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Abstract	Hypomethylation of repetitive DNA elements is a common epigenetics event in cancer. Although it is believed that this hypomethylation impacts chromosomal and transcriptional stability of the genome, the extent of repetitive sequences contribution to the development and progression of human cancers remains to be clarified. Repetitive sequences have largely been ignored by genome-wide studies, and thus little is known about the DNA methylation profiles of different repetitive DNA elements types. As a step toward investigating epigenetic landscape of repetitive DNA, we have developed a repeat-specific DNA microarray called RepArray. The RepArray comprises 236 prototypic oligonucleotides that span the main repetitive elements families found in the human genome. Combined to a methyl-DNA immunoprecipitation (MeDIP) approach, RepArray allows depicting simultaneously the global trends that affect multiple repeat classes through the analysis of a restricted number of targets. Here, we present the MeDIP-on-RepArray protocol as it was established in our laboratory to delineate DNA methylation changes after chemical or genetic disruption of DNA methyltransferase activity in cells. It might serve as a workflow guideline for screening DNA methylation changes on repetitive elements during development and aging, among tissues and in various types of stress or pathological situations.

Key words: (separated by '-')	DNA methylation - 5-Methylcytosine (5mC) antibody - DNA repetitive sequences - DNA repeats - Transposable element - DNA satellite - Oligonucleotide microarray - MeDIP-on-chip - Epigenetics

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Chapter 16 1

Comprehensive DNA Methylation Profiling of Human Repetitive DNA Elements Using an MeDIP-on-RepArray Assay 2 3 4

Eric Gilson and Béatrice Horard 5

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Abstract 6

Hypomethylation of repetitive DNA elements is a common epigenetics event in cancer. Although it is 7 believed that this hypomethylation impacts chromosomal and transcriptional stability of the genome, the 8 extent of repetitive sequences contribution to the development and progression of human cancers remains 9 to be clarified. Repetitive sequences have largely been ignored by genome-wide studies, and thus little is 10 known about the DNA methylation profiles of different repetitive DNA elements types. As a step toward 11 investigating epigenetic landscape of repetitive DNA, we have developed a repeat-specific DNA microarray 12 called RepArray. The RepArray comprises 236 prototypic oligonucleotides that span the main repetitive 13 [AU1] elements families found in the human genome. Combined to a methyl-DNA immunoprecipitation 14 (MeDIP) approach, RepArray allows depicting simultaneously the global trends that affect multiple repeat 15 classes through the analysis of a restricted number of targets. Here, we present the MeDIP-on-RepArray 16 protocol as it was established in our laboratory to delineate DNA methylation changes after chemical or 17 genetic disruption of DNA methyltransferase activity in cells. It might serve as a workflow guideline for 18 screening DNA methylation changes on repetitive elements during development and aging, among tissues 19 and in various types of stress or pathological situations. 20

Key words: DNA methylation, 5-Methylcytosine (5mC) antibody, DNA repetitive sequences, DNA 21 repeats, Transposable element, DNA satellite, Oligonucleotide microarray, MeDIP-on-chip, Epigenetics 22

1. Introduction 23

Aberrant DNA methylation is commonly observed in cancer 24 progression and development, aging and also in autoimmune and 25 neural disease (1). Carcinogenesis is frequently associated with 26 promoter hypermethylation of specific genes concomitant to an 27 overall demethylation. This hypomethylation largely affects the 28 intergenic and intronic regions of the genome, particularly repeat 29

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sequences and transposable elements. The significance of this hypomethylation of repetitive elements in cancer is still unclear, but it may play multiple roles in pathogenesis through enhanced chromosomal instability, increased mutation events and unwanted transcription by *cis*- or *trans*-effects (1–3). In this context, monitoring methylation level on repetitive sequences might be particularly important as it may pinpoint novel diagnostic or prognostic indicators.

Despite the large contribution of repetitive sequences to the human genome (≈50%), the majority of studies until recently have focused on regions where DNA methylation was assumed to have the greatest functional significance in the regulation of gene expression, such as CpG-rich promoters. In addition, although there are many different families of repetitive DNA elements in the genome, studies mainly focused on only few classes of repetitive elements (4–8). Therefore, the DNA methylation map in repetitive sequences remains poorly defined on large scale.

