Antibody anti-5mC (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 the MagMeDIP assay is important. Thus, we recommend using Diagenode XL GenDNA Extraction Module (included in this package) for the extraction and purification of genomic DNA from cultured cells. For the extraction of DNA from blood or tissue samples, 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-seq package V2 are validated for starting amounts from **10 ng to 1µg** of sheared double-stranded DNA fragments per IP reaction. These conditions provide high sequencing data quality. Nevertheless, the antibody quantity can be further optimized to your experimental conditions.

DNA Shearing

For an efficient and best resolution IP experiment, the gDNA has to be sheared into fragments with a mean size of 200 bp on the Bioruptor® Pico (B01080010) or Bioruptor® Plus (B01020001).

Before using the sheared DNA fragments for the MagMeDIP assay, we recommend analyzing the size of the fragments after shearing using a dedicated device such as the BioAnalyzer (Agilent) or the Fragment Analyzer (Agilent).

In case of low amount/volume of starting materials, we recommend using Bioruptor® Pico (B01080010) as it can accommodate shearing samples in 50 µl volume in the associated 0.2 ml tubes while Bioruptor® Plus (B01020001) works with 100 µl volume per sample.

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.**

Magnetic bead wash

When washing the MagBeads with wash buffer:

- Use enough wash solution to cover the pellet
- Do not disturb the captured beads attached to the tube wall
- Always briefly spin the tube to bring down liquid caught in the lid prior to positioning into the magnetic rack

Input

"INPUT"-labelled sample corresponds to the sheared DNA that undergoes the full MagMeDIP-seq procedure without incubation with the antibody and the MagBeads. The INPUT sample is used as a reference to calculate the IP recovery by qPCR. It is also used after sequencing during bioinformatic analysis for detection and removal of experimental bias. We recommend including one INPUT sample for each sheared DNA sample that undergoes immunoprecipitation.

Quality Controls

The MagMeDIP-seq package V2 contains one methylated and one unmethylated DNA spike-in controls and their associated qPCR primers. The spike-in controls can be added directly to each library sample before the MeDIP assay to verify the IP efficiency by qPCR. Those two spike-in controls are not homologous to any model species and will not interfere with the sample of interest. These spike-in controls will not be sequenced as they do not bear the Illumina adapters.

The MagMeDIP-seq package V2 also contains two qPCR primers targeting methylated (TSH2B) and unmethylated (GAPDH) regions on human genome. They can be used to monitor the IP success on the sample of interest by qPCR before the library amplification. Primers for control regions on mouse and rat genomes can be purchased separately (see **Optional supplies** paragraph). For each primer pair, run the INPUT sample alongside the IP sample during the qPCR.

DNA Isolation

For NGS analysis, we strongly recommend using the iPure kit V2 (included in this package) instead of the DNA Isolation Buffer (DIB) to isolate the IP sample before library amplification. The iPure kit V2 is the only DNA purification kit that is specifically optimized for extracting very low amounts of DNA after MeDIP and provides the necessary purity for sequencing.

Multiplexing advices

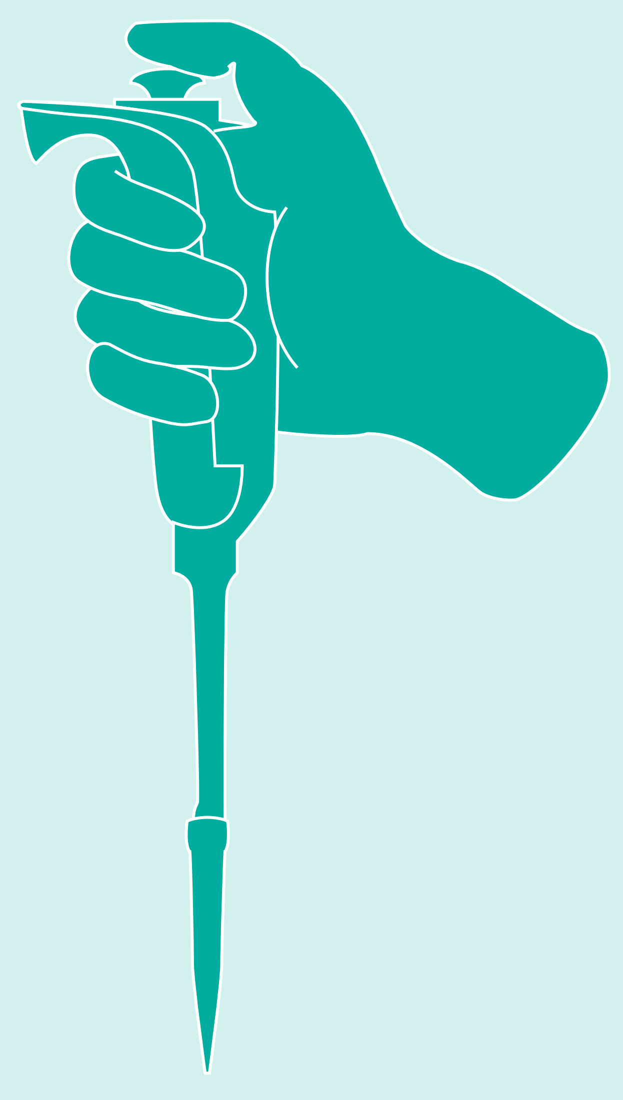
The iDeal UDI module (included in this package) contains unique dual index primers that are needed to make MagMeDIP-seq libraries. These UDIs were optimized and validated for use with the iDeal DNA IP Library Preparation kit (also included in this package).

The iDeal UDI module – Set A includes 24 primer pairs with unique dual barcodes (unique i5 and i7 indexes) for library multiplexing up to 24. The use of UDIs is highly recommended to mitigate errors introduced by read misassignment, including index hopping frequently observed with patterned flow cells such as Illumina's NovaSeq system.

For multiplexing we recommend the following index combinations:

Plex	Index number
2	1 & 2 3 & 4 5 & 6 7 & 8
≥3	Any 2-plex combination plus any other index

PROTOCOL



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STEP 1

Cell collection & lysis

Day 1-2 30 minutes, overnight

MAGMEDIP QPCR KIT (C02010020)

Starting material: cultured cells

> ***NOTE:*** *For DNA extraction from blood or tissue, other specific extraction kits should be used. Start directly with DNA shearing (Step 3) with the extracted DNA sample.*

1.1 Pellet suspension culture out of its serum-containing medium or trypsinize adherent cells and collect cells from the flask.

1.2 Centrifuge at 300 g at 4°C for 5 minutes.

1.3 Discard the supernatant.

1.4 Resuspend cells in **5 to 10 ml** ice-cold **PBS**.

1.5 Count cells.

1.6 Centrifuge at 300 g at 4°C for 5 minutes.

1.7 Discard the supernatant.

1.8 Repeat steps 1.4 to 1.7 for a total of 2 cell washes.

> ***NOTE:*** *If needed, cell pellets can be fresh frozen at this step and stored at - 80°C for several months.*

1.9 Meanwhile, place the **GenDNA Digestion Buffer** at room temperature and the **GenDNA Proteinase K** on ice.

1.10 Prepare the **Complete Digestion Buffer** by adding **5 µl of GenDNA Proteinase K** to **1 ml of GenDNA Digestion Buffer**.

