diagenode

Innovating Epigenetics Solutions

MethylCap kit

Methylated DNA Capture kit

Cat. No. C02020010 (48 rxns)

USER GUIDE

Version 6 10_2020

Contents

Introduction

Overview

Methylation of CpG dinucleotides is generally associated with epigenetic silencing of transcription and is maintained through cellular division. Multiple CpG sequences are rare in mammalian genomes, but frequently occur at the transcriptional start site of active genes, with most clusters of promoter CpGs being hypomethylated (1).

The binding specificity of the H6-GST-MBD fusion protein to un-, hemi- and fully methylated DNA was evaluated using synthetic DNA that either contained three methylated CpGs (GAM3), three hemimethylated CpG's or no methylated CpGs (GAM). Hemimethylated DNA does not stably interact with the MBD of MeCP2. A single fully methylated CpG is sufficient for the interaction between the H6-GST-MBD fusion protein and methylated DNA, whereas there is little binding to a hemimethylated target sequence (1).

Reference:
1. Kangaspeska S, Stride B, Métivier R, Polycarpou-Schwarz M, Ibberson D, Carmouche RP, Benes V, Gannon F, Reid G. 2008 Transient cyclical methylation of promoter DNA. Nature 452(7183):112-5.

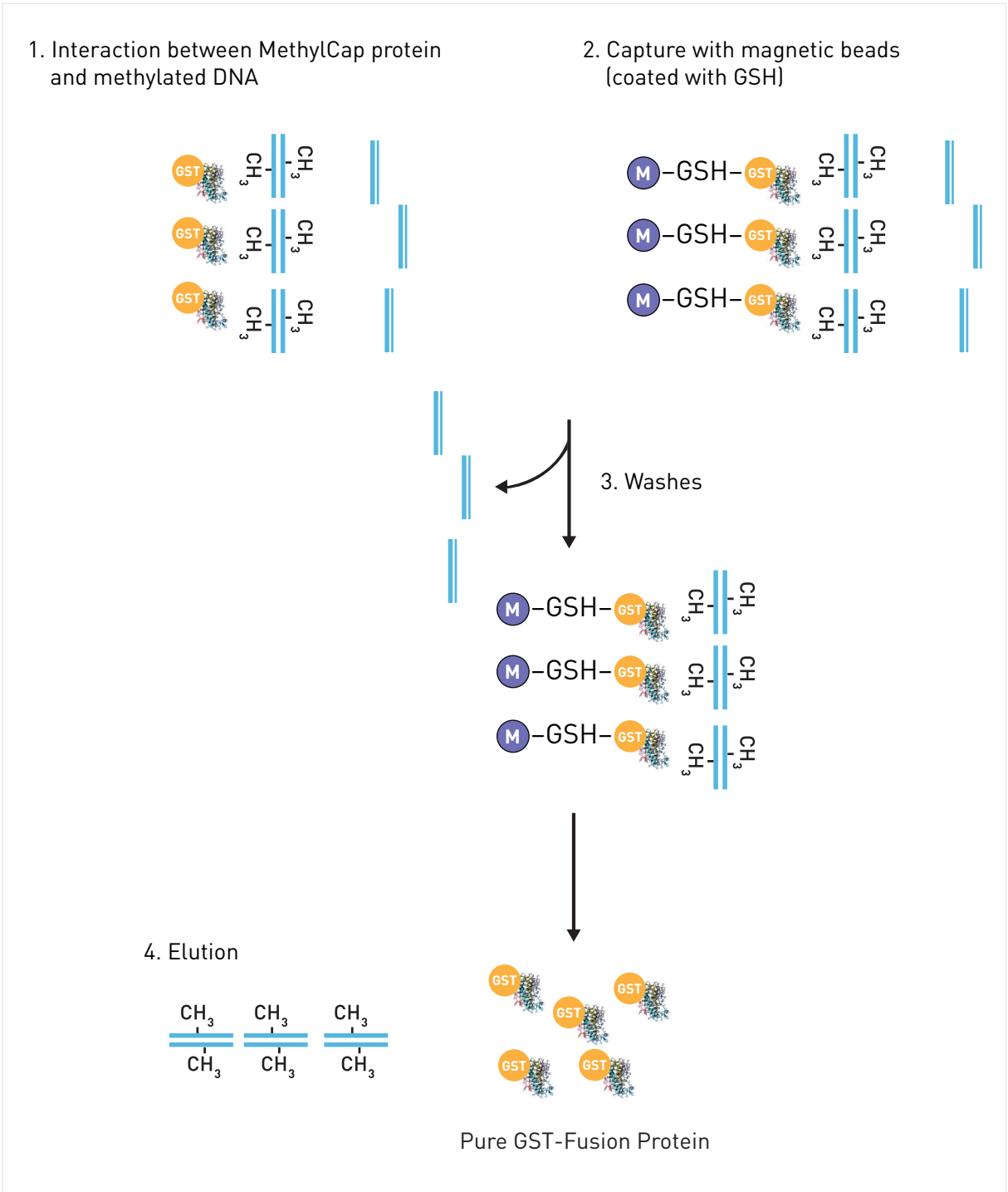
Product description

The MethylCap kit allows to specifically capture DNA fragments containing methylated CpGs. The assay is based on the affinity purification of methylated DNA using the MethylCap protein (Cat. No. C02020012) which has been extensively validated. The latter consists of the methyl-CpG-binding domain (MBD) of human MeCP2, as a C-terminal fusion with Glutathione-S-transferase (GST) containing an N-terminal His6-tag.

Libraries of captured methylated DNA can be prepared for next-generation sequencing (NGS) by combining MBD technology with the MicroPlex Library Preparation kit (Cat. No. C05010001 & C05010002).

Kit Method Overview

Prior to MethylCap, DNA is extracted and sheared using the Bioruptor® Sonicator (see our online DNA shearing Guide: https://www.diagenode.com/en/dna-shearing-guide).



Kit Materials

Kit content

The kit content is sufficient to perform 48 capture reactions (meDNA captures).