We have developed a microarray called RepArray for systematic and comprehensive analysis of all main families of DNA repetitive sequences found in the human genome (9). The RepArray comprises 236 prototypic oligonucleotides that span the four main categories of human repeats: tandem satellites found in centromeric, pericentromeric, and subtelomeric heterochromatin; interspersed transposable elements, such as LTR- and non-LTR retrotransposons as well DNA transposons (Table 1). Coupled to a methylated DNA immunoprecipitation approach (MeDIP), the RepArray platform provides information on the relative DNA methylation level of hundreds of repetitive sequences through the analysis of a restricted number of targets. Therefore, RepArray depicts relative prevalence of a particular DNA methylation pattern over a given repeat class and not the behavior of individual repetitive element in a given chromatin environment. MeDIP-on-RepArray is a relatively low cost, well-suited approach for rapid screening of DNA methylation changes occurring during development, aging as well as between diseased and healthy tissues or between genetically modified and unmodified control cells. As repeat-specific transcriptomic maps can also be established using RepArray (9), it enables a comprehensive epigenetic analysis on the repetitive compartment of the genome necessary to fully appreciate the roles of DNA repeats in disease initiation and progression.

In this chapter, we describe the MeDIP-on-RepArray assay as it is performed in our laboratory. The protocol we describe has been used successfully to study DNA methylation changes upon chemical or genetic disruption of DNA methyltransferase activity in cells (9). We provide detailed methods for DNA processing, MeDIP, labeling of enriched sequences and detection of enriched sequences using the RepArray as well as real-time PCR. Finally, we also provide a guide for primary data analysis of MeDIP-on-RepArray data.

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t1.1 **Table 1**
[AU2] t1.2 **Human repetitive DNA sequences represented on RepArray microarray**

Major classes	Subcategories	Oligos names
Satellite DNA		
	Centromeric	01A_003[a], 02A_005[a], 03A_012[a], 04C_005[a], 05I_005[a], 07D_004[a], 08A_008[a], 09A_002[a], 10E_002[a], 11A_003[a], 12A_003[a], 13D_003[a], 14A_001[a], 15J_003[a], 16A_004[a], 17M_005[a], 18C_008[a], 20C_002[a], 21H_008[a], 22B_002[a], XF06_004[a], chr_10_VERDIN[b], ALR, SATCONS[c], SN5
	Pericentromeric	48BP, BSR, CER, GSATX, HSATII, HSATI, SAT1, SAT3, PHUR98(99_159)[d], Phur98(1_60)[d], SatII_PUC1(101_161)[e]
	Sub- and telomeric	HEXA_TR_A1[f], REP522, TR_B1[g], TR_A6[g], TR_B2[g], TR_A16[g], TR_A19[g], TR_B5[g], TR_B6[g], TR_B9[g], TR_B13[g], TR_B16[g], TR_B19[g]
	Others	D4Z4, LSAU, MER122, MER22, MSR1, NBL2, SATR, R66, TETRA_NT_AATG[h], TETRA_NT_ACAG[i], HEPTA_TR_A4[j], NONA_TR_A17[k]
TEs—Class I		
Retroviruses and retrovirus-like	MaLR	MLT1A, THE1BR
	Others	HERV16, HERV18_2, HERV39, HERV3, HERVE, HERVH48I_1, HERVH48I_2, HERVI, HERVK, HERVL, HERVS71, HUERS_P1, HUERS_P2, MER21I_1, MER21I_2, MER41, MER4INT, MER57I, MER61I, MER65I, MER70I, MER89I
Long terminal repeats	MaLR LTRs	MLT1I, MSTA, THE1
	Other retrovirus LTRs	LOR1S, LTR10S, LTR12S, LTR13S, LTR16S, LTR17, LTR18_1, LTR18_2, LTR19, LTR1S, LTR21A_2, LTR22, LTR23, LTR24, LTR27, LTR29, LTR2, LTR30, LTR32, LTR33S, LTR34, LTR35, LTR36, LTR37, LTR3S, LTR40, LTR41, LTR42, LTR43, LTR44, LTR46, LTR47, LTR4S, LTR52, LTR53, LTR54, LTR55, LTR57, LTR62, LTR64, LTR66, LTR67, LTR68, LTR69, LTR6, LTR71, LTR72, LTR75, LTR7A, LTR9B, LTR9S, MER101, MER110, MER11, MER31, MER34, MER39, MER41E, MER48, MER49, MER4, MER50, MER51, MER61, MER65, MER66, MER67, MER68, MER70, MER73, MER76, MER83, MER84, MER87, MER89, MER90, MER92, MER93, MER95, MER9, MLT2E, PABL_A, TAR1
LINE	Non-LTR autonomous	CR1_HS, IN25, L1_3END, L1MC, L1MCC_5, L2
SINE	Non-LTR non-autonomous	ALU_ALL, MIR_MIR3, SVA

