Instruction Manual Version 6 - 01.14

MeDIP kit

Methylated DNA Immunoprecipitation kit

Cat. No. **C02010010** (mc-green-003)

- **Methyl DNA IP module:**
  catalog # mc-green-001 (one module)
- **GenDNA and qPCR modules:**
  catalog # mc-green-002 (two modules)
- **Complete MeDIP kit:**
  catalog # mc-green-003 (the three modules)

KIT KEY WORDS:

- Correlation between IP'd material and methylation status
- DNA internal IP controls provided
- Highly specific
- All in one tube
- Reliable, fast and user-friendly

diagenode

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Introduction

The Novel Diagenode MeDIP kit is designed to immunoprecipitate methylated DNA (Methyl DNA IP). This kit allows you to perform **DNA methylation analysis** of your sample together with **optimized internal IP** controls: **ALL IN ONE TUBE**.

This brand new Methyl DNA IP method provides you with methylated DNA (meDNA) and unmethylated DNA (unDNA) controls to be used together with your DNA sample allowing **direct CORRELATION between IP'd MATERIAL and METHYLATION STATUS**.

This methylation analysis is HIGHLY SPECIFIC and each IP is QUALITY controlled: essential keys for RELIABLE results. In addition, the kit protocol is FAST and USER-FRIENDLY.

The MeDIP kit includes three modules, they are used for: 1/ genomic DNA preparation, 2/ immunoprecipitation of methylated DNA and 3/ qPCR analysis of the immunoprecipitated (IP'd) DNA. Each module is provided with adapted buffers and detailed protocols.

1/ **A GenDNA module** is included and optimized for the preparation of DNA ready-to-use in Methyl DNA IP. An optimized protocol for DNA shearing is provided as well.

2/ **In the Methyl DNA IP module**, our antibody directed against 5-methyl Cytidine is provided as well as methylated and unmethylated DNA controls (positive meDNA control - that is IP'd - and negative unDNA control - that is not IP'd). The IP has been optimized to specifically select and precipitate the methylated DNA: by the use of our antibody, buffers and protocol. The IP efficiency can indeed be double-checked with the use of our internal controls.

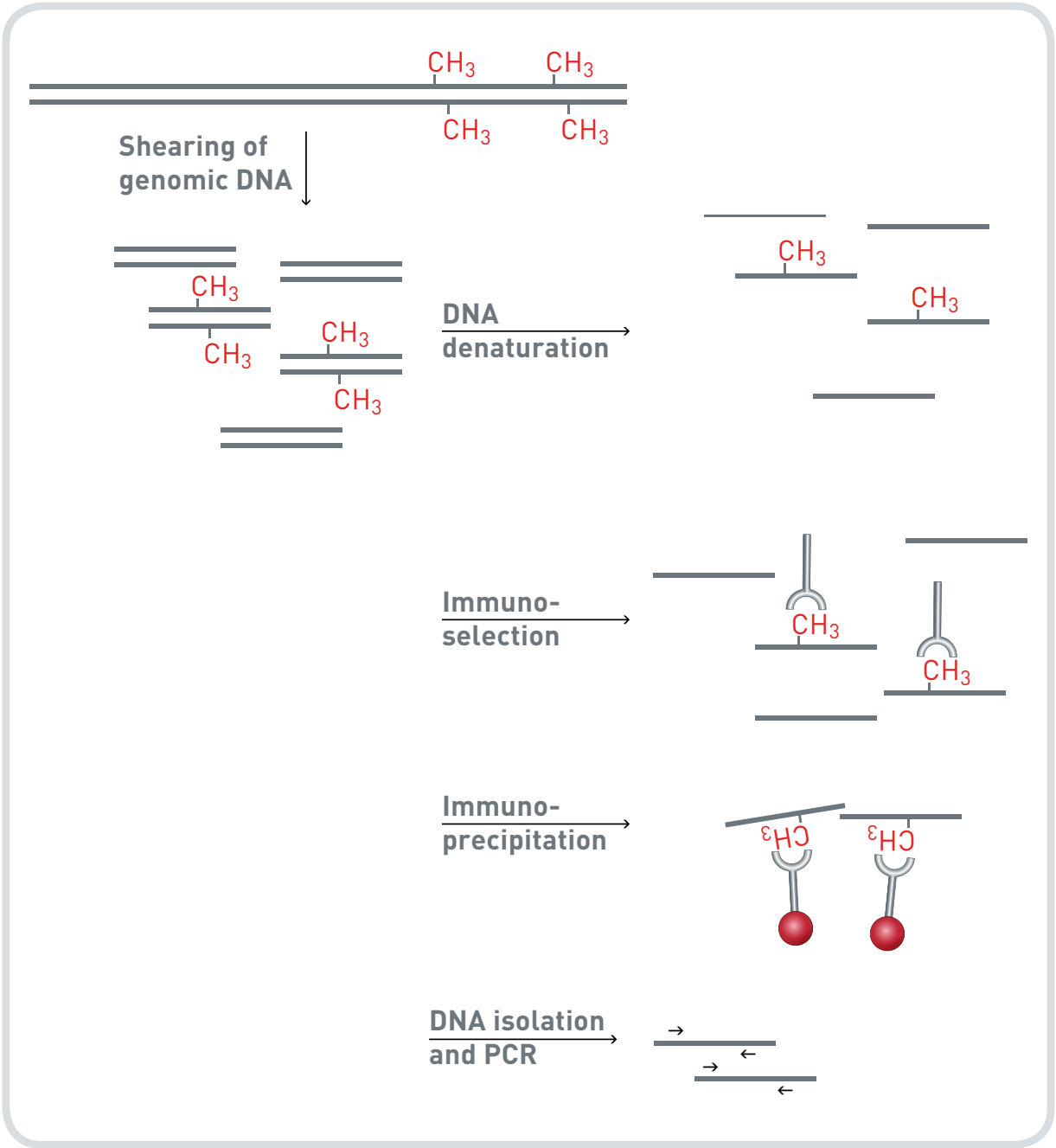
3/ **The qPCR module** includes validated primer pairs specific to four types of DNA: a: the methylated DNA control (meDNA positive ctrls #1 and #2), b: the unmethylated DNA control (unDNA negative ctrls #1 and #2), c: one methylated human DNA region (X-linked alpha satellites) and d: one unmethylated human DNA region (GAPDH promoter).

In the next few pages of this manual you will find the kit method overview, the kit content the short kit protocol and the detailed kit protocol. In the last sections of this manual, results obtained with this kit are shown. A troubleshooting guide and additional protocols are also present at the end of the manual.

One format: 10 IPs per kit.

For more information about DNA methylation, see our introduction to DNA methylation at the end of this manual.

Kit Method Overview



KIT KEY WORDS:
CORRELATION between IP'd MATERIAL and METHYLATION STATUS. HIGHLY SPECIFIC. ALL IN ONE TUBE. DNA INTERNAL IP CONTROLS PROVIDED. EACH IP is QUALITY CONTROLLED. RELIABLE, FAST and USER-FRIENDLY. NOVEL.

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Kit Materials

Kit Content

The content of the kit is sufficient to perform 10 Methylated DNA Immunoprecipitations (Methyl DNA IPs): from cell collection to qPCR.
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

- Gloves to wear at all steps
- Autoclaved tips
- RNase/DNase-free 1.5 ml (and 2 ml) tubes
- Other tubes: PCR tubes, 15 ml and 50 ml conical tubes
- Trypsin-EDTA
- Ice-cold PBS buffer
- Water
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24:1)
- Ethanol 100%
- Ethanol 70%
- Agarose and TAE buffer
- DNA molecular weight marker

Equipment

- Centrifuges for 1.5 ml tubes (4°C), 15 ml and 50 ml tubes
- Shaking platform
- Cell counter
- Bioruptor®: sonication apparatus from Diagenode (cat # UCD-200, website: http://www.diagenode.com/)
- Rotating wheel
- Fume hood
- Vortex
- Thermomixer (50°C, 65°C)
- Incubator (37°C)
- Cold room with microcentrifuge and rotating wheel
- Quantitative PCR facilities and reagents
- Agarose gel apparatus

Kit modules and components

Table 1 (Note: Upon receipt, store the components at the right temperature)

GenDNA Module			
Component	Comments	Quantity	Storage
GenDNA Digestion buffer	Detergent, salt and ion chelator included	3 ml	4°C
GenDNA Proteinase K (200x)	-	15 µl	-20°C
GenDNA precipitant	Salt included	3 ml	4°C
GenDNA TE	Ion chelator included	3 ml	4°C
GenDNA RNase (DNase free)	-	10 µl	-20°C

Methylated DNA Immunoprecipitation (Methyl DNA IP) Module			
Component	Comments	Quantity	Storage
Pre-blocked protein A/G beads for meDNA-IP	1:2 suspension for 10 IPs, 0.02% azide included	300 µl	4°C Do not freeze
Water	-	2 ml	4°C
Buffer A	Detergent mix, salt and ion chelator mix included	300 µl	4 °C
Buffer B	-	100 µl	4°C
Buffer C	-	40 µl	(4°C)/-20°C
Antibody anti-5meC	-	5 µl	-20°C/ (-80°C)
Positive meDNA control	Methylated DNA control	20 µl	-20°C
Negative unDNA control	Unmethylated DNA control	20 µl	-20°C
Wash buffer-1	Detergent mix, salt and ion chelator mix included.	12 ml	4°C
Wash buffer-2	Detergent mix, salt and ion chelator mix included.	10 ml	4°C
Wash buffer-3	Detergent mix and ion chelator mix included.	10 ml	4°C
Wash buffer-4	Ion chelator mix included	12 ml	4°C
Buffer D	-	6 ml	4°C
Buffer E	Detergent included. Need to be placed at room temperature 1 hour before use	1 ml	4°C/(RT)
Buffer F	Salt included	500 µl	4°C
meDNA-IP TE	Ion chelator included	5 ml	4°C
meDNA-IP co-precipitant	-	100 µl	-20°C
meDNA-IP precipitant	-	1 ml	4°C

qPCR Module			
Component	Comments	Quantity	Storage
hum meDNA primer pair (TSH2B)	10 µM each (Rv & Fw)	50 µl	-20°C
hum unDNA primer pair (GAPDH)	10 µM each (Rv & Fw)	50 µl	-20°C
meDNA positive control primer pair #1	10 µM each (Rv & Fw)	50 µl	-20°C
meDNA positive control primer pair #2	10 µM each (Rv & Fw)	50 µl	-20°C
unDNA negative control primer pair #1	10 µM each (Rv & Fw)	50 µl	-20°C
unDNA negative control primer pair #2	10 µM each (Rv & Fw)	50 µl	-20°C

Components available separately	
Component	Reference
GenDNA RNase (DNase free)	mcg-605-010
Pre-blocked protein A/G beads for meDNA-IP	mcg-606-300
5-mC monoclonal antibody 33D3	MAb-081-100
5-mC monoclonal antibody 33D3	MAb-081-500
5-mC monoclonal antibody cl. b	MAb-006-100
5-mC monoclonal antibody cl. b	MAb-006-500
Primer pairs	www.diagenode.com

Time Table

FIRST PREPARE DNA:

Table 2a

GenDNA MODULE			
MODULES		DAY	Time needed
STEP 1-	Cell collection and lysis - Starting material: culture cells - Cell lysis	DAY 1	40 minutes + O.N. incubation
STEP 2-	Nucleic acid extraction and DNA purification - DNA analysis	DAY 2	few hours
STEP 3-	DNA shearing	DAY 2	20 minutes

AND THEN PROCEED TO THE **Methylated DNA Immunoprecipitation:**

Table 2b

Methyl DNA IP MODULE			
MODULES		DAY	Time needed
STEP 4-	Immunoprecipitation	DAY 2	5 hours
STEP 5-	Washes	DAY 2	1 hour
STEP 6-	DNA elution & purification	DAY 2	30 minutes* (or 5 hours**)

OR

STEP 4-	Immunoprecipitation	DAY 2	1 hour + O.N. incubation
STEP 5-	Washes	DAY 3	1 hour
STEP 6-	DNA elution & purification	DAY 3	30 minutes** (or 5 hours***)

FINALLY, THE IP IS FOLLOWED BY THE IP'd DNA ANALYSIS:

Table 2c

qPCR MODULE			
MODULES		DAY	Time needed
STEP 7-	qPCR	DAY 2*, 3** or 4***	3 hours

- DNA preparation can be performed in one day (Day 1 above), and be ready for the following day (Day 2)
- IP (STEP 4) can be done either in 5 hours or in one night
- next, depending on the way the DNA is purified at STEP 6, the qPCR can be performed the earliest on Day 2*, the latest on Day 3** or Day 4*** (see *, **,*** indicating the link between DNA purification method and day of qPCR).