The kit content is described in Table 1. Upon receipt, store the components at the temperatures indicated in Table 1.

Required materials not provided

Reagents and equipment for capture and qPCR analysis

- Gloves to wear at all steps
- Autoclaved tips
- RNAse/DNase-free 1.5-ml (or 2-ml) tubes
- Water
- DNA at 0.1 µg/µl (see additional protocols for preparation)
- Diagenode Magnetic Rack (Cat. No. B04000001)
- Centrifuges for 1.5-ml tubes (4°C)
- Rotating wheel (4°C)
- Vortex
- Thermomixer (55°C, 95°C)

Reagents and equipment for the DNA preparation and shearing

- Tubes: 1.5-ml and 50-ml conical tubes
- Trypsin-EDTA
- Ice-cold PBS buffer
- Agarose gel reagent and equipment
- Centrifuges for 1.5-ml tubes and 50-ml conical tubes (4°C)
- Cell counter
- Bioruptor®: one of the sonication apparatus from Diagenode (website: http://www.diagenode.com/)

Reagents for captured DNA purification with Phenol/Chloroform/Isoamyl alcohol

- DNA Precipitant (Cat. No. C03030002)
- DNA Co-precipitant (Cat. No. C03030001)
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24:1)
- Ethanol 100%
- Ethanol 70%
- Fume hood

Note: *IPure kit V2 (Cat. No. C03010014 & C03010015)*
or MicroChIP DiaPure columns (Cat. No. C03040001)
can be used as an alternative for captured DNA purification.

Kit modules and components

Table 1: Kit content

Component	Description	Format	Storage
Buffer B (Capture)		40 ml	4°C
Wash Buffer 1		16 ml	4°C
Wash Buffer 2		32 ml	4°C
MethylCap Beads	Do not freeze	1700 µl	4°C
MethylCap Protein		55 µl	-20°C or -80°C
Low Elution Buffer		16 ml	4°C
Medium Elution Buffer		16 ml	4°C
High Elution Buffer		32 ml	4°C
hum meDNA primer pair (TSH2B)		500 µl	-20°C
hum unDNA primer pair (GAPDH)		500 µl	-20°C

Table 2: Components available separately

Component	Reference	Format	Storage
hum meDNA primer pair (TSH2B)	C17011041-500	500 µl	-20°C
hum unDNA primer pair (GAPDH)	C17011047-500	500 µl	-20°C
mouse meDNA primer pair (TSH2B)	C17021042-500	500 µl	-20°C
mouse unDNA primer pair (GAPDH)	C17021045-500	500 µl	-20°C
rat meDNA primer pair (TSH2B)	C17031043-500	500 µl	-20°C
rat unDNA primer pair (GAPDH)	C17031046-500	500 µl	-20°C
MethylCap protein	C02020012	50 rxns	-20°C or -80°C
DiaMag02 magnetic rack	B04000001	1 unit	RT