(continued)

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Table 1 (continued)

Major classes	Subcategories	Oligos names
TEs—Class II		
DNA transposons		CHARLIE1, CHARLIE2, CHARLIE6, CHARLIE7, CHARLIE8, CHESHIRE, GOLEM, HSMAR, LOOPER, MADE1, MARNA, MER103, MER104, MER105, MER106, MER107, MER112, MER113, MER115, MER116, MER117, MER119, MER121, MER1AS, MER2, MER20, MER28, MER3, MER30, MER45, MER53, MER5C, MER5, MER63, MER69, MER6, MER75, MER80, MER81, MER82, MER85, MER91, MER94, MER96, MER97, MER99, ORSL, TIGGER1, TIGGER5, TIGGER6, TIGGER9, ZOMBI
Unclassified		MER120, MER109
Others		
Ribosomal DNA		RDNA_EST[l], RDNA_KOND[m]

Oligos names are listed according to their affiliation. Oligos names as used in Repbase Update except for sequences referring to some satellite DNA

[a]Chromosome-specific subsets of human alphoid DNA were designed with the first number referring to the chromosome

[b]Chromosome 10 alpha satellite DNA corresponding to a preferential integration site for HIV virus (15)

[c]Human alpha satellite DNA consensus as defined by Vissel and Choo (16)

[d]Clone pHuR 98, a variant satellite 3 sequence, specific to chromosome 9qh (17)

[e]Clone Sat_II Puc 1a variant of satellite II from chromosome 1q (18)

[f]Telomere-specific hexanucleotide (TTAGGG)n (19)

[g]Subtelomeric minisatellites found at 1–840 kb of the telomeric tract

[h](AATG)n simple repeat

[i](ACAG)n simple repeat

[j](AACAAAAC)n simple repeat

[k](TTTTGTTTG)n simple repeat

[l]5′ end of the 28S component of ribosomal DNA (20, 21)

[m]ribosomal DNA (21)

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2. Materials

Prepare solutions using ultrapure water and analytical grade reagents (unless otherwise indicated). All reagents can be stored at room temperature (unless otherwise indicated).

2.1. Purification of Human Genomic DNA

1. DNeasy Blood & Tissue Kit (Qiagen #69581).
2. 1× Dulbecco's PBS Ca & Mg free (PAA #H15-002).
3. RNAse A 100 mg/ml (Qiagen #19101), store at −20°C.

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[AU3]

4. DNA resuspension Buffer: 10 mM Tris pH 7.4. 86
5. Agarose DNA grade (Euromedex #D5-D). 87

2.2. Sonication of Genomic DNA

1. 0.2 M Na-phosphate buffer pH 7.0 (stock buffer): 39 ml 0.4 M monobasic sodium phosphate (NaH_2PO_4), 61.0 ml dibasic sodium phosphate (Na_2HPO_4). Add H_2O to 200 ml. Sterile filtrate. 88 89 90
2. The protocol was established using a Standard Bioruptor Waterbath Sonication device (Diagenod Bioruptor® UCD-200), (see Note 1). 91 92 93
3. Agarose DNA grade (Euromedex #D5-D). 94

2.3. Methylated DNA Immunoprecipitation

1. IP Buffer: (10× stock buffer) 100 mM Na-phosphate buffer pH 7.0, 1,4 M NaCl, 0,5% TritonX-100. 95 96
2. Rabbit anti-5mC polyclonal antibody (MegabaseResearch #CP 51000) (20 µg per IP point), (see Note 2). 97 98
3. Purified rabbit IgG (Sigma-Aldrich #I5006) or mouse IgG (Sigma-Aldrich #I5381). 99 100
4. 1× Dulbecco's PBS Ca & Mg free (PAA #H15-002). 101
5. Bovine serum albumin (BSA) 30% (Sigma #A9576). Store at 4°C. 102
6. Protein-A Sepharose CL4B beads (GE Healthcare #17-0780-01). Store at 4°C, (see Note 3). 103 104
7. 5 M NaCl (stock solution) = 292 g NaCl. Add H_2O to 1 L. Sterile filtrate. 105 106
8. 2× TE buffer = 20mM Tris pH 8.0, 2 mM EDTA pH 8.0. Sterile filtrate. 107 108
9. Proteinase K (Roche Applied Science #03115887001). 109
10. Proteinase K digestion buffer = 50 mM Tris–Cl pH 8.0, 10 mM EDTA, 0.5% SDS. Sterile filtrate. 110 111
11. UltraPure Phenol:Chloroform:Isoamyl Alcohol (Invitrogen #15593031). 112 113
12. 3 M Na-acetate pH 5.2 (stock solution) = Dissolve 408 g sodium acetate $3H_2O$ in 800 ml H_2O Add H_2O to 1 L. Adjust pH to 5.2 with 3 M acetic acid. Sterile filtrate. 114 115 116
13. Glycogen 20 mg/ml (Roche Applied Science #10901393001). Store aliquots at −20°C. 117 118
14. Ethanol, absolute for molecular biology. 119
15. DNA resuspension buffer: 10 mM Tris pH 7.4. 120
16. NanoDrop spectrophotometer (Thermo Scientific). 121