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Protocol

Short protocol

STEP 1. Cell collection and lysis

1. Pellet suspension culture out of its serum-containing medium. Trypsinize adherent cells and collect cells from the flask. Centrifuge at 300 g for 5 min at 4°C.

2. Discard the supernatant. Resuspend cells in 5 to 10 ml ice-cold PBS. Centrifuge at 500 g for 5 min. Discard the supernatant. Repeat this resuspension and centrifugation step once more.

3. Meanwhile, place the GenDNA Digestion buffer at room temperature (RT) and the GenDNA proteinase K on ice.

4. Add GenDNA proteinase K to the GenDNA Digestion buffer before use. The stock of provided proteinase K is 200 X. e.g. add 5 µl per 1 ml of Digestion buffer.

5. Resuspend cells in complete Digestion buffer (point 4). For 3 million cells, use 300 µl complete Digestion buffer. For 10 million cells, use 500 µl complete Digestion buffer. If necessary: for 3 million cells, use up to 600 µl of buffer. For 10 million cells, use up to 1,000 µl of buffer.

6. Cell lysis: Incubate the samples with shaking at 50°C for 12 to 18 hours in tightly capped tubes.

STEP 2. Extraction of nucleic acids and DNA purification

1. Thoroughly extract the samples with an equal volume of phenol/chloroform/isoamyl alcohol. Add 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1). One volume is about 500 µl. It is possible to incubate the samples at RT for 10 min on a rotating wheel before centrifugation. Use gentle rotation and do not vortex. Work under a fume-hood.

2. Centrifuge at 1,700 g for 10 min in a swinging bucket rotor.

3. Transfer the aqueous (top) layer to a new tube. Increase volume if necessary (see above) and pipette slowly.

4. Add 1 volume of chloroform/isoamyl alcohol (24:1). It is possible to incubate the samples at RT for 10 min on a rotating wheel before centrifugation. Use gentle rotation and do not vortex. Work under a fume-hood.

5. Centrifuge at 1.700 g for 10 min in a swinging bucket rotor

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

7. Add 1/2 volume of GenDNA precipitant and 2 volumes of 100% ethanol. One volume is about 500 µl and corresponds to the original amount of top layer. Add therefore 250 µl of precipitant and 1,000 µl of 100% ethanol. The DNA should immediately form a stringy precipitate.

8. Recover DNA by centrifugation at 1,700 g for 2 min.

9. Rinse the pellet with 70% ethanol. Decant ethanol and air-dry the pellet. It is important to rinse extensively to remove any residual of salt and phenol.

10. Resuspend the pellet of DNA 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 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).

11. If necessary, residual RNA can 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 (similar to above).

12. For DNA analysis, run samples on a 1% agarose gel along with DNA size marker to visualize the DNA preparation efficiency.

STEP 3. DNA shearing

1. In a 1.5-ml tube, dissolve the DNA sample in TE to reach 0.1 µg/µl.

2. Use a final volume of 300 µl of DNA sample in 1.5-ml tubes.

3. Shear the DNA by sonication using the Bioruptor®.
   - Bioruptor Pico: 6 cycles of 30 sec ON / 90 sec OFF (1.5 ml microtubes with caps)
   - other Bioruptor models: 10 cycles of 15 sec ON / 15 sec OFF; Low Power

4. Sheared DNA can be analysed on agarose gel.

STEP 4 and 5. Methylated DNA Immunoprecipitation and washes

1. Prepare the IP incubation mix w/o DNA sample as follows. For one IP: 24.00 µl Buffer A, 6.00 µl Buffer B, 1.5 µl of positive meDNA control and 1.5 µl negative unDNA control and 45 µl water.

2. Label new 1.5-ml tubes. Add the DNA sample to the IP incubation mix.

3. Add per labeled "IP" tube: the IP incubation mix. Then, add 1 µg of DNA sample per tube. Using DNA samples at a concentration of 0.1 µg/µl: add 65 µl of IP incubation mix and 10 µl of DNA per tube. The total volume per IP is 75 µl.

4. Add per "input sample" tube: 20% of what is used per IP above. Using DNA samples at a concentration of 0.1 µg/µl: add 13 µl of IP incubation mix and 2 µl of DNA per tube. The total volume for 20% input is 15 µl.

5. Incubate at 95ºC for 3 min.

6. Quickly chill on ice (it is best to use ice-water).
7. Quickly perform a short spin at 4°C.

8. In a new tube, prepare the Diluted Antibody mix. For one IP: prepare a 1:10 antibody dilution as follows: (0.30 µl antibody, 0.60 µl Buffer A and 2.10 µl water). Then, add 2.00 µl of Buffer C. Final volume is 5.00 µl.

9. Add 5 µl of Diluted Antibody mix per IP tube (3. above). Antibody is added to the IP tubes, which contain IP incubation mix and your DNA sample.

10. Add 20 µl of beads (resuspend beads before use) to all tubes. That is the IP incubation which comprises the IP samples, the Diluted antibody mix and the beads. The final volume: 100 µl.

11. Place on a rotating wheel at 4ºC for 4 hours or overnight.

12. The IP samples are then washed as follows: add 450 µl of ice-cold Wash buffer to each IP tube, starting with Wash buffer-1. Place the four Wash buffers on ice and perform the washes in a cold room.

13. Rotate for 5 min at 4ºC.

14. Centrifuge at 6,000 rpm for 1 min at 4°C.

15. Discard the supernatant. Keep the pellet.

16. Wash the pellet again (as described above: Point 13. to 16.) as follows: perform one more wash with Wash buffer-1, then one wash with Wash buffer-2, and one wash with Wash buffer-3. Finally perform two more washes using the Wash buffer-4.

17. After the last wash, discard the last traces of Wash buffer (using a P200 pipet). Keep the bead pellets. These are the Methyl DNA IP samples. The Immunoprecipitated Methylated DNA is bound to the beads.

STEP 6. DNA elution and purification

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

2. Prepare the complete elution buffer by mixing Buffer D, E and F as follows. For one IP: 360 µl of Buffer D, 40 µl of Buffer E, 16 µl of Buffer F. The total volume is of 416 µl.

3. Add 416 µl of freshly prepared complete elution buffer to the bead pellets (the Methyl DNA IP samples).

4. Add 416 µl of freshly prepared complete elution buffer to the input samples.

5. Incubate in a thermo-shaker for 10 min at 65ºC at 1,000 to 1,300 rpm.

6. Cool down samples to room temperature, add 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1).

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

8. Add 1 volume of chloroform/isoamyl alcohol (24:1).

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

11. Per tube: add 5 µl of the provided meDNA-IP co-precipitant and 40 µl of the meDNA-IP precipitant.Then, add 1 ml of ice-cold 100% ethanol. Mix well. Leave at -80°C for 30 min.

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

13. Centrifuge for 10 min at 14,000 x g (13,000 rpm) at 4°C. Carefully remove the supernatant, leave tubes opened for 30 min at room temperature to evaporate the remaining ethanol. The pellets are: 1/ DNA that has been purified from the sheared DNA (input sample(s)) and 2/ DNA that has been isolated by IP (Methyl DNA IP samples).

14. Add 50 µl TE to the IP and input samples. Suspend the DNA evenly: place the tubes in a shaker for 30 min at 12,000 rpm at room temperature to dissolve the pellets.

STEP 7. qPCR analysis of IP'd DNA

1. Make aliquots of the purified DNA and prepare dilutions. Use the purified DNA from Methyl DNA IPs and DNA input(s). From the 50 µl of purified DNA: transfer 10 µl into a new tube (keep 40 µl for Methyl DNA IP-on-chip analysis, or further PCR analysis). For the first PCR analysis, dilute 10 µl of each purified DNA sample as follows: to 10 µl of purified DNA sample (from IP and input), add 35 µl of water. Final volume is 45 µl. Use 5 µl per PCR. Note: when testing the hum meDNA primer pair (AlphaX1): dilute the DNA sample 1:1,000.

2. Prepare your qPCR mix using SYBR PCR Green master mix. qPCR mix (total reaction volume is 25 µl: 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), 5.00 µl of diluted purified DNA sample (see above for DNA dilutions) and 6.50 µl of water.

3. PCR cycles: amplification: 1x 95°C for 7 min, 40 cycles of (95°C for 15 seconds, 60°C for 1 min and 95°C for 1 min).

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

Detailed protocol

Starting material- Cell culture or tissues
Each Methyl DNA IP assay requires 1 µg of DNA; scale accordingly.

- The GenDNA module provides you with a high excess of buffer for the preparation of DNA. Sufficient buffer is given for the preparation of four genomic DNA batches, each obtained from 3 to 10x 10e6 cultured cells (see STEP 1, and scale accordingly based on your starting material). Protocol is also given to prepare DNA from tissues (see STEP 1).

- From about 3 million cells, 20 to 30 µg of DNA can be expected (see Table 3 below).
- From about 10 million cells, 50 to 100 µg of DNA can be expected (STEP 1-pt. 4, STEP 2-pt. 12.)
- Scale volumes accordingly based on the starting material that is available.
- It is also possible to start with less cells, keeping in mind that 1 µg of DNA is needed per IP (Table 3).
- Cells can be submitted to inductions and/or treatments. Then DNA from treated as well as non-treated cells can be prepared and subsequently studied in Methyl DNA IP. DNA samples from normal cells (healthy individual) and cancer cells (patient) can also be studied in parallel and compared.
- It is also possible to purify DNA samples from reverse cross-linked sheared chromatin and to use it in Methyl DNA IP (STEP 1).

- After DNA preparation, most of the DNA is sheared to be used in the IP experiment, but remember that some of the sheared DNA is needed as control to double-check:
  1/ for DNA preparation efficiency (prior to Methyl DNA IP: STEP **2-pt.14: DNA analysis**)
  2/ for shearing efficiency (prior to Methyl DNA IP: STEP **3-pt. 18: DNA shearing**)
  3/ for the IP experiment efficiency: input sample (STEP **6- DNA elution and purification**)

Table 3

DNA preparation	Cell number needed	DNA amount expected	DNA to be used in IP
For 1 Methyl DNA IP	0.3x 10e6	2.0-3.0 µg	1 µg
For 10 Methyl DNA IP	3x 10e6	20-30 µg	10 µg (1 µg/IP)

Preparation of Genomic DNA for Methyl DNA IP (GenDNA Module)

The goal of this first step is to get high molecular weight genomic DNA.