Table 3. Modules available separately

Description	Comments	Reference	Quantity
XL GenDNA Extraction Module	For easy and fast DNA extraction	C03030020	60 rxns
IPure kit v2	For high-efficiency DNA purification	C03010014 & C03010015	24 rxns & 100 rxns
MicroChIP DiaPure columns	For high-efficiency DNA purification	C03040001	50 rxns
MicroPlex Library Preparation Kit v3	For DNA library preparation	C05010001 & C05010002	48 rxns & 96 rxns

Protocol

STEP 1. DNA shearing

Genomic DNA must be randomly sheared by sonication to generate fragments around 400 bp (see example below). To perform the MethylCap, at least 1 µg of sheared DNA is needed in a volume smaller than 20 µl.

To choose the best protocol for the sonication with Bioruptor, use our online DNA Shearing Guide: https://www.diagenode.com/en/dna-shearing-guide

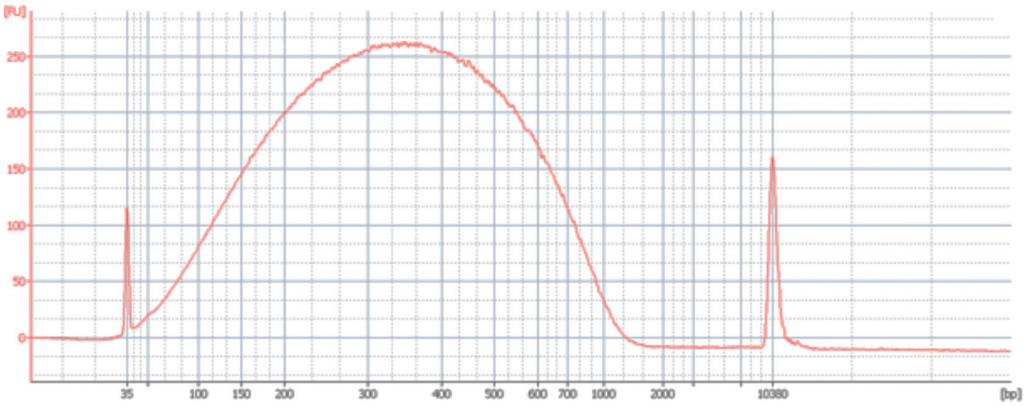
Only use the recommended tubes!

Example of shearing for MethylCap using the Bioruptor® Pico

The genomic DNA was diluted in TE buffer 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:

- Cycles: [15 seconds "ON" & 90 seconds "OFF"]
- 8 cycles



Agilent High Sensitivity DNA chip profile of sheared genomic DNA: smear around 400 bp

STEP 2. Capture of methylated DNA

- Before using the kit for the first time prepare aliquots of the MethylCap protein.

a. Thaw the MethylCap protein on ice.
b. Make 5 aliquots of 11 µl (10 capture reactions per aliquot) to avoid multiple freeze-thaw cycles.
c. Store at -80°C

Capture start

1. In a new 1.5-ml tube, prepare the capture reaction mix without MethylCap protein. For one reaction, see volume needed below (Table1). Vortex for 5 seconds, at medium power and keep on ice.

- DNA at 0.1 µg/µl. If not, adjust volumes of Buffer B.
- Note that the capture reaction mix contains an excess of 22.8 µl for 1 INPUT sample.

Table 1: Capture reaction mix without MethylCap protein

Reagent	Volume per capture reaction and INPUT sample (1 µg of DNA)
Sheared DNA (0.1 µg/µl)	12 µl
Buffer B	129.8 µl
TOTAL VOLUME	**141.8 µl**

2. Per capture reaction, dispense 119 µl of ice cold capture reaction mix in a new tube. Add 1 µl of MethylCap protein, each capture reaction to have a complete capture reaction mix of 120 µl.

- Use 8 x 200-µl tube strips
- Keep the excess at 4°C to be used later as input samples.

3. Incubate at 40 rpm on a rotating wheel for 2 hours at 4°C.

In the meantime: Bead Washes

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

4. Resuspend the provided meDNA Capture beads by pipetting and transfer the amount of beads needed for the number of capture reactions of the day into a new tube.