2.4. Real-Time PCR Analysis of MeDIP

1. QuantiTect SYBR Green PCR kit (Qiagen #204143). Store at −20°C. 122 123
2. 25 mM $MgCl_2$ stock solution (Roche Applied Science #11 699 113 001). Store at −20°C. 124 125

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3. Oligonucleotide primers to genomic regions of interest and control.
4. Real-time PCR machine: we used a Stratagene's Mx300OP™ qPCR System and the Agilent's MxPro qPCR software (Agilent Technologies Genomics), (see Note 4).
5. 96-well optical PCR plates and strip caps compatible with the Real-time PCR instrument (Agilent Technologies Genomics # 401333 and 401425).

2.5. Labeling of Immunoprecipitated DNA with Aminoallyl-dUTP

1. Random primers pd(N)6 (Roche Applied Science #11 034 731 001). Working solution 0.1 U/µl: dissolve 2 mg (50 A_{260} units) Random pd(N)6 Potassium Salt in 500 µl ultrapure water. Store in aliquots at −20°C.
2. Random prime reaction buffer (10× stock): 50 mM NaCl, 10 mM Tris–HCl pH 7.9, 10 mM $MgCl_2$, 1 mM Dithiothreitol (NEB #B7002S). Store at −20°C.
3. Klenow fragment (3′→5′ exo-) 50 U/µl (NEB # M0212M). Store at −20°C.
4. 100 mM dNTPs stock (NEB #N0446S). Store at −20°C.
5. 20× dNTPs working mix: 5 mM dATP, 5 mM dCTP, 5 mM dGTP, 2 mM dTTP.
6. 5-(3-aminoallyl)-dUTP: 10 mM aminoallyl ready-to-use solution (Ambion # 8438). Store frozen in aliquots below −70°C (see Note 5).
7. Qiaquick PCR Purification kit (Qiagen # 28104).
8. 1 M K-phosphate buffer pH = 8.0 (stock solution): mix 9.5 ml 1 M di-potassium hydrogen phosphate (K_2HPO_4) and 0.5 ml 1 M potassium di-hydrogen phosphate (KH_2PO_4). Sterile filtrate.
9. Column washing buffer: mix 20 ml 15 mM K-phosphate buffer pH 8.0 and 80 ml of absolute ethanol.
10. DNA elution buffer: 15 mM K-phosphate buffer pH 8.0.
11. DNA Speed Vacuum Concentrator (Thermo Scientific).

2.6. Coupling Monofunctional Reactive Cyanine Dyes to the Modified DNA

1. Fluorescent dye mono-reactive NHS-esters. We used Cy™ Dye Post Labelling Reactive Dye Pack (Amersham Bioscience #RPN 5661), (see Note 6).
2. Coupling buffer: dissolve 0.84 g $NaHCO_3$ in 90 ml ultrapure water. Adjust to pH = 9.0 with 1 M NaOH. Add water to 100 ml. Sterilize by filtration with a 0.45 µm filter and store aliquots at −20°C. Do not thaw and refreeze (see Note 7).
3. 4 M Hydroxylamine hydrochloride: dissolve 27.8 g hydroxylamine hydrochloride (Sigma Aldrich # H2391) in 100 ml ultrapure water.