The **GenDNA module from Diagenode** has been optimized for the preparation of Genomic DNA from cultured cells or mammalian tissues to be used in Methyl DNA IP.

Notes:
- It is also possible to prepare high molecular weight genomic DNA with your "in house" protocol and reagents. However, the quality of the DNA to be used in IP is very important, we therefore highly recommend the use of our GenDNA module.
- If you have DNA samples ready, and that have been prepared with your own protocol and reagents, be aware that it is important that the DNA has been purified using phenol-chloroform, chloroform extractions and ethanol precipitated with no co-precipitant added.
- It is also possible to purify DNA samples from reverse cross-linked sheared chromatin and to use it in Methyl DNA IP. The reversion is performed at 65°C for 4 hours. From decross-linked chromatin, the DNA is purified using phenol-chloroform extraction, chloroform extraction and ethanol precipitation. Then DNA is finally dissolved in TE (see our Red ChIP kit manual for detailed protocol).

STEP 1. Cell collection and lysis

Starting material: culture cells

1. Pellet suspension culture out of its serum-containing medium Trypsinize adherent cells and collect them from the flask. Centrifuge at 300 × g for 5 minutes at 4°C.
   - We advise using low speed such as 300 x g (about 1,800 rpm - depending on your microcentrifuge). For conversion from x g to rpm, see Troubleshooting guide.
   - Use gentle centrifugations when working with cells, avoid using higher speed than 500 x g (2,300 rpm).

2. Discard the supernatant. Resuspend cells in 5 to 10 ml ice-cold PBS. Centrifuge at 500 × 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 (RT) and the GenDNA proteinase K on ice (to be used at Point 3. below).

3. Add GenDNA proteinase K to the GenDNA Digestion buffer before use.
   - The stock of provided proteinase K is 200 x. e.g. add 5 µl per 1 ml of Digestion buffer.
   - That is the freshly prepared complete Digestion buffer to be used directly: Point 4..

4. Resuspend cells in complete Digestion buffer.
   - for 3 million cells, use 300 µl complete Digestion buffer.
   - for 10 million cells, use 500 µl complete Digestion buffer.
   - It might be necessary to use more buffer to avoid problems when performing the extractions at Point 7. below. If necessary: for 3 million cells, use up to 600 µl of buffer. For 10 million cells, use up to 1,000 µl of buffer.
   - Proceed to Point 5. below (cell lysis).

Alternatively: starting material: whole tissue

1B. As soon as possible after excision, quickly mince tissue and freeze in liquid nitrogen.
   - **Be aware that some tissues contain high levels of degrading enzymes, when possible remove them (e.g: gallbladder in liver).**
   - **Start with 200 mg to 1 g, grind tissue with a prechilled mortar and pestle**

2B. Prepare the complete Digestion buffer as shown at Point 3. above. Suspend the powdered tissue in 1.2 ml complete Digestion buffer per 100 mg of tissue.
   - **Avoid clumps.**
   - **Scale volumes accordingly, based on the amount of available starting material.**
   - **Proceed to Point 5. below (cell lysis).**

Cell lysis

5. Incubate the samples with shaking at 50°C for 12 to 18 hours in tightly capped tubes.
   - At this stage samples are viscous. After 12 hours incubation the tissue should be almost indiscernible, a sludge should be apparent from the organ samples and tissue culture cells should be relatively clear.

STEP 2. Extraction of nucleic acids and DNA purification

6. Thoroughly extract the samples with an equal volume of phenol/chloroform/isoamyl alcohol.
   - Add 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1).
   - One volume is about 500 µl (see Point 4. above).
   - It is possible to incubate the samples at RT for 10 minutes on a rotating wheel before centrifugation. Use gentle rotation and do not vortex.
   - Work under a fume-hood.

7. Centrifuge at 1,700 × g for 10 minutes in a swinging bucket rotor.
   - Equivalent to 4,700 rpm in a microcentrifuge.
   - For conversion from x g to rpm (see Troubleshooting guide).
   - Do not use higher speed.
   - It must be a very low speed because it is genomic DNA.
   - 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 (see Point 4. above).

8. Transfer the aqueous (top) layer to a new tube.
   - Increase volume if necessary (see Point 4. above) and pipette slowly.
   - At this stage, before proceeding to the next Point, it is possible to perform a chloroform extraction
   - In order to perform the chloroform extraction repeat steps 6. and 7. and add chloroform/isoamyl alcohol (24:1) instead of phenol/chloroform/isoamyl alcohol.
   - Work under a fume-hood.

It is possible to omit Points 9. to 12. below and perform a dialysis instead.
Although dialysis is time consuming it is a good alternative and allows the prevention of possible shearing of high molecular weight DNA.

In brief, to dialyse: remove organic solvents and salt from the DNA by at least two dialysis steps against a minimun of 100 volumes of TE buffer. Because of the high viscosity of the DNA, it is necessary to dialyze for a total of at least 24 hours.

9. Add **1/2 volume of GenDNA precipitant** and 2 volumes of 100% ethanol. That is to purify the DNA.
   - One volume corresponds to the original amount of top layer (500 µl - see Point 4.above).
   - Add therefore 250 µl of precipitant and 1,000 µl of 100% ethanol.
   - The DNA should immediately form a stringy precipitate.

10. Recover DNA by centrifugation at 1700 × g for 2 minutes.
    - Do not use higher speed.
    - It must be a very low speed because it is genomic DNA.
    - 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.

11. Rinse the pellet with 70% ethanol. Decant ethanol and air-dry the pellet.
    - It is important to rinse extensively to remove any residual of salt and phenol.

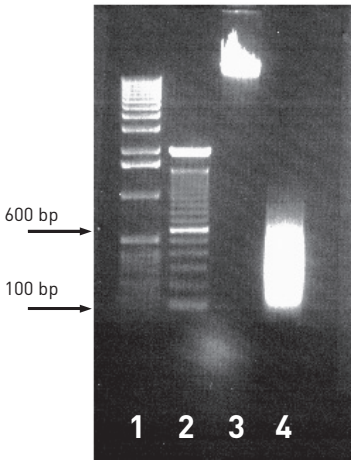
12. Resuspend the pellet of DNA at ~1 mg/ml in GenDNA TE until dissolved. Shake gently at room temperature or at 65°C for several hours to facilitate solubilization. Store at 4°C.
    - From 3 million cells, ~20 to 30 µg of DNA can be expected (in a volume of 20 to 30 µl).
    - From 10 million cells, ~50 to 100 µg of DNA can be expected (in a volume of 200 to 300 µl).
    - 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 STEP 3/ DNA shearing).

13. If necessary, residual RNA can 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 (similar to Points 6. to 11. above).

DNA Analysis

14. Run samples in a 1% agarose gel along with DNA size marker to visualize the DNA preparation efficiency (lane 3, figure below). Note that in this figure the shearing efficiency of the obtained DNA sample is shown as well (lane 4), protocol and optimization are described in the figure legend and in the next pages.

Bioruptor and **GenDNA module** from **Diagenode:**
Prepare DNA and shear DNA ready-to-use in Methyl DNA IP



Genomic DNA preparation:
- start of STEP 1- using 30x million cells
- STEP 1- use 500 µl complete digestion buffer
- STEP 2- (after phenol chloroform extraction): volume is 350 µl
- end of STEP 2- (after precipitation): volume is 200 µl
- STEP 3- measurement Nanodrop: 1.4 µg/µl (OD 260/280 = 1.8)

Shearing of the genomic DNA using the Bioruptor:
- Bioruptor® Pico: 6 cycles of 30 sec ON / 90 sec OFF (1.5 ml microtubes with caps)
- other Bioruptor models: 10 cycles of 15 sec ON / 15 sec OFF; Low Power

1: molecular weight marker (1 kb)
2: molecular weight marker (100 pb)
3: genomic DNA preparation (not sheared, 10 µg)
4: sheared genomic DNA (10 µg): average size of 300 bp

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STEP 3. DNA shearing

15. In a 1.5 ml tube, dissolve the DNA sample in TE to reach 0.1 µg/µl
- When preparing the sheared DNA sample to be used in Methyl DNA IP, make sure you use the DNA concentration of 0.1 µg/µl.
- Keep in mind that 1 µg of DNA is needed per IP.

16. Use a final volume of 300 µl of DNA sample in 1.5 ml tubes
- It is recommended to work with 30 µg of DNA per 300 µl (when enough material is available).
- e.g. for one IP done in duplicate and the input (corresponding to 20% of one IP), you will need 24 µl of sheared DNA sample (see Methyl DNA IP STEP 4-Table 5).
- Scale accordingly based on your sample size and number of IPs to do.

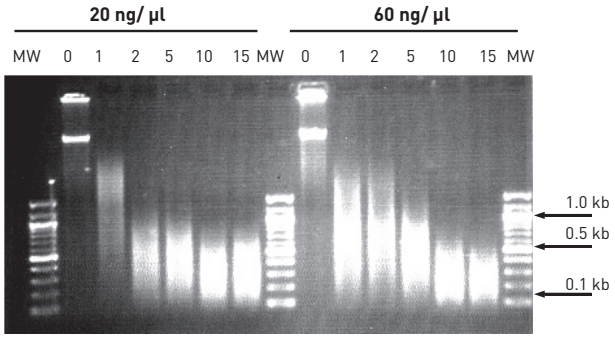
17. Shear the DNA using the BioruptorTM (http://www.diagenode.com/)
- Bioruptor® Pico: 6 cycles of 30 sec ON / 90 sec OFF (1.5 ml microtubes with caps)
- other Bioruptor models: 10 cycles of 15 sec ON / 15 sec OFF; Low Power

18. Sheared DNA can be analysed on agarose gel as shown in the above picture.

Additional notes:
- **It is advised to optimize the shearing conditions "in house" with your own sample, e.g.: it is possible to shear DNA using for instance: lower volumes of sample in smaller tubes; lower DNA concentrations can also be tested when setting up your shearing "in house"; ... (see pictures below).**
- **The expected size of the DNA fragments is 300-500 bp.**

Figures: analysis of sheared DNA and shearing optimization.

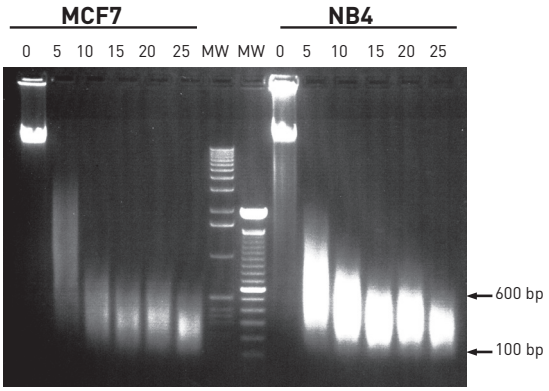
DNA Shearing: sample volume of 75 µl in 0.5 ml tubes



MW: molecular weight marker (100 bp)
0: not sheared genomic DNA (0.2 µg)
sheared genomic DNA for a total time of:
1: 1 minute
2: 2 minutes
5: 5 minutes
10: 10 minutes (best)
15: 15 minutes
DNA on gel: 0.2 µg

Shearing of the genomic DNA from NB4 cultured cells using the Bioruptor from Diagenode:
for 10 minutes at Low power (best shearing conditions) using cycles of 15 seconds « ON » and 15 seconds « OFF », volume sample of 75 µl (in 0.5 ml tube), and DNA concentration of 20-60 ng/µl.