1.11 Resuspend cells in **Complete Digestion Buffer**.

- For 1 to 1.5 million cells, use up to **500 µl of Complete Digestion Buffer**.

- It might be necessary to use more buffer to avoid viscosity when performing the extraction.

1.12 Incubate with shaking (500 rpm) at 50°C for 12 to 18 hours in tightly capped tubes.

STEP 2

DNA extraction & purification

MAGMEDIP QPCR KIT (C02010020)

2.1 Add **1 volume** (500 µl according to step 1.11) **of Phenol/ Chloroform/ Isoamyl alcohol (25:24:1)** to the cell sample to extract the genomic DNA.

CAUTION: *Work under a fume-hood.*

2.2 Incubate at room temperature for 10 minutes on a rotating wheel.

- Use gentle rotation, do not vortex.

2.3 Centrifuge at 1700 g at room temperature for 10 minutes.

- If the phases do not resolve properly, add **1 volume of GenDNA Digestion Buffer** without Proteinase K, and repeat the centrifugation.
- If there is a thick layer of white material at the interface between the phases, repeat the extraction.

2.4 Transfer the aqueous (top) layer to a new 2 ml nuclease-free tube.

2.5 Increase volume with **GenDNA Digestion Buffer** without Proteinase K to avoid viscosity if necessary and pipette slowly.

2.6 Add **1 volume** (500 µl) of **Phenol/Chloroform/Isoamyl alcohol (25:24:1)** to extract the genomic DNA.

CAUTION: *Work under a fume-hood.*

2.7 Incubate at room temperature for 10 minutes on a rotating wheel.

2.8 Centrifuge at 1700 g at room temperature for 10 minutes.

2.9 Transfer the aqueous (top) layer to a new 2 ml nuclease-free tube.

2.10 Add **½ volume** (250 µl) **of GenDNA Precipitant and 2 volumes (1 ml) of**

100% Ethanol.

2.11 Centrifuge at 1700 g at room temperature for 5 minutes to precipitate gDNA.

- 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.

2.12 Discard the supernatant.

2.13 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.

2.14 Resuspend the pellet of gDNA at ~ **1mg/ml in GenDNA TE** until dissolved.

2.15 Shake gently at room temperature or at 65°C for several hours to facilitate solubilization.

- 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 at 37°C for 1 hour, followed by Phenol/Chloroform/Isoamyl extraction and Ethanol precipitation (same protocol as above, starting from step 2.1).
- 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).

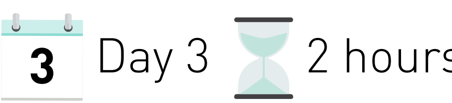
NOTE: *At this step the DNA quality can be analyzed by electrophoresis on 0.8% agarose gel or with a Fragment Analyzer (Agilent) and Genomic DNA Analysis Kit (DNF-487, Agilent) (please refer to manufacturer's instructions).*

2.16 Store the gDNA at 4°C until DNA shearing (Step 3).

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

STEP 3

DNA shearing

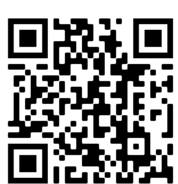


MAGMEDIP QPCR KIT (C02010020)

Genomic DNA must be randomly sheared by sonication on Diagenode Bioruptor® to generate DNA fragments around 200 bp (see Figure 1 in **Example of Results** section). For MagMeDIP assay, the intended amount of sheared DNA is needed in a total volume smaller than 50 µl. We recommend that you plan a surplus in DNA quantity of 10% for inputs and of 10% for pipetting errors.

3.1 Dilute the required amount of DNA in **GenDNA TE** and transfer the DNA sample into appropriate sonication tubes (see below).

3.2 Shear DNA by sonication using the Bioruptor® Pico (B01080010) or Bioruptor® Plus (B01020001). Choose the protocol and consumables which are adapted to your sample volume.



Please visit https://www.diagenode.com/en/protocols

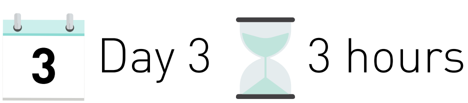
- For high amount/high volume samples: shear your DNA in 100 µl volume on Bioruptor® Pico (B01080010) using 0.65ml tube holder (B01201143) and dedicated 0.65ml Pico Microtubes (C30010011) for 13 cycles (30"" ON / 30"" OFF) OR on Bioruptor® Plus (B01020001) using 0.5/0.65ml tube holder (B01200043) and dedicated 0.5 ml Bioruptor Plus Microtubes (C30010013) for 30 cycles (30"" ON / 90"" OFF).

- For low amount/low volume samples: shear your DNA in 50 µl volume on Bioruptor® Pico (B01080010) using 0.2ml tube holder (B01201144) and dedicated 0.2ml Pico Microtubes (C30010020) for 13 cycles (30"" ON / 30"" OFF).

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

STEP 4

Library preparation



IDEAL DNA IP LIBRARY PREPARATION KIT (C05010024) IDEAL UDI MODULE (C05010025)

END PREPARATION

4.1 Prepare your sample in a total volume of 50 µl in a 0.2 ml nuclease-free tube as described in the following table:

Component	Quantity/Volume
Sheared DNA	1 µg to 10 ng + 10% surplus for inputs + 10% surplus for pipetting errors
Nuclease-free water	Up to 50 µl
Total Volume	**50 µl**

4.2 Add the following components into the reaction tube:

Component	Volume
iDeal DNA IP End Prep Enzyme Mix ●	3 µl
iDeal DNA IP End Prep Reaction Buffer ●	7 µl
Total Volume	**60 µl**

4.3 Mix thoroughly by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

4.4 Place in a thermal cycler with heated lid on (≥75°C) and run the following program:

Temperature	Time
20°C	30 min
65°C	30 min
4°C	Max 1 h hold

ADAPTER LIGATION

4.5 Determine **iDeal DNA IP Adapter ●** concentration necessary for the DNA quantity and dilute accordingly, with **iDeal Adapter Dilution Buffer ●**:

DNA quantity	Adapter Diltuion	Working adapter concentration
> 100 ng	No dilution	15 µM
100 ng – 10 ng	1/10	1.5 µM

4.6 Add the following components into the reaction tube:

Component	**Volume**
iDeal DNA IP Adapter ● at proper dilution for DNA quantity	2.5 µl
iDeal DNA IP Ligation Master Mix ●	30 µl
iDeal DNA IP Ligation Enhancer ●	1 µl
Total Volume	**93.5 µl**

NOTE: *Mix the iDeal DNA IP Ligation Master Mix by pipetting before use as it is very viscous.*

4.7 Mix thoroughly by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

4.8 Incubate at 20°C for 15 minutes in a thermal cycler with heated lid off.

4.9 Add **3 µl of iDeal DNA IP Uracil Excision Reagent ●** into the reaction tube.

4.10 Mix thoroughly by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

4.11 Incubate at 37°C for 15 minutes in a thermal cycler with heated lid on (≥47°C).

NOTE: *If necessary, samples can be stored overnight at -20°C but we recommend to carry on.*

SIZE SELECTION

CAUTION: If the starting material is > 50 ng, follow the protocol for ***size selection*** *only from steps 4.12 to 4.27. If the starting material is ≤ 50 ng, size selection is not recommended to maintain library complexity, thus follow the protocol for* ***clean-up*** *only from steps 4.28 to 4.39.*

CAUTION: The following size selection protocol is based on a starting volume of 96.5 µl per sample.