- 30 µl of beads are needed per capture reaction. Transfer 35 µl of capture beads per capture reaction to new tube.

5. Centrifuge at 1,300 rpm for 5 minutes at 4°C, discard the supernatant and keep the bead pellet.

6. Wash the provided meDNA Capture beads twice with ice-cold Buffer B (Capture) as follows: add Buffer B, resuspend the beads by pipetting, then centrifuge at 1,300 rpm for 5 minutes at 4°C, discard the supernatant and keep the bead pellet.

- To 35 µl of beads (one capture reaction), add 100 µl of Buffer B.
- To 280 µl of beads (8 capture reactions), add 800 µl of Buffer B.

7. After washing, resuspend the beads in Buffer B to the volume originally used. Keep on ice.

   - 35 µl of Buffer B to perform one capture reaction.
   - 280 µl of Buffer B to perform 8 capture reactions.

Capture continuation

8. Resuspend the beads by pipetting and add 30 µl of washed beads per capture reaction in 120 µl of complete capture reaction mix.

   - Keep the beads homogenously in suspension at all times by pipetting. Variation in the amount of beads will lead to lower reproducibility.

9. Incubate at 40 rpm on a rotating wheel for 1 hour at 4°C.

STEP 3. Bead washes

- We recommend the use of the Diagenode Magnetic Rack (Cat. No. C03030001).
- Do not disturb the beads attached to the tube wall.
- Always briefly spin the tubes to bring down liquid caught in the lid prior to positioning in the Diagenode Magnetic Rack.

10. Place the Wash Buffer 1, Wash Buffer 2 and Magnetic Rack on ice.

11. After capture reaction, spin the tubes and place in the ice-cold Magnetic Rack. Wait 1 minute and discard the buffer with a pipette. This buffer contains DNA unbound to the magnetic beads (flow-through).

- To analyse DNA present in this fraction, do not discard this buffer but transfer in a new 1.5-ml tubes for the purification (STEP 4 - Point 20).

12. Wash the Beads as follows. Add per capture reaction tube, 150 µl ice-cold Wash Buffer 1, close the tube caps, invert the 8-tube strip to resuspend the beads, incubate for 5 minutes at 4°C on a rotating wheel (40 rpm), spin, place in the Magnetic Rack, wait 1 minute and discard the buffer with a pipette. Keep the beads.

13. Wash twice the beads (as described above: Point 12) with 150 µl ice-cold Wash Buffer 2. After the last wash, proceed to the elution step.

- Spin the tubes to bring down liquid caught in the lid before to place in the ice-cold Magnetic Rack.
- To analyse DNA present in the wash- 1 and wash-2 fractions, do not discard these buffers but transfer them in a new 1.5-ml tubes for the purification (STEP 4 - Point 20).

STEP 4. Elution of captured DNA

- 150 µl of any elution buffer are needed per capture reaction.

We propose two options and corresponding buffers.

Option 1: One single total Elution
Needed: 150 µl of the High Elution Buffer per capture reaction.

Option 2: Three intermediate Salt Elutions
Perform 3 elutions, using sequentially 150 µl of Low Elution Buffer, Medium Elution Buffer, High Elution Buffer per capture reaction.

- Our three elution buffers are ready to use in order to have a differential fractionation of double-stranded DNA based on CpG methylation density.

To elute proceed as follows:

- Decide buffers you need (option 1 or 2) and place all these buffers on ice.
- Start with lower salt buffer and go progressively to high salt concentration.

14. After the last wash with Wash Buffer 2, remove the tubes from the Magnetic Rack. Add 150 µl of the first elution buffer per bead pellets. Resuspend the beads with a pipette.

15. Take the INPUT DNA sample and spin the tube to bring down liquid caught in the lid before to place in the ice.

16. Take out 11.9 µl (that is 10% INPUT), transfer to a new 1.5-ml labelled tube and add 138.1 µl of High Elution Buffer.

17. Incubate the captured DNA and INPUT DNA at 40 rpm on a rotating wheel for 10 minutes at 4°C.

18. Spin captured DNA, place in the Magnetic Rack, wait 1 minute and transfer the suspension into a new 1.5-ml tubes.

- This tubes contain the first fraction ready for purification.