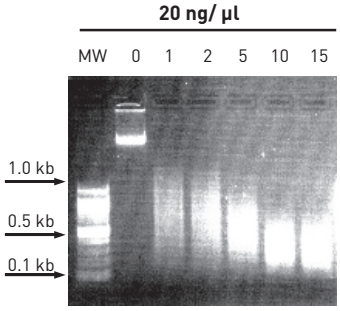
Bioruptor and **GenDNA module** from **Diagenode:**
Prepare DNA and shear DNA ready-to-use in Methyl DNA IP



MW: molecular weight markers
(1 kb and 100 bp ladders)
0: not sheared genomic DNA (0.2 µg)
sheared genomic DNA for a total time of:
1: 5 minutes
2: 10 minutes
5: 15 minutes
10: 20 minutes
15: 25 minutes

Shearing of the genomic DNA from MCF7 and NB4 cultured cells using the Bioruptor from Diagenode:
for 10 minutes at Low power (best shearing conditions) using cycles of 15 seconds « ON » and 15 seconds « OFF », volume sample of 300 µl (in 1.5 ml tube), and a DNA concentration of 20-60 ng/µl.

DNA Shearing: sample volume of 75 µl in 0.5 ml tubes

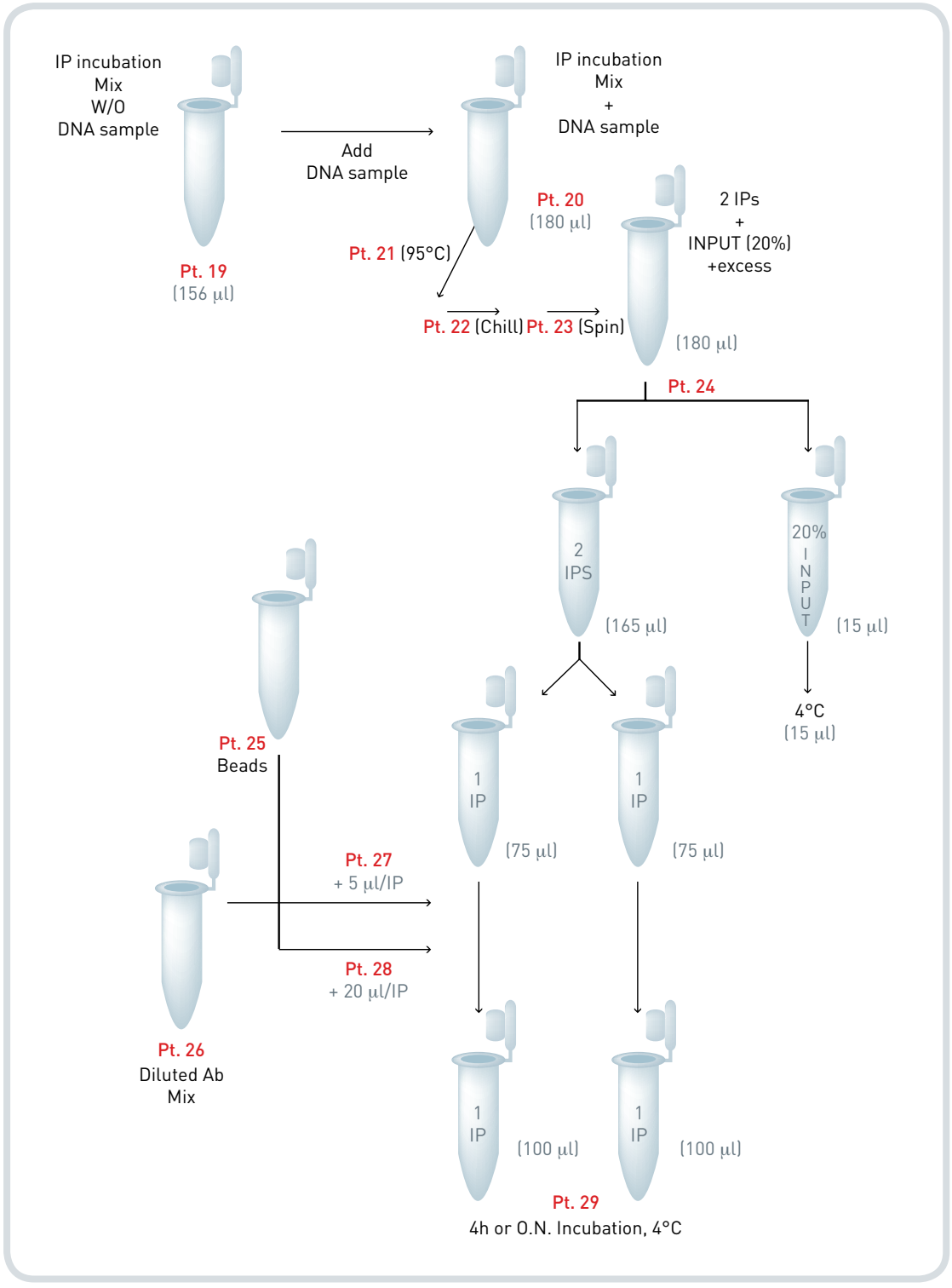


MW: molecular weight marker (100 bp ladder)
0: not sheared genomic DNA (0.2 µg)
sheared genomic DNA for a total time of:
1: 1 minute
2: 2 minutes
5: 5 minutes
10: 10 minutes (best)
15: 15 minutes
DNA on gel: (0.2 µg)

Shearing of the genomic DNA from MCF7 and NB4 cultured cells using the Bioruptor from Diagenode:
for 10 minutes at Low power (best shearing conditions) using cycles of 15 seconds « ON » and 15 seconds « OFF », volume sample of 300 µl (in 1.5 ml tube), and a DNA concentration of 20-60 ng/µl.

MANUAL

STEP 4. Immunoprecipitation of methylated DNA



19. Prepare the **IP incubation mix w/o DNA sample**.

- When preparing the IP incubation mix (w/o DNA sample added), take into account the following: - 1/ the input sample must also be used as control next to the IP, - 2/ when possible it is best to perform the IP at least in duplicate, and -3/ include an excess (see Table 5, right columns). Note that this mix includes the 2 kit DNA internal IP controls (unme and me).
- It is advised to perform each IP in duplicate (see right column).
- An input sample represents 20% of the IP sample, which includes the 2 DNA internal IP ctrls.
- The right column gives the volume of IP incubation mix to prepare for 2 IPs and 20% input. (Note that the final volume includes 20% excess).
- At this stage, the IP incubation mix contains the methylated and unmethylated DNA IP internal controls, the DNA sample will be added at the next Point.

Table 5. IP incubation mix without DNA sample added

#	Reagent	Volume per IP + Input (1)	Volume to be prepared for 2 IPs and input sample (2)
1	**Water** (adjust to sample*)	45 µl*	90 µl
2	**Buffer A**	24 µl	48 µl
3	**Buffer B**	6 µl	12 µl
4	**positive meDNA control**	1.5 µl	3 µl
5	**negative unDNA control**	1.5 µl	3 µl
	TOTAL VOLUME	**78 µl**	**156.00 µl**

* If the DNA sample is at a concentration of 0.1 µg / µl, use 45 µl of water per IP. If the concentration of the DNA sample is not at 0.1 µg / µl, adjust the volumes of water to add. Keep the volume of incubation mix without DNA sample at 78 µl / IP.

20. Label new 1.5 ml tubes. Add the **DNA sample** to the IP incubation mix (see below).
Start either with (IP DUPLICATES and input) combined in one tube (2) or not (1):

- (2) It is best to perform each IP in duplicate and to combine in one single tube both the input DNA and the DNA sample for duplicated IPs (see below).
- (1) If you do not have enough sample to perform duplicates of each IP, proceed to the alternative section (see below).
- In any case: 1 µg of DNA sample is needed per IP.

(2) If starting with combined (IP DUPLICATES and input):
(IP duplicates and input) are combined in one single tube until Point 6.
- Add in one tube: the IP incubation mix. Then, add 2.4 µg of DNA sample per tube.

- Using DNA samples at a concentration of 0.1 µg/µl: add 156 µl of IP incubation mix prepared at Point 1. (Table 5) and 24 µl of DNA sample per tube.
- **The total volume is then 180 µl corresponding to 2x IPs (2x 75 µl), 20% input (15 µl) and 20% excess (15 µl).**
- Using DNA samples at a concentration that is not 0.1 µg/µl: adjust the volumes as described in Table 5.

(1) Alternative
If starting with ISOLATED samples:
- Add per labeled "IP" tube: the IP incubation mix. Then, add 1 µg of DNA sample per tube.

- Using DNA samples at a concentration of 0.1 µg/µl: add 65 µl of IP incubation mix and 10 µl of DNA per tube. **The total volume per IP is 75 µl.**
- Using DNA samples at a concentration that is not 0.1 µg/µl: adjust the volumes as described in Table 5.

- Add per "input sample" tube: 20% of what is used per IP above.

- Using DNA samples at a concentration of 0.1 µg/µl: add 13 µl of IP incubation mix and 2 µl of DNA per tube. **The total volume for 20% input is 15 µl.**
- **Using DNA samples at a concentration that is not 0.1 µg/µl: use 20% of the volumes used per IP.**

21. Incubate at 95ºC for three minutes.

22. Quickly chill on ice (it is best to use ice-water).

23. Quickly perform a short spin at 4°C.

24. (*) If working with (IP Duplicates and input) combined in one single tube: first, take out **15 µ**l (that is **20% input**) and transfer to a new labeled tube.
Then, transfer what is left equally into 2 labeled IP tubes: **75 µl** per tube (these are **the 2 IP** tubes).

- Keep the input samples at 4°C. To be used later (STEP 6-). The input sample is to be used as a control of starting material and it is therefore not to be used in IP.
- Use the two IP tubes at Point 27. below.

(**) If working with isolated samples from the start, then proceed to Point 28.

25. In a new tube, prepare the **Diluted Antibody mix** as shown in Table 6 below.

For 4 IPs (Table 6: third column) and 10 IPs (Table 6: fourth column):

- Prepare a 1:10 antibody dilution (antibody, Buffer A and water).
- Then, add Buffer C.

Table 6. Diluted Antibody mix

Reagents (#7 and 8)	One IP	Volume to prepare for 4 IPs	Volume to prepare for 10 IPs
Antibody (as provided)	0.30 µl	1.50 µl	4.00 µl
Buffer A	0.60 µl	3.00 µl	8.00 µl
Water	2.10 µl	10.50 µl	28.00 µl
Buffer C	2.00 µl	10.00 µl	27.00 µl
FINAL VOLUME	**5.00 µl**	**25.00 µl**	**67.00 µl**

- In the table above, final volumes include an excess.
- Scale the volumes accordingly, based on the number of IPs that are performed on the day.
- Do not omit the dilution step (Point 8.) as the amount of antibody to be used is critical.
- You need **3 µl** of antibody at dilution **1:10** per IP and also **2 µl** of Buffer C per IP (Table 4).
- Antibody is first diluted using Buffer A and water, then Buffer C is added as shown in Table 6.
- Then 5 µl of Diluted Antibody mix per IP will be used (Point 9.).

26. Add **5 µl** of **Diluted Antibody mix** per **IP** tube (from Point 24. above).

- Antibody is added to the IP tubes, which contain IP incubation mix and your DNA sample
- Then, this will be transferred to the tubes, which contain the beads (Point 28.)
- The Diluted Antibody mix that is left can be discarded.

27. Add 20 µl of **beads** to all tubes (resuspend before use).
    - That is the IP incubation as described in Table 4. The final volume in each tube is 100 µl.
    - The IP incubation comprises the IP samples, the Diluted antibody mix and the beads.