4.12 Carefully resuspend the **AMPure XP beads** at room temperature by shaking and light vortexing until no pellet is visible at the bottom of the container.

4.13 Add **40 µl of AMPure XP** beads and mix by pipetting 10 times until the mixture is homogeneous.

4.14 Incubate at room temperature for at least 5 minutes.

4.15 Briefly spin the tube, place it on the **Magnetic rack – DiaMag 0.2ml** and wait until the beads are completely bound to the magnet (~2 minutes).

4.16 Without disturbing the pellet, carefully aspirate and transfer the supernatant to a new 0.2 ml nuclease-free tube. Discard the beads that contain the unwanted large fragments.

4.17 Add **20 µl of AMPure XP beads**. Mix by pipetting 10 times until the mixture is homogeneous.

4.18 Incubate at room temperature for at least 5 minutes.

4.19 Briefly spin the tube, place it on the magnetic rack and wait until the beads are completely bound to the magnet (~2 minutes).

4.20 Carefully remove and discard the supernatant without disturbing the beads that contain the desired DNA targets.

4.21 Wash the beads pellet 2 times as follows:

- With the tube on the magnetic rack, add **200 µl** of freshly prepared **80% Ethanol** without disturbing the beads pellet and wait for 30 seconds.

- Carefully remove and discard the supernatant without disturbing the pellet.
- Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube, place it back on the magnetic rack and remove traces of ethanol with a pipette.

4.22 Leaving the tube open, let dry the beads on the magnetic rack for 3 minutes.

CAUTION: *Do not over dry the beads as it will decrease the DNA elution efficiency.*

4.23 Remove the tube from the magnetic rack and elute DNA by resuspending thoroughly the beads in 25 µl of Resuspension Buffer ● .

4.24 Incubate at room temperature for at least 2 minutes.

4.25 Briefly spin the tube, place it on the magnetic rack and wait until the beads are completely bound to the magnet (~ 2 minutes).

4.26 Without disturbing the pellet, carefully aspirate and transfer the supernatant to a new 1.5 ml nuclease-free tube. Label the tube as "LIBRARY".

4.27 Place the LIBRARY tubes on ice until further processing.

- The libraries are now ready for enrichment of methylated DNA by immunoprecipitation (Step 5).

NOTE: *If necessary, libraries can be stored at -20°C until further processing, but we advise to carry on with the protocol.*

CLEAN-UP

CAUTION: *The following clean-up protocol is based on a starting volume of 96.5 µl per sample.*

4.28 Carefully resuspend the **AMPure XP beads** at room temperature by shaking and light vortexing until no pellet is visible at the bottom of the container.

4.29 Add **87 µl of AMPure XP** beads and mix by pipetting 10 times until the

mixture is homogeneous.

4.30 Incubate at room temperature for at least 5 minutes.

4.31 Briefly spin the tube, place it on the **Magnetic rack – DiaMag 0.2ml** and wait until the beads are completely bound to the magnet (~2 minutes).

4.32 Carefully remove and discard the supernatant without disturbing the beads that contain the desired DNA targets.

4.33 Wash the beads pellet 2 times as follows:

- With the tube on the magnetic rack, add **200 µl** of **freshly prepared 80% Ethanol** without disturbing the beads pellet and wait for 30 seconds.
- Carefully remove and discard the supernatant without disturbing the pellet.
- Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube, place it back on the magnetic rack and remove traces of ethanol with a pipette.

4.34 Leaving the tube open, let dry the beads on the magnetic rack for 3 minutes.

CAUTION: *Do not over dry the beads as it will decrease the DNA elution efficiency.*

4.35 Remove the tube from the magnetic rack and elute DNA by resuspending thoroughly the beads in **25 µl of Resuspension Buffer ●** .

4.36 Incubate at room temperature for at least 2 minutes.

4.37 Briefly spin the tube, place it on the magnetic rack and wait until the beads are completely bound to the magnet (~ 2 minutes).

4.38 Without disturbing the pellet, carefully aspirate and transfer the supernatant to a new 1.5 ml nuclease-free tube. Label the tube as “LIBRARY”.

4.39 Place the LIBRARY tubes on ice until further processing.

- The libraries are now ready for enrichment of methylated DNA by

immunoprecipitation (Step 5).

NOTE: *If necessary, libraries can be stored at -20°C until further processing, but we advise to carry on with the protocol.*

STEP 5

Methylated DNA immunoprecipitation

MAGMEDIP QPCR KIT (C02010020)

BEADS PREPARATION

5.1 Determine the **number of IP reactions** to be run according to your number of samples. For each IP, 10% of the volume will be collected and will not undergo immunoprecipitation. This will correspond to the INPUT associated to the IP.

5.2 Prepare the **MagBuffer A 1x** in a 1.5 ml nuclease-free tube as described in the following table and keep it on ice.

Reagent (µl)	1 IP	2 IPs	3 IPs	4 IPs	5 IPs	6 IPs	7 IPs	8 IPs	9 IPs	10 IPs
MagBuffer A (5x)*	20	40	60	80	100	120	140	160	180	200
Nuclease-free water	80	160	240	320	400	480	560	640	720	800

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

5.3 Take the required amount of **MagBeads** and transfer it to a clean 1.5 ml nuclease-free 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.*

5.4 Place the tube on the **Magnetic rack – DiaMag 1.5ml** and wait until the beads are completely bound to the magnet (~1 minute).

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

5.6 Wash the beads pellet 2 times with ice-cold **MagBuffer A 1x** as follows:

- Take the required amount of ice-cold **MagBuffer A 1x** and transfer it to the tube containing the beads. **27.5 µl of MagBuffer A 1x** 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.

5.7 Take the required amount of ice-cold **MagBuffer A 1x** and transfer it to the tube containing the beads. **22 µl of MagBuffer A 1x** are needed per IP.

5.8 Resuspend the beads by pipetting up and down several times.

5.9 Keep the beads tube on ice until further processing.

CAUTION: *Do not freeze the beads.*

DNA IMMUNOPRECIPITATION

5.10 Prepare the **MagMaster Mix** in a 1.5 ml nuclease-free tube as described in the following table:

Reagent (µl)	1 IP	2 IPs	3 IPs	4 IPs	5 IPs	6 IPs	7 IPs	8 IPs	9 IPs	10 IPs
MagBuffer A (5x)*	27.6	55.2	82.8	110.4	138	165.6	193.2	220.8	248.4	276
MagBuffer B	6.9	13.8	20.7	27.6	34.5	41.4	48.3	55.2	62.1	69
Methylated spike-in control	0.35	0.7	1	1.4	1.7	2.1	2.4	2.8	3.1	3.5
Unmethylated spike-in control	0.35	0.7	1	1.4	1.7	2.1	2.4	2.8	3.1	3.5
Nuclease-free water	39.6	79.1	118.7	158.2	197.8	237.4	276.9	316.5	356	395.6

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

5.11 Take your **LIBRARY sample** from step 4.39. The volume should be 25 µl. If not, adjust by adding nuclease-free water.