19. For DNA captured samples, repeat Point 14, 17 and 18 with Medium and High Elution Buffer.

20. Purification of all fractions and INPUT by using one of the following techniques:

- Purification using Phenol/Chloroform/Isoamyl alcohol (see additional protocols for instructions).
- MicroChIP DiaPure columns (Cat. No. C03040001).
- IPure Kit v2 (Cat. No. C03010014).

21. Use the instructions and elute with 50 µl of water.

NOTE: *After elution in the High Elution Buffer (or other elution buffers), we recommend to follow the IPure v2 protocol directly at the DNA binding step by adding 150 µL of isopropanol, 2 µL of carrier and 10 µL of IPure beads to each sample. You can then follow the standard IPure v2 protocol starting from Step 2 to Step 4.*

At this stage, you obtain purified DNA from: 1/ the sheared DNA (input sample(s)) and 2/ DNA that was isolated by Methyl DNA capture (meDNA captured sample(s)).

The purified samples can be processed by qPCR and/or library preparation for Next-Generation Sequencing.

STEP 5. qPCR

qPCR analysis of the following fractions: Flow-through, wash 1, wash 2, Low, Medium and High.

Control primers available at Diagenode

Primer pairs	Specificity	Input DNA sample (which includes Ctrls) amplification :	meDNA capture (which includes Ctrls) amplification :
Human meDNA primer pair (TSH2B)	Human DNA	Yes (if sample is human DNA)	Yes
Human unDNA primer pair (GAPDH)			No
Mouse meDNA primer pair (TSH2B)	Mouse DNA	Yes (if sample is mouse DNA)	Yes
Mouse unDNA primer pair (GAPDH)			No
Rat meDNA primer pair (TSH2B)	Rat DNA	Yes (if sample is Rat DNA)	Yes
Rat unDNA primer pair (GAPDH)			No

22. Prepare your qPCR mix using SYBR PCR Green master mix and start out qPCR. qPCR mix (total volume of 25 µl/reaction):

- 1.00 µl of provided primer pair (stock: 10 µM each: reverse and forward)
- 12.50 µl of master mix (e.g.: iQ SYBR Green supermix)
- 2.50 µl of purified DNA sample
- 9.00 µl of water

qPCR cycles:

	Temperature	Time	Cycles
PCR Amplification	95°C	7 minutes	x1
	95°C	15 seconds	x40
	60°C	60 seconds	
	95°C	1 minute	x1
Melting curve	65°C and increment of 0.5°C per cycle	1 minute	x60

23. When the PCR is done, analyse the results.

Data interpretation

The efficiency of methyl DNA capture (meDNA-CAP) of particular genomic locus can be calculated from qPCR data and reported as a percentage of starting material: % (meDNA-CAP/ Total input).

% (meDNA-CAP/ Total input)= 2^[(Ct(10%input) - 3.32) - Ct(meDNA-CAP)]x 100%

Here 2 is the AE (amplification efficiency), Ct (meDNA-CAP) and Ct (10% input) are threshold values obtained from exponential phase of qPCR for the methyl DNA sample and input sample respectively; the compensatory factor (3.32) is used to take into account the dilution 1:10 of the input. The recovery is the % (meDNA-CAP/ Total input).

STEP 6. Sequencing

The samples collected in the fractions of interest and the input samples can also be analyzed by Next-Generation Sequencing (NGS).

1. Prepare DNA libraries for Illumina® NGS platforms with the MicroPlex Library Preparation Kit v3 (Cat. No. C05010001) by following Diagenode's instruction for kit handling and protocol.

2. Quantify the libraries and check their profiles on a BioAnalyzer or Fragment Analyzer (Agilent) as explained in the manual of the MicroPlex Library Preparation Kit v3 (Cat. No. C05010001).

3. Prepare equimolar pools of the samples that will be sequenced in the same lane. We recommend to target 50 million of raw reads per sample. The requirements for a final library concentration depend on a sequencer and may vary between different sequencing service providers. The usual range is between 5-20 nM in a final volume 10-15 µl but we recommend inquiring with your sequencing platform.

4. Sequence the libraries on Illumina® NGS platforms and analyze the results.

Additional Protocols

DNA preparation for MethylCap

1. Use the XL GenDNA Extraction module (Cat. No. C03030020) from Diagenode and resuspend the pellet of DNA at ~1 mg/ml in GenDNA TE until dissolved.