28. Place on a rotating wheel at 4°C for 4 hours or overnight.

STEP 5. Washes

30. The Methyl DNA IP samples are then washed as follows: add 450 µl of ice-cold Wash buffer to each IP tube, starting with Wash buffer-1.
    - Place the four Wash buffers on ice and perform the washes in a cold room.

31. Rotate for 5 minutes at 4ºC.

32. Centrifuge at 6,000 rpm for 1 minute at 4°C.

33. Discard the supernatant. Do not disturb the pellet. Keep the pellet.

34. Wash the pellet again (as described above: Point 30. to 33.) as follows: perform one more wash with Wash buffer-1, then one wash with Wash buffer-2, and one wash with Wash buffer-3. Finally perform two more washes using the Wash buffer-4.

35. After the last wash, discard the last traces of Wash buffer (using a P200 pipet). Keep the bead pellets. Proceed to STEP 6-.
    - These are the Methyl DNA IP samples.
    - The Immunoprecipitated Methylated DNA is bound to the beads.

STEP 6. DNA Elution & purification

Option 1: Elution & purification using QIAquick PCR purification columns (QIAGEN # 28106)

36a. Take the input samples, centrifuge briefly and from now onwards treat the input DNA samples and IP samples in parallel.
- input samples (STEP 4- Point 24.).
- Methyl DNA IP samples (STEP 4- Point 35.).

37a. Prepare the complete elution buffer by mixing Buffer D, E and F as follows.
- The right column gives the volume of complete elution buffer to prepare for 2 IPs and 20% input. (Note that the final volume includes about 10% excess).

Table 7. Complete elution buffer

Complete elution buffer	1 Methyl DNA IP	2 IPs + input
Buffer D	103.50 µl	335 µl
Buffer E	11.50 µl	37 µl
Buffer F	5.00 µl	16 µl
Total volume	**120.00 µl**	

38a. Add 120 µl of freshly prepared complete elution buffer to the bead pellets (the Methyl DNA IP samples).

39a. Add 120 µl of freshly prepared complete elution buffer to the input samples.

40a. Incubate in a thermo-shaker for 10 minutes at 65°C at 1,000 to 1,300 rpm.

41a. Then, use the QIAquick instructions. In brief: add 600 µl PBI, vortex, and apply sample to column, centrifuge at 4,000 rpm for 1 minute (possibility to combine the two methyl DNA IP duplicate samples per column).

42a. Wash with 700 µl PE, centrifuge at 4,000 rpm for one minute, get rid of the flow-through.

43a. Spin at 13,000 rpm for one minute.

44a. Elute with 50 µl EB.
The purified DNA can also be eluted in TE buffer, but the EDTA may inhibit subsequent enzymatic reactions.

45a. Incubate at 50°C for 5 minutes.

46a. Centrifuge at 13,000 rpm for one minute.

At this stage, you obtain purified DNA from: 1/ the sheared DNA (input sample(s)) and 2/ DNA that was isolated by Methyl DNA IP (**Methyl DNA IP** sample(s)). Proceed to STEP 7-.

Option 2: Elution & purification using Phenol/Chloroform/Isoamyl alcohol

36b. Take the input samples, centrifuge briefly and from now onwards treat the input DNA samples and IP samples in parallel.
- Input samples (STEP 4- Point 24.).
- IP samples (STEP 4- Point 35.).

37b. Prepare the complete elution buffer by mixing Buffer D, E and F as follows.
- The right column gives the volume of complete elution buffer to prepare for 2 IPs and 20% input. (Note that the final volume includes about 10% excess).

38b. Add 416 µl of freshly prepared complete elution buffer to the bead pellets (the Methyl DNA IP samples).

Table 8. Complete elution buffer

Complete elution buffer	1 Methyl DNA IP	2 IPs + input
Buffer D	360 µl	1,188 µl
Buffer E	40 µl	132 µl
Buffer F	16 µl	53 µl
Total volume	**416 µl**	**1373 µl**

39b. Add 416 µl of freshly prepared complete elution buffer to the input samples.

40b. Incubate in a thermo-shaker for 10 minutes at 65ºC at 1,000 to 1,300 rpm.

41b. Cool down samples to room temperature, 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. Use gentle rotation.**

42b. 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.

43b. 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 on ice.

44b. 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.

45b. Per tube: add 5 µl of the provided meDNA-IP co-precipitant and 40 µl of the meDNA-IP precipitant. Then, add 1 ml of ice-cold 100% ethanol. Mix well. Leave at -80°C for 30 minutes.

46b. 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.

47b. 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 IP (Methyl DNA IP 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.

48b. Add of 50 µl TE to the IP and input 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 IP (Methyl DNA IP sample(s)). Proceed to STEP 7-.

STEP 7. qPCR analysis of IP'd DNA

The qPCR module includes validated primer pairs specific to four types of DNA:
a: one methylated human DNA region (X-linked alpha satellites)
b: one unmethylated human DNA region (GAPDH promoter)
c: the methylated DNA control (meDNA positive ctrls #1 and #2)
d: the unmethylated DNA control (unDNA negative ctrls #1 and #2).

See in the Table 8, the description of the primer pairs provided in the MeDIP kit: names, specificity, and expected amplification with input DNA and IP'd DNA are given. The color code in the left column refers to the graph given in the Results section.

Table 8. qPCR module following Methyl DNA IP

	Primer pairs (10 µM each)	Specificity	input DNA sample (which includes Ctrls) amplification :	Methyl DNA IP (which includes Ctrls) amplification :
	hum meDNA primer pair (AlphaX1)	Human DNA	Yes	Yes
	hum unDNA primer pair (GAPDH)	Human DNA	Yes	No
	meDNA pos control primer pair #1	Kit Positive Ctl	Yes	Yes
	meDNA pos control primer pair #2	Kit Positive Ctl	Yes	Yes
	unDNA neg control primer pair #1	Kit Negative Ctl	Yes	No
	unDNA neg control primer pair #2	Kit Negative Ctl	Yes	No

* If sample is human DNA

1/ Make aliquots of the **purified DNA** and make dilutions as follows:
- Use the purified DNA from Methyl DNA IPs and DNA input(s) (end of STEP 2-).
- From the 50 µl of purified DNA: transfer 10 µl into a new tube (keep 40 µl for Methyl DNA IP-on-chip analysis, or further PCR analysis).
- For the first PCR analysis, **dilute 10 µl of each purified DNA sample** as follows:
  to 10 µl of purified DNA sample (from IP and input), add 35 µl of water (find volume: 45µl).
  Use 5 µl per PCR (see below).
  Note: when testing the hum meDNA primer pair (AlphaX1): dilute the DNA sample 1:1,000.

2/ 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)
5.00 µl of diluted purified DNA sample (see above for DNA dilutions)
6.50 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

3/ When the PCR is done, analyse the results. Some major advices are given below.

- **Your own primer design**
  - Self-complementarity and secondary structure of the primers can be tested using primer design programs. Annealing temperature of 60°C is recommended for qPCR primers.
  - 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.

- **Advantages of the qPCR**
  qPCR or Real time PCR enables fast, quantitative and reliable results. Visit: http://www.gene-quantification.info/. The Gene Quantification page describes and summarises all technical aspects involved in quantitative gene expression analysis using real-time qPCR & qRT-PCR. It presents a lot of applications, chemistries, methods, algorithms, cyclers, kits, dyes, analysis methods, meetings, workshops, and services involved.

- **Validation of your primers**
  - 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 by formula(1): 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.

- **Data interpretation**
  The efficiency of Methyl DNA Immunoprecipitation of particular genomic loci can be calculated from qPCR data and reported as a percentage of starting material: % (MeDNA-IP/ Total input).

% (MeDNA-IP/ Total input)= 2^[(Ct(20%input) - 2.322) - Ct(MeDNA-IP)]x 100%

Here 2 is the AE (amplification efficiency) as calculated above(1); $Ct^{(MeDNA\text{-}IP)}$ and $Ct^{(20\%input)}$ are threshold values obtained from exponential phase of qPCR for the Methyl DNA sample and input sample respectively; the compensatory factor (2.322) is used to take into account the dilution 1:5 of the input. The recovery is the % (MeDNA-IP/ Total input).

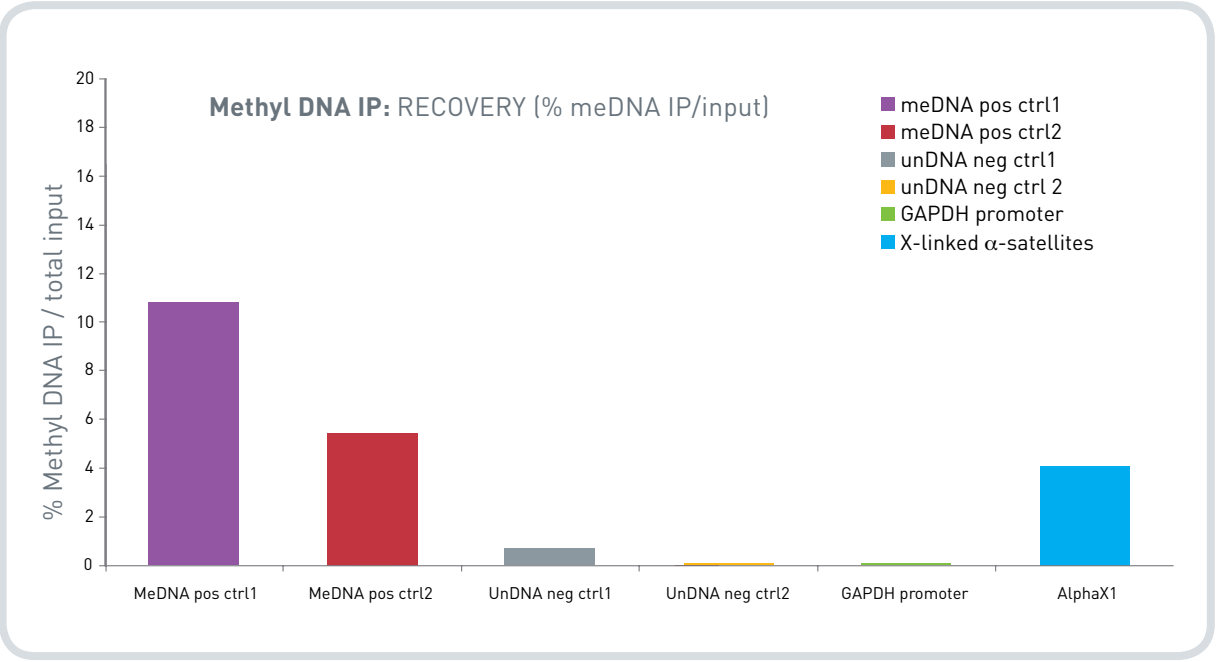
- **Background determination**
  The final goal of IP is to calculate the enrichment in the same IP sample of: 1/ the specific DNA fragments (corresponding to the methylated DNA) in comparison with 2/ non-methylated DNA (i.e. negative unDNA control).

- **Relative occupancy** can be calculated as a ratio of specific signal over background.

Occupancy= % input (specific loci) / % input (background loci)

Relative occupancy is then used as a measure of the methylation of a specific locus; it provides clues about specificity of the IP. (background loci) corresponds to the signal obtained with one of the unmethylated DNA kit control (unDNA neg ctrl 1 or 2).

Results



Methyl DNA IP results obtained with the Diagenode MeDIP Kit (cat# mc-green-03).
Methyl DNA IP assays were performed using DNA from NB4 cells, the Diagenode antibody directed against 5-methyl Cytidine and optimized PCR primer pairs for qPCR. The DNA was prepared with the GenDNA module. The IP on the human DNA sample, together with the internal kit controls. The internal positive and negative DNA controls included in the IP assay are methylated DNA (meDNA) and unmethylated DNA (unDNA). The DNA is then purified from the IP'd material and analysed by PCR using the primer pairs included in the kit (see below).