5.12 Add **65 µl of MagMaster Mix** into each LIBRARY tube.

5.13 Incubate at 95ºC for 3 minutes in a thermal cycler to denature the DNA, and then cool down on ice.

5.14 Briefly spin the LIBRARY tube and place it back on ice for at least 10 minutes and maximum 30 minutes.

CAUTION: *Be careful to keep the denaturized DNA sample on ice to keep the DNA single-stranded and avoid re-annealing to double-stranded DNA.*

5.15 Check that your sample volume is still at least 85 µl. If it is not the case, fill it up to 85 µl by adding ice-cold **MagBuffer A 1X**.

NOTE: *For easier handling, please use the 0.2 ml tube in strips provided for INPUT and IP samples from this point on.*

5.16 Transfer **7.5 µl** from each LIBRARY tube into a new 0.2 ml nuclease-free tube.

- Label the tube as **INPUT** as this will be used as a control of your starting material and will NOT be used for immunoprecipitation.
- Keep at 4°C until further processing (step 6).

5.17 Transfer **75 µl** from each LIBRARY tube into a new 0.2 ml nuclease-free tube.

- Label the tube as **IP** as this will be the reaction tube for immunoprecipitation.
- Keep on ice until further processing (step 5.20).

5.18 Prepare the **Diluted Antibody 1:2** in a 0.2 ml nuclease-free tube with ice-cold 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	9 IPs	10 IPs
Antibody anti-5mC (33D3 clone)*	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0
Nuclease-free water	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0

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

5.19 Prepare the **Diluted Antibody Mix** in a 0.2 ml nuclease-free tube as described in the following table. Mix sequentially by pipetting the **Diluted Antibody 1:2** with **MagBuffer A 5x**, then with **Nuclease-free water** and finally with **MagBuffer C**.

Reagent (µl)	1 IP	2 IPs	3 IPs	4 IPs	5 IPs	6 IPs	7 IPs	8 IPs	9 IPs	10 IPs
Antibody diluted 1:2	0.35	0.7	1	1.4	1.7	2.1	2.5	2.7	3.1	3.5
MagBuffer A 5X	0.7	1.4	2.1	2.8	3.5	4.1	4.8	5.5	6.2	6.9
Nuclease-free water	2.4	4.8	7.2	9.7	12	14.5	16.9	19.3	21.7	24.1
MagBuffer C	2.3	4.6	6.9	9.2	11.5	13.8	16.1	18.4	20.7	23

5.20 Add **5 µl of Diluted Antibody Mix** into each IP tube from step 5.17.

5.21 Mix and add **20 µl** of washed **MagBeads** (from step 5.9) into each IP tube.

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

5.22 Place the IP tube on a rotating wheel at 4°C overnight.

DNA WASHES

CAUTION: *For washing steps (from steps 5.23 to 5.28), keep the IP tube on ice .*

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

5.24 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).

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

5.26 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.

5.27 Wash the beads once with 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.

5.28 Place the IP tube with beads pellet on ice until further processing.

- The samples are now ready for DNA isolation (Step 6).

STEP 6

DNA isolation

IPURE KIT V2 (C03010014)

DNA ELUTION

CAUTION: *For elution steps (from steps 6.1 to 6.10), keep the samples and buffers at room temperature to avoid crystals formation. If some crystals remain in solution, heat up to room temperature and mix gently until complete disappearance of such crystals.*

6.1 Take **Buffer A** from iPure kit V2 out and keep it at room temperature until it is clear before use at step 6.4.

6.2 Take the INPUT tube from step 5.16 and centrifuge briefly.

6.3 Take the IP tube with beads pullet from step 5.28.

- From now on, treat the INPUT sample and the IP sample in parallel.
- 1 IPure reaction corresponds to the purification of 1 IP sample **OR** 1 INPUT sample.

6.4 Prepare the **Elution Buffer** in a 1.5 ml nuclease-free tube as described in the following table:

Reagent (µl)	2 rxns	4 rxns	6 rxns	8 rxns	10 rxns	12 rxns	14 rxns	16 rxns	18 rxns	20 rxns
Buffer A	230.8	461.6	692.4	923.2	1154	1384.8	1615.6	1846.4	2077.2	2308
Buffer B	9.2	18.4	27.6	36.8	46	55.2	64.4	73.6	82.8	92

6.5 Add **92.5 µl of Elution Buffer** to the INPUT tube.

6.6 Add **50 µl of Elution Buffer** to the IP tube.

6.7 Incubate at room temperature for 15 minutes on a rotating wheel (40 rpm).

6.8 Spin down the tubes, place it on the magnetic rack, and wait until the beads are completely bound to the magnet (~1 minute).

6.9 Without disturbing the pellet, carefully aspirate and transfer the supernatant to new labelled tubes.

6.10 For the IP sample **only**, repeat steps 6.6 to 6.9 for a total of 2 elutions in **50 µl of Elution Buffer** (total volume of 100 µl).

- Elutions of IP and INPUT samples are now completed in 100 µl.

DNA BINDING

6.11 Add **2 µl of carrier** per tube, vortex briefly and perform a short spin.

6.12 Add **100 µl of 100% Isopropanol** (not supplied) per tube, vortex briefly and perform a short spin.

NOTE: *Following the addition of isopropanol, the solution may become cloudy. This is not detrimental to your experiment and will not influence the quality or quantity of your purified DNA.*

6.13 Resuspend the **iPure Beads V2**.

6.14 Add **10 µl of iPure Beads V2** per tube, vortex regularly the beads to ensure homogeneity and uniform distribution.

NOTE: *Keep iPure Beads V2 in liquid suspension during storage at 4°C and at all handling steps, as drying will result in reduced performance.*

6.15 Incubate at room temperature for 10 minutes on a rotating wheel (40rpm).

DNA WASHING

6.16 When using the iPure kit V2 for the first time, prepare the **Wash Buffer 1** containing **50% Isopropanol** (not supplied) as follows:

Kit component	Volume
Wash Buffer 1 w/o isopropanol	1.5ml
Isopropanol (100%)	1.5ml
Total Volume	**3 ml**

NOTE: *Never leave the bottle open to avoid evaporation.*

6.17 Spin down the tubes from step 6.15, place them on the magnetic rack, and wait until the beads are completely bound to the magnet (~1 minute).

6.18 Carefully remove and discard the supernatants without disturbing the beads.

6.19 Add **100 µl of Wash Buffer 1** per tube, close the tube cap, and invert the tube to resuspend the beads.

6.20 Incubate at room temperature for 5 minutes on a rotating wheel (40 rpm).

6.21 When using the iPure kit V2 for the first time, prepare the **Wash buffer 2** containing **50% isopropanol** (not supplied) as follows:

Kit component	Volume
Wash Buffer 2 w/o isopropanol	1.5ml
Isopropanol (100%)	1.5ml
Total Volume	**3 ml**

CAUTION: *Never leave the bottle open to avoid evaporation.*

6.22 Spin down the tubes from step 6.20, place them on the magnetic rack, and wait until the beads are completely bound to the magnet (~1 minute).