   - From 1-1.5 million cells, ~8 to 12 µg of DNA can be expected (in a volume of 8 to 12 µl).
   - From 3 million cells, ~20 to 30 µg of DNA can be expected (in a volume of 20 to 30 µl).
   - From 10 million cells, ~50 to 100 µg of DNA can be expected (in a volume of 200 to 300 µl).

2. Measure the DNA concentration.

   - If possible, it is recommended to get at least 30 µg of DNA (when enough material is available) to be able to work with 30 µg of DNA: see 2/ DNA shearing protocol).

3. Run samples in a 1% agarose gel along with DNA size marker to visualize the DNA preparation efficiency.

Using Phenol / Chloroform / Isoamyl alcohol

1. Add 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1)
   - It is possible to incubate the samples at RT for 10 minutes on a rotating wheel before centrifugation.

2. Centrifuge for 2 minutes at 14,000x g (13,000 rpm) at RT. Transfer the top aqueous phase into a new 1.5 ml tube.

3. Add 1 volume of chloroform/isoamyl alcohol (24:1).
   - It is possible to incubate the samples at RT for 10 minutes on a rotating wheel before centrifugation. Use gentle rotation. Meanwhile, thaw the DNA co-precipitant (Cat. No. C03030001) on ice.

4. Centrifuge for 2 minutes at 14,000x g (13,000 rpm) at RT. Transfer the top aqueous phase into a new 1.5 ml tube. Thaw on ice the co-precipitant.

5. Per tube: add 5 µl of the provided meDNA-IP co-precipitant and 40 µl of the meDNA-IP precipitant (Cat. No. C03030002). Then, add 1 ml of ice-cold 100% ethanol. Mix well. Leave at -80°C for 30 minutes.

6. Centrifuge for 25 minutes at 14,000x g (13,000 rpm) at 4°C. Carefully remove the supernatant and add 500 µl of ice-cold 70% ethanol to the pellet.

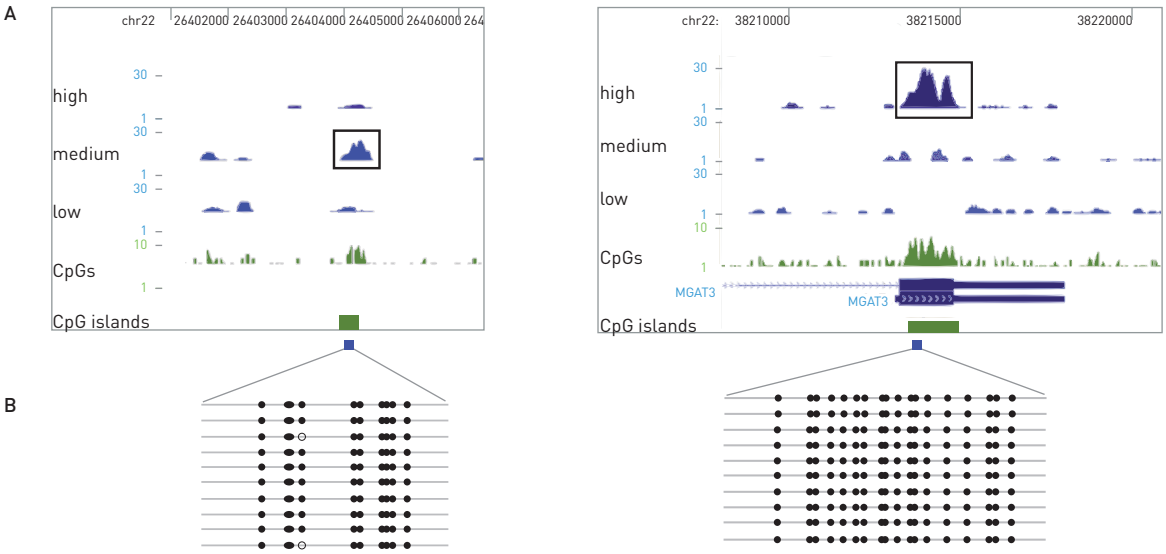
7. Centrifuge for 10 minutes at 14,000x g (13,000 rpm) at 4°C. Carefully remove the supernatant, leave tubes opened for 30 minutes at room temperature to evaporate the remaining ethanol. The pellets are: 1/ DNA that was purified from the sheared DNA (input sample(s)) and 2/ DNA that was isolated by capture reaction (meDNA captured samples).
   - Avoid leaving ethanol on tube walls. The sheared DNA that is taken as input sample must correspond to the same preparation of sheared DNA used in the IP assay.