Each "primer pair" targets a specific DNA and expected results are as follows:
- Internal DNA controls
"meDNA pos ctrl1": meDNA control (positive signal is obtained for methylation).
"meDNA pos ctrl2": meDNA control (positive signal is obtained for methylation).
"unDNA neg ctrl1": unDNA control (no signal is obtained for 0% methylation).
"unDNA neg ctrl2": unDNA control (no signal is obtained for 0% methylation).
- Human DNA sample:
"GAPDH promoter": no signal is expected as this region is not methylated.
"X-linked alpha-satellites": a positive signal is expected as it is a methylated region.

The Methyl DNA IP controls reveal IP efficiency.

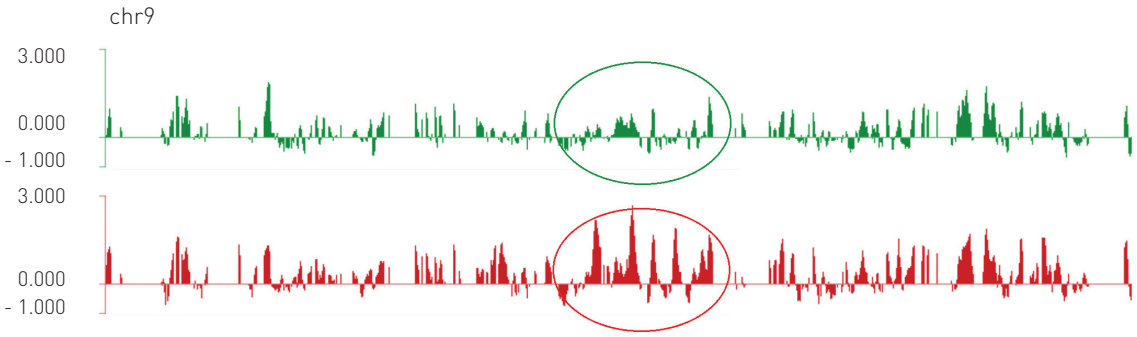
Additional Protocol

Methyl DNA IP-on-chip and results

The DNA obtained following a Methyl DNA IP must be submitted to amplification before hybridization to DNA array. The method of choice is T7 based linear amplification described by Liu and al, in 2003 (2)

Results

Methyl DNA IP-on-chip starting with the MeDIP Kit from Diagenode for the IP.



Methyl DNA IP has been performed with the MeDIP Kit from Diagenode (cat# : mc-green-03), prior to amplification and hybridization.
Analysis of the DNA methylation from two samples: one from an healthy individual (top) and one from a patient (bottom). Array peaks indicate methylated DNA regions in and around genes on one piece of chromosome 9. There is a clear enrichment of methylated DNA in one sample compared to the other (as indicated, circles).

Troubleshooting Guide

Critical steps	Troubles	Solutions
Cell lysis	Cells are not completely disrupted.	Do not use too many cells per amount of lysis buffer (W/V). Follow the instructions in the protocol.
Cell number	The amount of cells required for one preparation of genomic DNA is important.	It is important to keep in mind that enough cells must be available to start with, to be able to use then one µg of DNA per IP.
DNA shearing	Buffer composition.	Use the buffers provided in the kit. They are optimized containing essential key components. Keep samples cold.
Picture of Sheared DNA	The migration of large quantities of DNA on agarose gel can lead to poor quality pictures, which do not reflect the real DNA fragmentation.	Do not load too much on a gel. Do not load more than 5 µg/lane. Also treat the sample with RNAse.
	Agarose concentration.	Do not use more than 1% agarose gel and run slowly
	Running buffer concentration.	1X TAE or TBE is preferred to 0.5X TAE which can lead to smears on gel..
Beads in the IP	Beads centrifugation.	Don't spin the beads at high speed. Use gentle centrifugation (500x g for 2-3 minutes) as described in the manual protocol. g = 11.18 x r x (rpm/1000)^2; knowing that r is the radius of rotation in mm. It is possible to centrifuge the 1.5 ml tubes at 1,000 – 2,000 x g, for 20 seconds.
	Bead storage	Store at 4°C. Do not freeze.
	Bead binding capacity	pAb from rabbit, guinea pig, pig, human IgG. MAb from mouse (IgG2), human (IgG1,2 and 4); and rat (IgG2c).
Antibody in IP	Can I use another antibody?	This kit has been validated with the provided antibody. It is essential to use the provided antibody together with buffers. Follow the kit instructions.
	Amount of antibody per IP to use?	To ensure efficient IP it is important to use the diluted antibody as described in the protocol. The lack of antibody can result in low IP efficiency whereas large excess of antibody might lead to lower specificity.
	Your own primers.	- length: 18 to 24 nucleotides - Tm: 60°C (+/- 3.0°C) - % GC: 50% (+/- 4%)
	PCR controls: –ve and +ve	-ve PCR control: PCR with primers specific for a DNA region that is not methylated (kit control and human) +ve PCR control: PCR with primers specific for a DNA region that is methylated (kit control and human) +ve PCR control: PCR on input DNA.
	Methyl DNA IP: qPCR primer pairs.	The provided qPCR primers are targeting human genomic loci (unmethylated and methylated) as well as control DNA (unmethymated and methylated).
	qPCR primers are provided for rapid checking of the Methyl DNA IP efficiency.	The provided qPCR primers are targeting human genomic loci (unmethylated and methylated) as well as control DNA (unmethymated and methylated).
Freezing	Samples can be frozen at several steps of the protocol.	- genomic DNA - sheared DNA - input DNA - IP'd DNA
	Avoid freeze/thawingTTT	Snap freeze cells and thaw on ice.

References

1. Pfaffl M.W.2001 *Nucleic Acids Res.* 29(9):e45.
2. Liu C.L., Schreiber S.L. and Bernstein B.E. 2003 *BMC Genomics* 4(1):1-11

Ordering information

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Diagenode website: http://www.diagenode.com/

MANUAL

Bringing it all together: Diagenode's ChIP-seq workflow

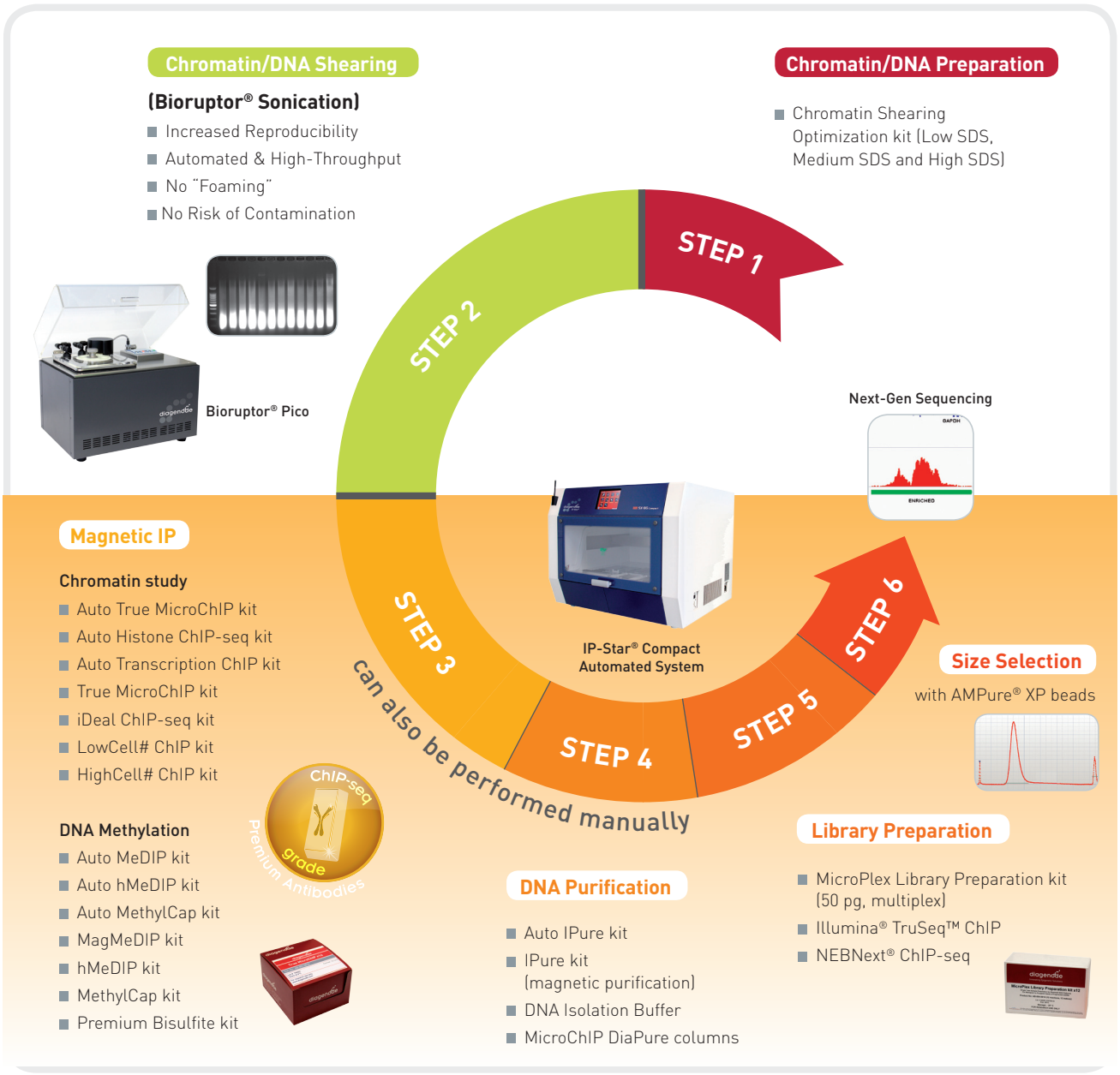


Figure 1. Diagenode provides a full suite of manual and automated solutions for ChIP experiments.

For Step 1, we offer products to isolate nuclei and chromatin. Step 2 describes reproducible sample shearing with the Bioruptor® product line. In Step 3 and Step 4, the Diagenode IP-Star® Compact provides error-free, walk-away automation for all your immunoprecipitation and antibody capture needs.

DNA Methylation, Cancer and Methods Overview

In addition to the MeDIP Kit manual:

Intoduction to DNA Methylation

What is DNA Methylation?

DNA methylation and CpG islands

DNA methylation is an **epigenetic** event that affects cell function by altering gene expression. It involves the addition of a methyl group to cytosine residues at CpG dinucleotides, a reaction that is catalyzed by DNA methyltransferase (DNMT) enzymes.

5-methyl cytosine is produced by the action of the DNA methyltransferases (DNMT 1, 3a or 3b), which catalyses the transfer of a methyl group (CH3) from S-adenosylmethionine (SAM) to the carbon-5 position of cytosine. DNA methylation occurs exclusively at CpG dinucleotides. The biological significance of methylation status seems to be important at **CpG islands**, where CpG sites cluster. It is required for embryonic development and implicated with genomic imprinting and X-chromosome inactivation.

CpG is under-represented throughout most of genome but enrichment of CpG is found at expected frequencies in short ~1 KB stretches called CpG islands. There are about 45,000 CpG islands in genome, and these are mostly located at promoters within first exons of genes. CpG islands are unmethylated in normal cells. Alteration of DNA methylation is associated with diseases.