6.23 Carefully remove and discard the supernatants without disturbing the beads.

6.24 Add **100 µl of Wash buffer 2** per tube, close the tube cap, and invert the tube to resuspend the beads.

6.25 Incubate at room temperature for 5 minutes on a rotating wheel (40 rpm).

DNA ELUTION

6.26 Spin down the tubes, place them on the magnetic rack, and wait until the beads are completely bound to the magnet (~1 minute).

6.27 Carefully remove and discard the supernatants without disturbing the beads.

6.28 Add **25 µl of Buffer C** per tube, close the tube cap, and invert the tube to resuspend the beads.

6.29 Incubate at room temperature for 15 minutes on a rotating wheel (40 rpm).

6.30 Resuspend the beads by pipetting.

6.31 Spin down the tubes, place them on the magnetic rack, and wait until the beads are completely bound to the magnet (~1 minute).

6.32 Without disturbing the beads pellet, carefully aspirate and transfer the supernatants to new labelled tube.

6.33 Place the tubes on ice until further processing or store them at -20°C or -80°C until further use.

- The samples are now ready for library amplification (Step 8). We recommend to perform a quality control by qPCR to check the MeDIP efficiency (Step 7).

STEP 7

Quality control & MeDIP efficiency

MAGMEDIP QPCR KIT (C02010020)

At this step, it is possible to check the MeDIP efficiency by performing qPCR on a few microliters of each sample.

The MagMeDIP qPCR kit includes 4 validated primer pairs that target different DNA sequences:

1. Primer pair for external methylated DNA spike-in control
2. Primer pair for external unmethylated DNA spike-in control
3. Primer pair for internal human TSH2B gene (also known as HIST1H2BA or H2BC1) (methylated region)
4. Primer pair for internal human GAPDH gene (unmethylated region)

NOTE: *Primer pairs for mouse and rat samples are also available.*

Please visit https://www.diagenode.com/en/categories/primer-pairs

NOTE: *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.*

7.1 Take few microliters of each of the IP and INPUT samples and dilute them 10 times.

- **5 µl of 1:10 diluted sample** are needed per qPCR reaction.
- We advise to control the 4 DNA sequences and to perform PCR in duplicates for a total of 8 qPCR reactions per sample.

CAUTION *Keep IP and INPUT tubes from Step 6 on ice during qPCR.*

7.2 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.

Component	For 1 qPCR rxn
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**

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

7.4 Add **5 µl of 1:10 diluted sample.**

7.5 Seal the strip or plate, briefly vortex, and run the following program on the qPCR machine:

Step	Temperature	Time	Cycles
Denaturation*	95°C	7 min	1
Amplification*	95C	15 sec	40
	60°C	1 min	
	95°C	1 min	
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.*

7.6 Data analysis:

IP efficiency

The efficiency of methylated DNA immunoprecipitation can be calculated for each targeted DNA sequence 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 and INPUT samples 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). 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. The real amplification efficiency, if known, should be used.*

IP enrichment and specificity

To determine immunoprecipitation success, enrichment of methylated DNA over background noise can be calculated:

Enrichment= %recovery specific locus/ % recovery background locus

In this formula, methylated DNA spike-in & TSH2B are the specific loci, while unmethylated spike-in & GAPDH are the corresponding background loci detected during the qPCR.

IP specificity can be calculated:

Specificity = 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%

STEP 8

Library amplification

IDEAL DNA IP LIBRARY PREPARATION KIT (C05010020)
IDEAL UDI MODULE (C05010025)

OPTIMAL CYCLE NUMBER

Before the amplification, you will have to determine for each IP and INPUT sample the optimal number of PCR cycles by performing a qPCR.

NOTE: *For this step, only 1 µl of each library from Step 6 will be used.*

8.1 Prepare the **Quantification Mix** as described in the following table for the number of desired reactions. Mix by pipetting and keep on ice until use.

Component	For 1 qPCR rxn
iDeal DNA IP PCR Master Mix ●	5 µl
iDeal Primer UDI #** ●	0.2 µl *
100X SYBR ●	0.1 µl *
Nuclease-free water	3.7 µl
Total Volume	**9 µl**

**If the number of reactions is small, an intermediate dilution can be used if the needed volume is too low for pipetting. The volume of nuclease-free water should be adapted to keep a final volume of 9 µl per reaction.*

***All iDeal Primer UDI to be subsequently used at library amplification step to label individual libraries can be pooled in equal proportions to create an index mix for the qPCR. This will help to determine the average number of cycles needed to amplify each sample regardless of which index is assigned to the sample.*

8.2 Transfer **9 µl of the Quantification Mix** into a qPCR appropriate 0.2 ml nuclease-free tubes or strips.

8.3 Add **1 µl of IP or INPUT sample** in each tube and mix by pipetting.

8.4 Briefly spin the tubes and run the following program on the qPCR machine:

Step	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	20
Annealing/extension	65°C	75 sec	
Melting curve	Follow qPCR instrument manufacturer recommendations		

***CAUTION**: Keep IP and input samples from Step 6 on ice during qPCR.*

8.5 Analyse the Ct values. The optimal cycle number for the amplification of the MeDIP samples is typically Ct (rounded up) + 1 (e.g. for a Ct of 6.82 use 8 amplification cycles).

***NOTE:** The Ct value is highly dependent on the thermocycler you use, as well as the way you analyse the qPCR results. Thus, when using the kit for the first time you may need to verify that the Ct+1 rule applies well in your conditions.*

PCR AMPLIFICATION

8.6 Take the IP and INPUT tubes from Step 6.

8.7 Check the volume of each tube and fill up to 24 µl by adding **nuclease-free water** if necessary.

8.8 Add **25 µl of iDeal DNA IP PCR Master Mix** ● per tube.

8.9 Add **1 µl of iDeal Primer UDI** ● per tube, using a different UDI for each sample you plan to sequence on the same lane of the sequencing flowcell.

8.10 Briefly spin down the tubes and run the following program with heated lid on (105°C):

Step	Temperature	Time	Cycles00
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	Rounded-up Ct+1
Annealing/extension	65°C	75 sec	
Final Extension	65°C	5min	1
Hold	4°C	∞	

CLEAN-UP

8.11 Carefully resuspend the **AMPure XP beads** at room temperature by shaking and light vortexing until no pellet is visible at the bottom of the container.

8.12 Add **45 µl of AMPure XP** beads to each tube and mix by pipetting 10 times until the mixture is homogenous.

8.13 Incubate at room temperature for at least 5 minutes.

8.14 Briefly spin down the tubes, place them on the magnetic rack and wait until the beads are completely bound to the magnet (~ 2 minutes).

8.15 Carefully remove and discard the supernatants without disturbing the beads that contain the desired DNA targets.

8.16 Wash the beads pellet twice as follows:

- With the tube on the magnetic rack, **add 200 µl of freshly prepared 80% Ethanol** without disturbing the beads pellet and wait for 30 seconds.
- Carefully aspirate and discard the supernatant without disturbing the pellet.