8. Add of 50 µl of DNAse free water to the captured DNA and input DNA samples.
   - Suspend the DNA evenly: place the tubes in a shaker for 30 minutes at 12,000 rpm at room temperature to dissolve the pellets.

At this stage, you obtain purified DNA from: 1/ the sheared DNA (input sample(s)) and 2/ DNA that was isolated by Methyl DNA capture (meDNA captured sample(s)).

Results

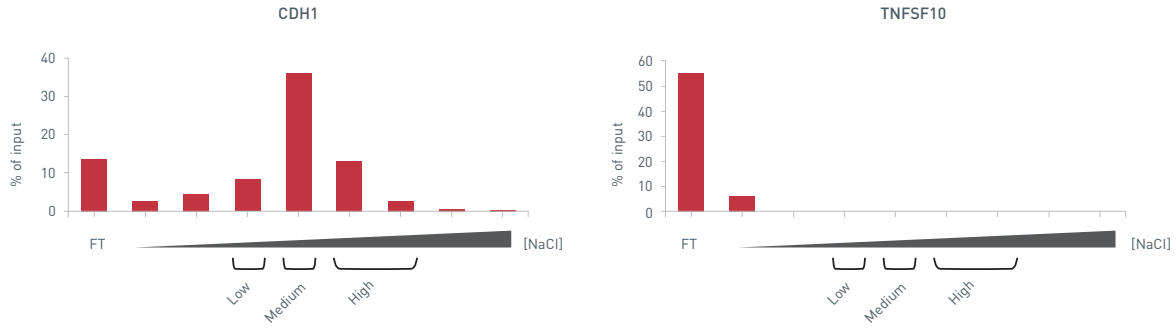
MBD-seq (Methylbinding domain - sequencing) allows for detection of genomic regions with different CpG density



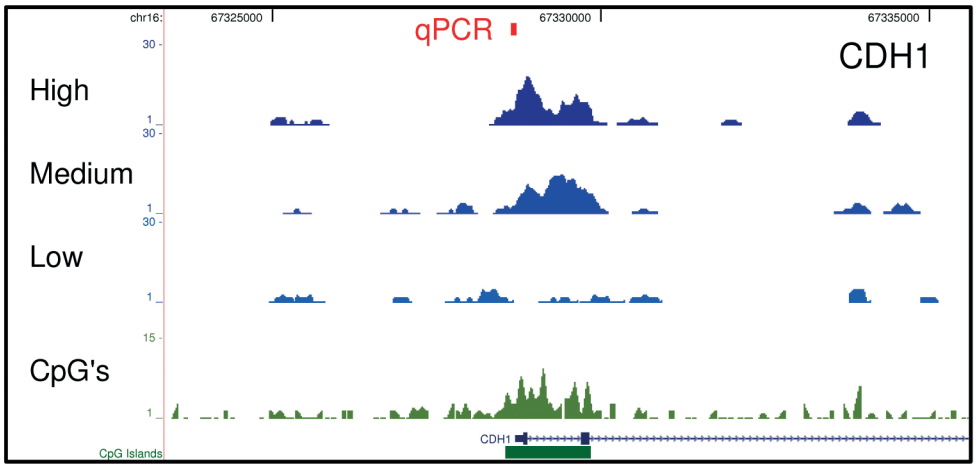
Data provided by Henk Stunnenberg (Nijmegen Center for Molecular Life Sciences - The Netherlands)

Figure 1.
Using MBD-seq, two methylated regions were detected in different elution fractions according to their methylated CpG density (A). Low, Medium and High refer to the sequenced DNA from different elution fractions with increasing salt concentration. Methylated patterns of these two different methylated regions were validated by bisulfite conversion assay (B).

A



B



Data provided by Henk Stunnenberg (Nijmegen Center for Molecular Life Sciences - The Netherlands).

Figure 2.

MethylCap assays were performed using DNA from NB4 cells and the MethylCap kit (Diagenode). Differential fractionation of double stranded DNA based on CpG methylation density was performed using increasing salt concentration during the elution steps. (A) qPCR results in a methylated (CDH1-CpG) and a unmethylated (TNFSF10) region show the % of recovery of captured DNA compared to the input in the different fractions. (B) Results have been confirmed by sequencing the captured DNA in the different elution fractions. FT represents the unbound fraction.

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