CpG islands located in the 5' region of a gene are known as molecular switches that can turn-off or turn-on the expression of the downstream gene. For the majority of genes, the CpG islands in their 5' regions are not methylated and they are ready to be expressed.
In some cancers, CpG islands in the 5' regions of tumor-suppressor genes are methylated and their expressions are switched-off (e.g. methylation of CpGs near tumor suppressor genes (p53 or p16) is often related to silencing of these genes in tumors). This mechanism of gene silencing is known to be one of the major mechanisms of tumor-suppressor gene inactivation.

CpG islands are thought to be normally unmethylated and are associated with 75% of human genes (Ioshikhes and Zhang 2000). CpG islands are defined operationally as sequences that have G+C contents of →0.55, observed versus expected CpG densities of →0.5, and a length of →300 bp (although very few are ←500 bp) (Aerts et al. 2004). The CpG island compartme nt has been held to be constitutively unmethylated (Rollins et al. 2006).

What is the role of DNA methylation?

DNA methylation is the process by which cytosines are methylated after DNA replication. In higher organisms from plants to humans, methylation protects DNA from endonuclease degradation and plays a critical role in regulating gene expression, making it essential for normal development and function.

The cytosine at CpG sites is methylated or is unmethylated. When CpGs are methylated, they are methylated on both strands by the DNA methyltransferase that methylates hemi-methylated CpG sites. Upon DNA replication, the methylated or unmethylated status is inherited on both of the two newly synthesized DNA molecules. DNA methylation is a marker on genomic DNA that can persist over cell generations.

It is suggested that cytosine methylation acts to maintain the accessible compartment of the genome at a constant level and thereby buffers the genome against large changes in size that result from sequence duplications and the accumulation of transposons (Rollins et al. 2006).This suggestion in consistent with the finding that all known large genome (→5 -108 bp) organisms have methylated DNA (Kidwell 2002) and contain genes for members of the DNA methyltransferase 1 and 3 families (Goll and Bestor 2005), while many small genome eukaryotes lack recognizable DNA methyltransferase genes altogether.

What is the link between DNA Methylation and cancer?

DNA methylation and cancer

Genome stability and correct gene expression is maintained to a great extent thanks to a pre-established pattern of DNA methylation and histone modifications. DNA methylation is therefore an important epigenetic mechanism of transcriptional control. DNA methylation plays an essential role in maintaining cellular function and changes in methylation patterns may contribute to the development of cancer. Cancer is a process driven by the accumulation of abnormalities in gene function. The fact that tumor-suppressor genes can be silenced by aberrant DNA methylation was first shown in 1993.The role of DNA methylation in carcinogenesis has been a topic of considerable interest in the last few years. Alterations in DNA methylation are common in a variety of tumors as well as in development. Of all epigenetic modifications, hypermethylation, which represses transcription of the promoter regions of tumor suppressor genes (such as BRCA1, hMLH1, p16INK4a and VHL) leading to gene silencing, has been most extensively studied. However, global hypomethylation has also been recognized as a cause of oncogenesis. New information concerning the mechanism of methylation and its control has led to the discovery of many regulatory proteins and enzymes (Das and Singal 2004).

Aberrant methylation of DNA (global hypomethylation accompanied by region-specific hypermethylation) is frequently found in tumor cells. Global hypomethylation can result in chromosome instability, and hypermethylation has been associated with the inaction of tumor suppressor genes. Preclinical and clinical studies suggest that part of the cancer-protective effects associated with several bioactive food components may relate to DNA methylation patterns (Davis and Uthus 2004).
Aging and chronic inflammation are known to cause DNA methylation. There are many carcinogens that do not cause mutations but some of these could induce aberrant DNA methylation. DNA methylation serves as a mark on DNA molecules that cannot be erased over cell generations. However, the fidelity of the DNA methylation mark started to be analyzed in detail a few years ago. The fidelity in inheriting unmethylated or methylated status of a CpG site has been measured and it is essential to analyze possible changes in this fidelity in cancer cells (Ushijima et al. 2003).
Epigenetic alterations play a crucial early role in cancer that can substitute for genetic variation later in tumour progression. Therefore, epigenetically disrupted stem/progenitor cells might be a crucial target for cancer risk assessment and chemoprevention (Feinberg et al. 2006).Embryonic stem cells rely on Polycomb group proteins to reversibly repress genes required for differentiation. It was reported that stem cell Polycomb group targets are up to 12-fold more likely to have cancer-specific promoter DNA hypermethylation than non-targets, supporting a stem cell origin of cancer in which reversible gene repression is replaced by permanent silencing, locking the cell into a perpetual state of self-renewal and thereby predisposing to subsequent malignant transformation (Widschwendter et al. 2007).

Histone modifications, DNA methylation and cancer

Histone modifications and DNA methylation integrate nuclear function. Cancer is characterised by the breakdown of both DNA methylation and histone modification patterns.
It is also widely accepted that methyl-CpG binding proteins (**MBD**s) couple DNA methylation to gene silencing through the recruitment of histone deacetylase and chromatin remodelling activities that modify chromatin structure (Esteller).
The laboratory of Adrian Bird has an interest in the study of the methyl-CpG binding repressors.
Although some histone modifications are general to active genes (acetylation and trimethylation at lysine 4 of H3), genes are regulated at many different levels including methylation of argine 17 of H3, specific use of histone H3.3 variant and nucleosomal remodeling (Esteller and Almouzni 2005). The aberrant pattern of epigenetic modifications in cancer cells can be as follows: promoter CpG islands of tumour-suppressor genes undergo DNA hypermethylation and there is a loss of histone acetylation and trimethylation of H3–lysine 4, while repetitive sequences present DNA hypomethylation and loss of monoacetylation of H4–lysine 16 and trimethylation of H4–lysine 20 (Esteller and Almouzni 2005).

How is DNA Methylation studied?

Methods to study DNA methylation are listed below and some are described in details as well.

Hypermethylation of CpG island sequences is a nearly universal somatic genome alteration in cancer. Rapid and sensitive detection of DNA hypermethylation would aid in cancer diagnosis and risk stratification.
In the field of epigenomics, there has been much activity in researching the additional layer of regulation that DNA methylation adds to the genetic code. Many techniques have been developed to analyze the genome-wide methylation content and the methylation status of specific loci. The majority of methylation screening protocols utilizes methylation-sensitive endonuclease digestion or bisulfite treatment of the template followed by subsequent PCR amplification of a specific sequence. Methylated DNA sequencing is also a critical method being used to understand an array of developmental diseases and cancers. Here we also describe the methylated DNA immunoprecipitation method (Methyl DNA IP).

Methods used for detection of methylated DNA are:

1. Methylation sensitive enzymatic digestion
2. MBD methods
3. Bisulfite method
4. Methyl DNA IP

Main characteristics:

1. Methylation sensitive enzymatic digestion: DNA sequence dependent, not HTP
2. MBD methods: sequence dependent, low specificity
3. Bisulfite method: involves sequencing, laborious, not HTP
4. Methyl DNA IP: highly efficient enrichment of methylated DNA dose-dependent and sequence-independent, high specificity

DNA methylation possible approaches are:

- Gene-specific: use methylation sensitive restriction enzymes to digest DNA followed by Southern detection or PCR amplification.

- Global: measure the overall level of methyl cytosines in genome such as with chromatographic methods and methyl accepting capacity assay.

Bisulfite reaction based methods have become very popular such as methylation specific **P**CR (**MSP**), bisulfite genomic sequencing PCR. Several of genome-wide screen methods have been invented such as restriction landmark **g**enomic **s**canning for **M**ethylation (**RLGS-M**) and CpG island microarray.

While cytosine methylation is a major component of epigenetic regulation of gene expression, many of the techniques used to test cytosine methylation at multiple loci are not suitable for comparing methylation levels at different loci within a genome (Khulan et al. 2006). In contrast, analogous intragenomic profiling has been successfully developed for studying chromatin organization using chromatin immunoprecipitation with genomic microarrays (ChIP-on-chip) (Kim et al. 2005) and DNA copy number using array comparative genomic hybridization (aCGH) (Selzer et al. 2005). The establishment of a platform for intragenomic profiling is a prerequisite for integrating studies of cytosine methylation with other whole-genome studies of epigenetic regulation. Comprehensive reviews of cytosine methylation analytical techniques describe the various approaches used to date (Laird 2003; Ushijima 2005), not including several recent reports of note (Weber et al. 2005).

1. Methylation sensitive enzymatic digestion:

MS-RDA (methylation-sensitive representational difference analysis)
A genome-scanning method for aberrant DNA methylation.
A genome-scanning technique that enables to search for differences in DNA methylation was developed in order to identify new tumor-suppressor genes by searching for aberrant DNA methylation throughout the genome and by locating the genes silenced by aberrant DNA methylation (Ushijima 1997)

Trying to identify new players in cancer using DNA methylation.
The MS-RDA method enables to isolate DNA fragments that were differentially methylated between normal and cancer cells (Ushijima 1997). The next step is to clarify what genomic regions those DNA fragments were derived from and which genes were located nearby. This step is extremely time-consuming before the release of the human genome sequence but can now be done in 10 to 20 minutes by computer analysis.

Methylation-sensitive representational difference analysis (**MS-RDA**) was previously established to detect differences in the methylation status of two genomes. This method uses the digestion of genomic DNA with a methylation-sensitive restriction enzyme, HpaII, and PCR to prepare «HpaII-amplicons,» followed by RDA. An HpaII-amplicon prepared using betaine and reverse electrophoresis was enriched 3.6-fold (compared with the HpaII-amplicon prepared by the original method) with DNA fragments originating from CpG islands. As for the specificity of MS-RDA, it was shown that DNA fragments that are unmethylated in the tester and almost completely methylated in the driver are efficiently isolated. This indicated that genes that are in biallelic methylation or in monoallelic methylation with loss of the other allele are efficiently isolated. Further, by use of two additional methylation-sensitive six-base recognition restriction enzymes, SacII and NarI, more DNA fragments were isolated from CpG islands in the 5′ regions of genes. After analysis of human lung, gastric, and breast cancers, 12 genes were seen to be silenced and additional genes seen to show decreased expression in association with methylation of genomic regions outside CpG islands in the 5′ regions of genes. **MS-RDA** is effective in identifying silenced genes in various cancers (Kaneda et al. 2003).

The principle of a new approach is to combine methylation-sensitive enzyme digestion with the comparative genomic hybridization (CGH) technique to develop an array-based method to screen the entire genome for changes of methylation pattern. The new technique will serve as an efficient tool in understanding the nature of epigenetic changes and their significance to the aging process and cancer development (Wojdacz and Hansen 2006).

The advantage of **MS-RDA** is that it focuses on unmethylated and CpG-rich regions of the genome, and thus differential methylation of unique CpG islands can be intensively screened, avoiding interference by repetitive sequences.

MSRE-PCR
MSRE-PCR is an assay for simultaneous analysis of DNA methylation of many promoters, based on digestion of genomic DNA with methylation-sensitive restriction enzyme and multiplexed PCR with gene-specific primers (Melnikov et al. 2005).

MSRE-PCR includes extensive digestion of genomic DNA (uncut fragments cannot be identified by PCR), can be applied to dilute samples (←1 pg/microl), requires limited amount of starting material (42 pg or genomic equivalent of seven cells) and can identify methylation in a heterogeneous mix containing ←2% of cells with methylated fragments (Melnikov et al. 2005).