8.17 Leaving the tubes open, let dry the beads on the magnetic rack for 3 minutes. If necessary, briefly spin down the tubes, place them back on the magnetic rack and remove traces of ethanol with a pipette.

8.18 Remove the tubes from the magnetic rack and elute DNA by resuspending thoroughly the beads in **20 µl of Resuspension Buffer** ●

NOTE: *If the DNA concentration is critical, it is possible to reduce the elution volume down to 15 µl.*

8.19 Incubate at room temperature for 5 minutes.

8.20 Briefly spin down the tubes, place them on the magnetic rack and wait until the beads are completely bound to the magnet (~2minutes).

8.21 Without disturbing the pellet, carefully aspirate and transfer the

supernatants to new labelled nuclease-free tubes.

8.22 Place the IP and INPUT tubes on ice until further processing or store at -20°C for longer storage.

- The samples are now ready for sequencing. We recommend to perform a quality control first on the libraries (Step 9).

STEP 9

Quality control &
Next-Generation Sequencing

9.1 For quantification, measure the concentration of each library using a fluorescence-based assay, such as the QuBit® High Sensitivity assay kit, according to the manufacturer's instructions.

9.2 For library size estimation, run a DNA High Sensitivity assay kit for BioAnalyzer® or on Fragment Analyzer (Agilent) according to the manufacturer's instructions.

NOTE: *In some cases, for example if adapter dimers (around 150 bp) are still present, a new 0.9X bead clean-up can be performed.*

- The libraries are now ready for pooling and sequencing.

Sequencing Recommendations

We recommend to sequence individual MagMeDIP-seq libraries with a **minimum of 50 million raw reads** in case of human samples in order to sufficiently cover the library content for downstream analysis. The read depth should be adjusted depending on the genome size of your species of interest.

We recommend paired-end sequencing with 50 bp read length as it is the most cost-effective solution. However, it is also possible to use longer reads or single-end sequencing.

MagMeDIP-seq libraries can be pooled together at desired molar ratios to allow multiplex sequencing. Such libraries should have been built with different indexes. The minimal molar concentration needed to sequence the pool depends on the requirements of the sequencing platform.

MagMeDIP-seq libraries can be sequenced as regular Illumina Dual Indexed libraries, meaning that they do not require any specific considerations regarding cluster density optimization. However, we recommend to spike 1-5% of PhiX Control in the library pool that is about to be sequenced for a quality monitoring purpose.

Data Analysis Recommendation

Reads trimming, alignment, deduplication and filtering

Recommended reads processing:

- Perform trimming of the potential adapters and low-quality bases with cutadapt.
- Assess the quality of the reads before and after trimming using FastQC.
- Align the trimmed reads to the reference genome using Burrows-Wheeler Aligner.
- Filter the resulting alignment files (e.g. removal of PCR duplicates) using Samtools.

MagMeDIP-seq data analysis

Following the alignment to the reference genome, we recommend the use of the R package MeDIPS for quality control, genomic coverage estimation and differential coverage analysis. Unlike other software, MEDIPS normalizes for CpG density that can introduce bias towards the identification of regions with higher CpG frequency.

When generating MEDIPS datasets, we recommend using the following parameters:

- uniq=1: to remove PCR duplicates if this filtering was not performed on the alignment BAM file.
- extend: not used as the fragment length will be determined from the paired-end reads.
- shift=0: number of nucleotides the reads need to be shifted for.
- window size=100: size of the adjacent windows (100 bp) the reference genome will be divided into. Further calculation such as coverage and differential analysis will be calculated from the information stored in each window.

CpG density over the reference genome was generated using a MEDIPS dataset and the appropriate MEDIPS.couplingVector() function. MEDIPS

datasets from replicates can optionally be concatenated into a single dataset and the correlation between replicates can be assessed using the MEDIPS.correlation() functionality. An estimation of the sufficient sequencing depth can be obtained with MEDIPS.saturation() function.

MeDIP-seq specific quality controls include CpG coverage and CpG enrichment scores that can be calculated with the MEDIPS.seqCoverage() and MEDIPS.CpGenrich() functions, respectively.

Differentially methylated windows between an IP sample and its corresponding INPUT or between two IP samples can be calculated with the MEDIPS.meth() function using the edgeR method and Bonferroni adjusted p-values, and considering only the windows with a coverage higher than 10 (minRowSum=10). Windows with significant differences, considering an adjusted p-value threshold of 0.1, can then be selected and adjacent windows merged into regions defined as differentially methylated.

Links for the tools used in the example pipeline:

Tool	Link
Website	https://www.diagenode.com/en
Cutadapt	https://github.com/marcelm/cutadapt
FastQC	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Burrows-Wheeler Aligner	http://bio-bwa.sourceforge.net/bwa.shtml
Samtools	http://www.htslib.org/download
MeDIPS	https://bioconductor.org/packages/release/bioc/html/MEDIPS.html

Example of Results

Figure 1. BioAnalyzer (Agilent) High Sensitivity DNA chip profile of sheared human gDNA: smear around 200 bp. gDNA 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).

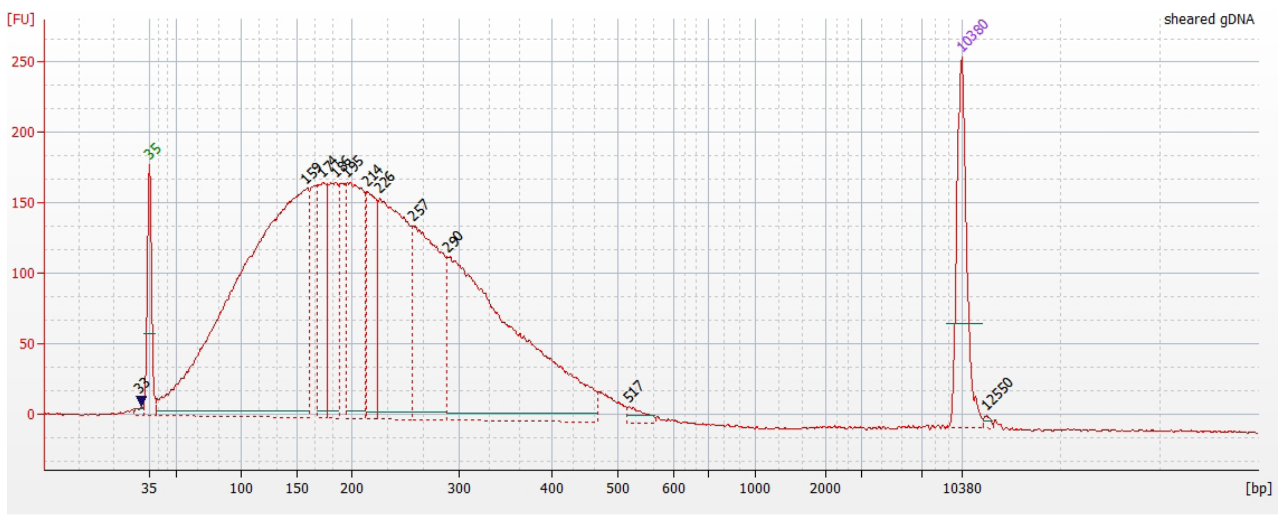


Figure 2. qPCR analysis of external spike-in DNA controls (methylated and unmethylated) after IP. Samples were prepared using 1µg – 100ng -10ng sheared human gDNA with the MagMeDIP-seq Package V2.

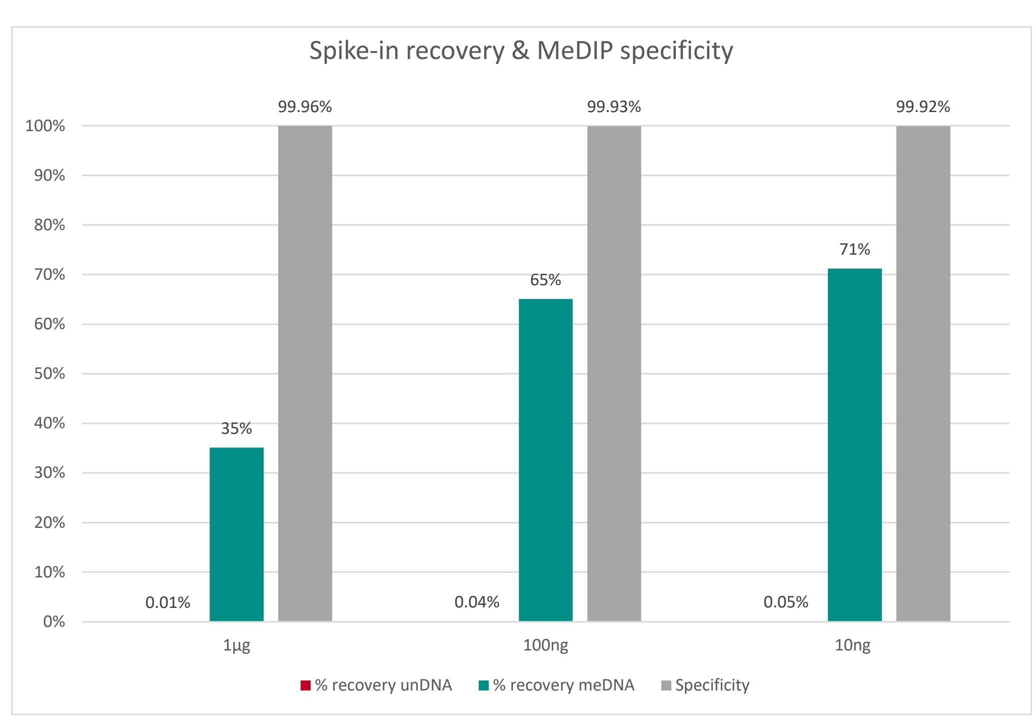


Figure 3. BioAnalyzer (Agilent) High Sensitivity DNA chip profile of IP (red) and corresponding INPUT (blue) libraries prepared from 100 ng sheared human gDNA with the MagMeDIP-seq Package V2.

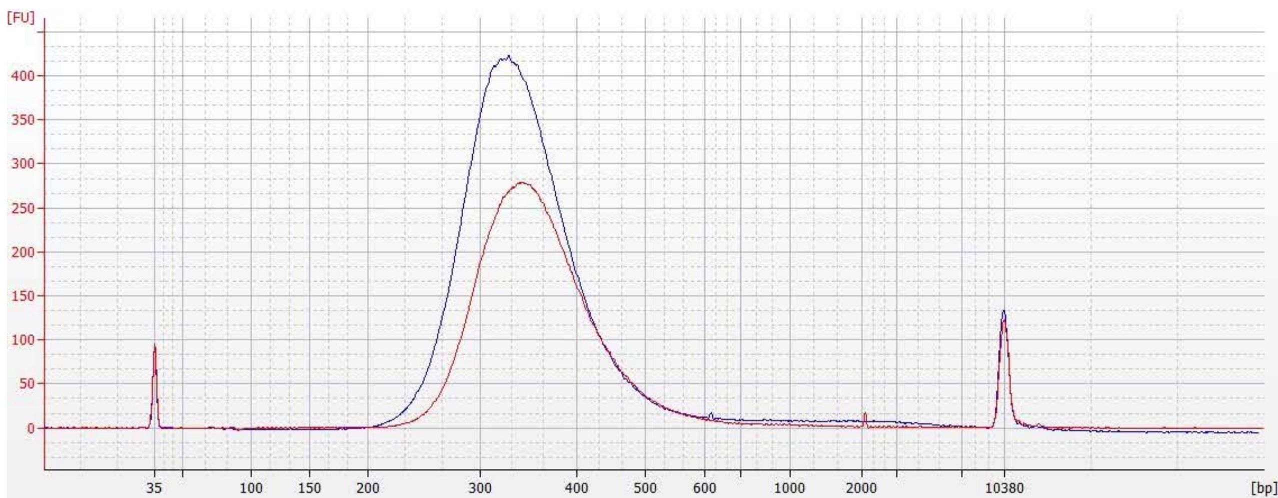
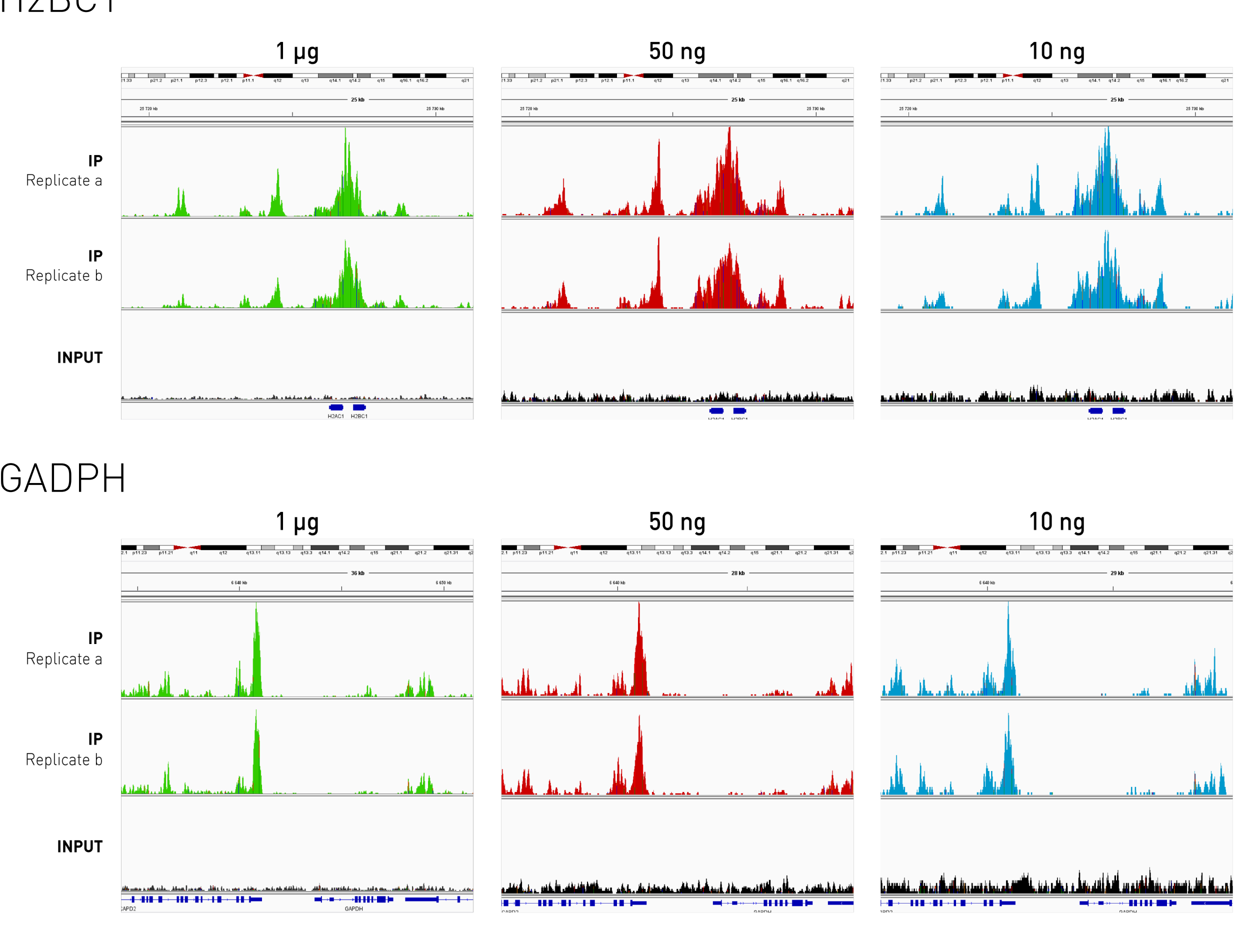