HELP (HpaII tiny fragment Enrichment by Ligation-mediated PCR)

The use of restriction enzymes that are sensitive to cytosine methylation has allowed many of the early insights into the distribution of methylated CpG dinucleotides in the mammalian genome. For example, the use of HpaII revealed that most of the genome remains high molecular weight following digestion despite the short recognition motif (5'-CCGG-3') at which the enzyme cuts (Singer et al. 1979). It was subsequently recognized that 55%–70% of HpaII sites in animal genomes are methylated at the central cytosine (Bird 1980; Bestor et al. 1984), which is part of a CpG dinucleotide. The minority of genomic DNA that cuts to a size of hundreds of base pairs was defined as HpaII Tiny Fragments (HTFs) (Bird 1986), revealing a population of sites in the genome at which two HpaII sites are close to each other and both unmethylated on the same DNA molecule. Cloning and sequencing of these HTFs revealed them to be (G+C)-rich and CpG dinucleotide-rich, allowing base compositional criteria to be created to predict presumably hypomethylated CpG. These criteria remain in use for genomic annotations today, defining sequences that tend to localize with transcription start sites, especially of genes active constitutively or during embryogenesis (Khulan et al. 2006).

Genome sequencing project data have revealed that ←12% of HpaII sites in the human genome (and ←9% in mouse) are located within annotated CpG islands (Fazzari and Greally 2004).

This raised the question of whether a substantial proportion of HTFs is, in fact, derived from non-CpG island sequences and could be used to examine many non-CpG island sites in the genome for cytosine methylation status (Khulan et al. 2006). A technique was described that is based on HTF enrichment by ligation-mediated PCR, creating the acronym HELP that gives a name to this assay (Khulan et al. 2006). HELP enrichment, used as part of comparative isoschizomer profiling and in combination with customized genomic microarrays, allows robust intragenomic profiling of cytosine methylation. It was show that in primary mouse tissues 28%–34% of annotated CpG islands are categorized as methylated, that the technique reveals large numbers of tissue-specific differentially methylated regions (T-DMRs), and that some of the hypomethylated sites are located at repetitive sequences. These surprising patterns of cytosine methylation indicate that the intragenomic profiling capability of the HELP assay will allow insights into this major mediator of epigenetic regulation that were not possible with single-locus studies or intergenomic comparisons.

MethylLight

MethyLight is a quantitative real-time PCR technique to measure the DNA methylation. The technology measures the frequency of molecules in which a series of CpG sites in a given CpG region are methylated. The individual measurements are normalized for the total amount of DNA present and the proportion of fully methylated molecules from an enzymatically methylated sample (SssI-treated DNA) using two different control genes (Siegmund 2004). The resulting measurement is the percentage of methylated reference (PMR).

Main characteristics and drawbacks of the methylation sensitive enzymatic digestion method

DNA methylation detection assays using methylation sensitive restriction enzymes to digest unmethylated DNA while leaving methylated DNA intact can be used followed by the detection by Southern blot analysis, PCR or real-time PCR.

- The Southern blot strategy requires large amounts of high molecular weight DNA and is not compatible with high-throughput analysis.
- Enzymatic digestion followed by PCR is sensitive, but is limited to interrogating methylation only at the enzyme recognition sites (e.g. HpaII digestion is limited by sequence content) and incomplete digestion is often the cause of false-positive results.

Other ways to detect methylated DNA:

2. MBD-methods:

Another strategy for in vitro methylation detection, first introduced in 1994 by Cross, uses column- or bead-immobilized recombinant methylated-CpG binding domain (MBD) proteins, particularly MECP2 and MBD2, to enrich for methylated DNA fragments for subsequent detection by Southern blot, PCR or microarray hybridization.

COMPARE-MS

COMPARE-MS has been described as a new method that can rapidly and quantitatively detect CpG island hypermethylation with high sensitivity and specificity in hundreds of samples simultaneously. To quantitate CpG island hypermethylation, COMPARE-MS uses real-time PCR of DNA that was first digested by methylation-sensitive restriction enzymes and then precipitated by methyl-binding domain polypeptides immobilized on a magnetic solid matrix (Yegnasubramanian et al. 2006).

MB-PCR (methyl-binding (MB)-PCR)

A novel technique for detecting CpG-methylated DNA termed methyl-binding (MB)-PCR has been developed (Gebhard C. et al. 2006). MB-PCR utilizes a recombinant protein with high affinity for CpG-methylated DNA that is coated onto the walls of a PCR vessel and selectively captures methylated DNA fragments from a mixture of genomic DNA. The retention and, hence, the degree of methylation of a specific DNA fragment (e.g. a CpG island promoter of a specific gene) is detected in the same tube by gene-specific PCR. MB-PCR does not require bisulfite treatment or methylation-sensitive restriction and provides a quick, simple and extremely sensitive technique allowing the detection of methylated DNA, in particular in tumor tissue or tumor cells from limited samples, but lack of specificity and HTP compatibility. Using this novel approach, the methylation status of several established and candidate tumor suppressor genes was determined (Gebhard C. et al. 2006).

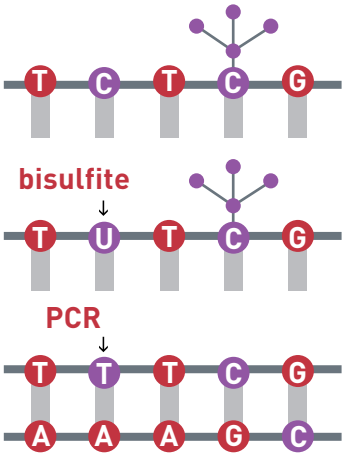
A recent version of this strategy, called methylated-CpG island recovery assay (MIRA), MIRA (Rauch and Pfeifer 2005), uses full-length MBD2 immobilized on magnetic beads to enrich for methylated DNA with subsequent detection of candidate methylated genes by PCR. MIRA-assisted microarray analysis has also been developed and has a low false-positive rate and the capacity to catalogue methylated CpG islands on a genome-wide basis (Rauch and Pfeifer 2006).

Drawbacks of the MBD methods

The use of each the MBD technique has been limited by one or more of the following: a requirement for relatively large amounts of input genomic DNA, a potential for false-positive results due to capture of unmethylated DNA, incompatibility with high-throughput platforms and lack of quantitative data.

3. Bisulfite method

The bisulfite method for determining the methylation status of cytosine residues (C) in a DNA molecule depends on the reaction of bisulphite with cytosines (C) in single stranded DNA. Cytosines (C) are converted to uracils (U) whereas 5-methyl cytosines (5-meC) are unreactive. The modified DNA strands can be amplified using PCR and sequenced to give methylation data from single DNA molecules.



An understanding of DNA methylation and its potential role in gene control during development, aging and cancer has been hampered by a lack of sensitive methods which can resolve exact methylation patterns from only small quantities of DNA. A genomic sequencing technique has been developed which is capable of detecting every methylated cytosine on both strands of any target sequence, using DNA isolated from fewer than 100 cells (Clark et al. 1994). In this method, sodium bisulfite is used to convert cytosine residues to uracil residues in single-stranded DNA, under conditions whereby 5-methylcytosine remains non-reactive. The converted DNA is amplified with specific primers and sequenced. All the cytosine residues remaining in the sequence represent previously methylated cytosines in the genome (Clark et al. 1994).

Optimization of the methods based on bisulfite modification of DNA permits the analysis of limited CpGs in restriction enzyme sites (e.g. combined bisulfite restriction analyses and methylation-sensitive single nucleotide primer extension) and overall characterization based on differential methylation states (e.g. methylation-specific PCR (MSP), MethyLight, and methylation-sensitive single-stranded conformational polymorphism). Very specific patterns of methylation can also be revealed by bisulfite DNA sequencing. In addition, novel methods designed to search for new methylcytosine hot spots have yielded further data without requiring prior knowledge of the DNA sequence (Fraga and Esteller 2002).

MSP (Methylation-specific PCR):
DNA is denatured then bisulfite-treated before MSP. Bisulfite treated DNA is then purified. As described above, after treatment, the unmethylated cytosine will be converted to uracil, whereas the methylated cytosine is unchanged. Uracil is recognized as thymine by Taq polymerase. The treated DNA is subjected to MSP using unmethylation-specific or methylation-specific primers. An untreated DNA from a normal individual is used as negative (Yang et al. 2004).

Some other ways to detect methylated DNA:

- FastStart High Fidelity PCR system (Paduch 2005).
- COBRA (combined bisulfite restriction analysis) method as described previously. Methylated DNA remains capable of cleavage while unmethylated DNA is resistant.

Characteristics and some drawbacks of the bisulfite method:
Many of the current DNA methylation detection methods use sodium bisulfite to deaminate cytosine to uracil while leaving 5-methylcytosine intact, such as: methylation-specific PCR (MSP) uses PCR primers targeting the bisulfite induced sequence changes to specifically amplify either methylated or unmethylated alleles. There are quantitative variations of this technique, such as: MethyLight, HeavyMethyl and MethylQuant, which employ methylation-specific oligonucleotides in conjunction with Taqman probes or SYBR Green based real-time PCR amplification to quantitate alleles with a specific pattern of methylation.
These techniques are highly sensitive and specific for detection of DNA methylation, but:
- the PCR primer design is quite difficult due to the reduction in genome complexity after bisulfite treatment, leading to an inability to interrogate the methylation pattern at some or all CpG dinucleotides in a genomic locus of interest.
- all of the bisulfite-based techniques are quite cumbersome, involving time- and labor-intensive chemical treatments that damage DNA and limit throughput.

Diagenode developed tools to overcome the difficulties inherent to "in house" protocols, see below (Diagenode's Tools section below).

4. Methyl DNA IP (Methylated DNA Immunoprecipitation)

Methylated DNA can be immunoprecipitated using an antibody directed against 5-methyl Cytidine (5-meC). Immunoprecipitation of methylated DNA (Methyl DNA IP, also called MeDIP) was first described in 2004 (Mukhopadhyay et al. 2004, Weber et al. 2005). The method is described in the overview shown below. In brief, Methyl DNA IP is performed as follows: from cultured cells or tissues, genomic DNA is prepared, genomic DNA is then sheared and denatured. Then, immunoselection and immunoprecipitation can take place using the antibody directed against 5-methyl Cytidine and antibody binding beads. DNA isolation and purification is performed to proceed to qPCR and analyse the IP'd methylated DNA (see Diagenode's Tools section below).

Characteristics of the Methyl DNA IP method:
Methyl DNA IP uses bead-immobilized anti-5-methyl cytosine antibodies, instead of MBD proteins, to enrich for methylated DNA. However, the use of the Methyl DNA IP technique is compatible with high-throughput platforms and can directly give reliable qualitative results as well as semi-quantitative data. In addition, the use of a standard methylation curve should render the method quantitative.

The MBD domain of MeCP2 has been used instead of an antibody to pulldown methylated sequences, but:
1. A monoclonal antibody is produced with far less lot-to-lot variation in comparison with a fusion protein expressed in E. coli. We indeed demonstrated the specificity of our antibody directed against 5-methyl Cytidine and Methyl DNA IP kit at Diagenode.

2. The MBD domain of MeCP2 is known to have sequence preference besides its recognition of methyl-CpGs (Klose et al., 2005). There is an AT-run next to the methyl-CpGs that really boosts binding activiy. This activity is not located elsewhere in MeCP2 but within the MBD itself. Using the MBD of MeCP2 for pulling-down methylated DNA is very likely to be biased in some way.
3. The MBD method and the antibody method have been compared: both methods give similar but yet different results : the MBD method does require higher methyl-CpG density (Zhang et al., 2006).

Finaly, methods involving the use of restriction enzymes to digest DNA for methylation analysis introduce another type of bias, or may result in a poorer resolution if these sites are absent in a particular DNA sequence.

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