Figure 4. Sequencing profiles of MeDIP-seq libraries prepared from different starting amounts of sheared gDNA on the positive and negative methylated control regions. MeDIP-seq libraries were prepared from decreasing starting amounts of gDNA (1 µg (green), 50 ng (red), and 10ng (blue)) originating from human blood with the MagMeDIP-seq Package V2. 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 H2BC1 (also known as HIST1H2BA or TSH2B) gene (marked by blue boxes in the bottom track) and its surroundings. The H2BC1 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 assays?

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 50-600bp, after library preparation and IP we observed fragment size between 50bp and maximum 450bp (thus libraries including adaptors around 200-600bp). On Illumina sequencers, clustering of libraries above 500bp is not very efficient, thus you can expect a resolution between 50bp and 350bp.

Can I use another DNA extraction kit than the XL GenDNA extraction? Can we clean up the genomic DNA using columns instead of using Phenol/ Chloroform/Isoamyl alcohol?

The XL GenDNA extraction module allows the extraction of high molecular weight DNA from cultured cells. For such samples, you can use columns instead of Phenol/ Chloroform/Isoamyl alcohol for clean-up, as long as you are confident with the quality of the genomic DNA you will get at the end. For blood or tissue samples, you should use other specific kits for gDNA extraction.

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

As long as you obtain DNA fragments from 50-600 bp, you can use the fragmentation technique of your choice. We usually do not recommend using restriction enzymes because the fragmentation is less random than with sonication.

If I have sheared DNA fragments with a mean size other than 200bp, how do I proceed with the size-selection during the library preparation (step 4)?

Recommended conditions for bead-based size selection, starting from a sample volume of 96.5 µl are provided below:

Approximated mean sheared DNA size	150bp	200bp	250bp	300-400bp	400-500bp	500-700bp
1st bead addition	50	40	30	25	20	15
2nd bead addition	25	20	15	10	10	10

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. Note that a lack of antibody can result in lower IP efficiency, whereas an excess of antibody might lead to lower IP specificity.

What is the lowest DNA starting amount that we can use with the kit?

We recommend using at least 10 ng of sheared DNA per IP. Note that a lack of antibody can result in lower IP efficiency, whereas an excess of antibody might lead to lower IP specificity. These conditions provide high sequencing data quality. Nevertheless, the antibody quantity can be further optimized to your experimental conditions.

Can I use another antibody with the kit?

The kit has been extensively validated with Diagenode highly specific 5-mC antibody (clone 33D3, C15200081) for high quality sequencing, therefore we do not recommend changing the antibody.

Why should I use the spike-in controls?

The methylated and unmethylated DNA spike-in controls and their associated qPCR primers 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 using these controls is to confirm the immunoprecipitation specificity.

Is it mandatory to use the spike-in controls for MeDIP-seq experiments?

The DNA spike-in controls are designed for qPCR validation that you may want to do before sequencing. If you do not perform qPCR validation, there is no need to add them because they will not be sequenced.

Is it mandatory to add the spike-in controls in all samples?

We recommend to add the DNA spike-in controls to each sample, but you can also choose to add them in only one sample per experimental condition.

Can I buy spike-in controls separately?

The kit contains enough spike-in controls for all the reactions. If you need more spikein controls, you can buy the corresponding DNA Methylation Control Package V2 (C02040019) as a separate product.

Note that the DNA Methylation Control Package V1 (C02040012) provides other methylated and unmethylated DNA spike-in controls and their associated qPCR primers. Those controls are different since they are produced from the genome of Arabidopsis thaliana and may therefore interfere with plant samples. Nevertheless, they were validated with Diagenode MagMeDIP assays and are compatible with every other sample species.

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 control products?

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

- unDNA: 110 pb
- meDNA: 103 pb
- TSH2B: 170 pb
- GAPDH: 64 pb

Why do I need to add the adapters before the immunoprecipitation step? Would it be possible to do the IP first and then make the libraries from the resulting library?

Before MeDIP, the DNA has to be denaturated in order to allow the antibody to target the 5-mC. As the standard Illumina adapters are double-stranded, for the ligation to work, the DNA template has to be double-stranded as well. This is why adapter ligation has to be performed before DNA denaturation and thus before MeDIP for MeDIP-seq protocols.

I would like to use the methylated DNA spike-in control as a positive control for sequencing. Has this methylated DNA spike-in control control been used as internal sequencing control before?

In the Diagenode MagMeDIP-seq protocol, the DNA spike-in controls are added after the library preparation step, therefore they do not have the adapters that are required for sequencing. For a use in sequencing, the spike-in controls should be added before the library preparation step, but it has not been validated so far.

Related Products

Product	Cat. No.
MagMeDIP qPCR kit	C02010020
iPure kit V2	C03010014
iDeal DNA IP Library Preparation kit	C05010024
iDeal Unique Dual Indexes module	C05010025
XL GenDNA Extraction Module	C03030020
DNA Methylation control package V2	C02040019
5-methylcytosine (5-mC) monoclonal antibody 33D3	C15200081
Human meDNA primer pair (TSH2B)	C17011041-500
Human unDNA primer pair (GAPDH)	C17011047-500
DiaMag 0.2ml – magnetic rack	B04000001
DiaMag 1.5ml – magnetic rack	B04000003
DiaMag rotator	B05000001
Bioruptor® Pico sonication device	B01080010

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