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4. NucAway™ Spin Column (Ambion # AM10070). 167
5. DNA Speed Vacuum Concentrator (Thermo Scientific). 168

2.7. Preparation of DNA Microarray

1. Library of long oligonucleotides DNA probes representative of a set of human repetitive DNA sequences, including satellites (centromeres, pericentromeres, telomeres, megasatellites, minisatellites), retrotransposons (LTR or non-LTR), DNA transposons and also rDNA intergenic region (Table 1), (see Note 8a). Additionally, unique sequences from *Arabidopsis thaliana*, *Drosophila melanogaster*, *Bacillus subtilis*, *Escherichia coli*, *Nicotiana tabacum*, *Photonis pyralis* were also added on the array as irrelevant (negative) controls. Long oligonucleotides are synthesized at 1 or 2 nmol scale and packaged in lyophilized form in 384-well plates by Eurogentec S.A., France, (see Note 8b). 169–180
2. Pronto!™ Universal Spotting Solution (Corning # 40027), (see Note 9). 181–182
3. UltraGAPS™ Coated Slides without Bar code (Corning #40016). 183
4. Arraying equipment and printing pins (see Note 9). 184

2.8. Array Hybridization

1. Hybridization chambers for 25×75 mm microarray slides (Corning #2551). 185–186
2. Bovine Serum Albumin 30% (Sigma #A9576). Store at 4°C. 187
3. Deionized Formamide (Sigma Aldrich #F9037). 188
4. 20% SDS stock solution (Euromedex # EU0660-A). 189
5. 20× SSC stock solution (Euromedex # EU0300-D). 190
6. Coverglass 24×60 mm (Corning # 2935-246). 191
7. Prehybridization solution (Fresh): 5× SSC, O.1% SDS (w/v), 1% BSA(w/v). 192–193 [AU4]
8. 2× Hybridization stock solution: 50% formamide, 10× SSC, 0.2% SDS (see Note 10). 194–195
9. Waterbath at 42°C. 196
10. Post-hybridization wash solution 1 (fresh): 1× SSC, 0.2% SDS. 197
11. Post-hybridization wash solution 2(fresh): 0.1× SSC, 0.2%SDS. 198
12. Post-hybridization wash solution 3 (fresh): 0.1× SSC. 199
13. MilliQ Water. 200
14. Propanol-2 (VWR # 8.18766.1000). 201
15. Plastic containers for washing slides. BD Falcon™ conical tubes are ideal for individual washing of hybridized slides. 202–203
16. Scientific cleaning wipes (KIMTECH SCIENCE* Precision Wipes Tissue Wipers # 05511). 204–205

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206 17. Tabletop centrifuge equipped with a swinging bucket rotor
207 that can accommodate 50 ml tubes (Beckman Coulter #
208 Allegra® X-15R).

209 *2.9. Scanning*
210 *and Analysis*

1. Microarray scanner for imaging two-color microarrays: We opted for the robust and easy-to-use GenePix® 4000B microarray scanner that allows data acquisition at two wavelengths simultaneously. The GenePix® 4000B microarray scanner includes one license of GenePix® Pro Acquisition and Analysis software (Molecular Devices # GenePix 4000B).
2. RepArray specific array list (File extension ".gal").
3. Software for bioinformatic: Bioconductor (http://www.bioconductor.org/) and R packages (http://www.r-project.org/).

3. Methods

The complete protocol for MeDIP-on-RepArray starting from cell pellets up to data acquisition takes ≈7 days (Fig. 1). [AU5]

3.1. Isolation of Genomic DNA

Typically, genomic DNA is purified from fresh or frozen cell pellets (≈ 5×10^6 cells) using DNeasy Blood and Tissue Kit according to manufacturer's recommendation. Briefly, adherent cells were grown to confluency, harvested after dispersion by trypsin treatment, washed in 1× PBS and pelleted by centrifugation ($300 \times g$).

1. Fresh or frozen cell pellets were resuspended in 200 µl 1× PBS. When using frozen pellets, before adding PBS allow cells to thaw until the pellets can be dislodged by gently flicking the tube.
2. Because the antibody also recognizes 5mC in the context of RNA molecules, it is important to completely remove RNA from the genomic preparation. Add RNase A to a final concentration of 300 µg/ml and incubate 10 min at room temperature.
3. Proceed to genomic DNA extraction according to manufacturer's instructions. Elution of purified DNA from DNeasy Mini spin column is performed in two successive steps using 150 µl of 10 mM Tris pH 7.4.
4. Determine DNA concentration with NanoDrop Spectrophotometer. Yields of genomic DNA will vary depending on cell type and quality of starting material. Approximately 6 µg of DNA should be obtained from 10^6 mammalian cells.
5. To assess DNA integrity, resolve 250 ng of DNA by 1% agarose gel electrophoresis.

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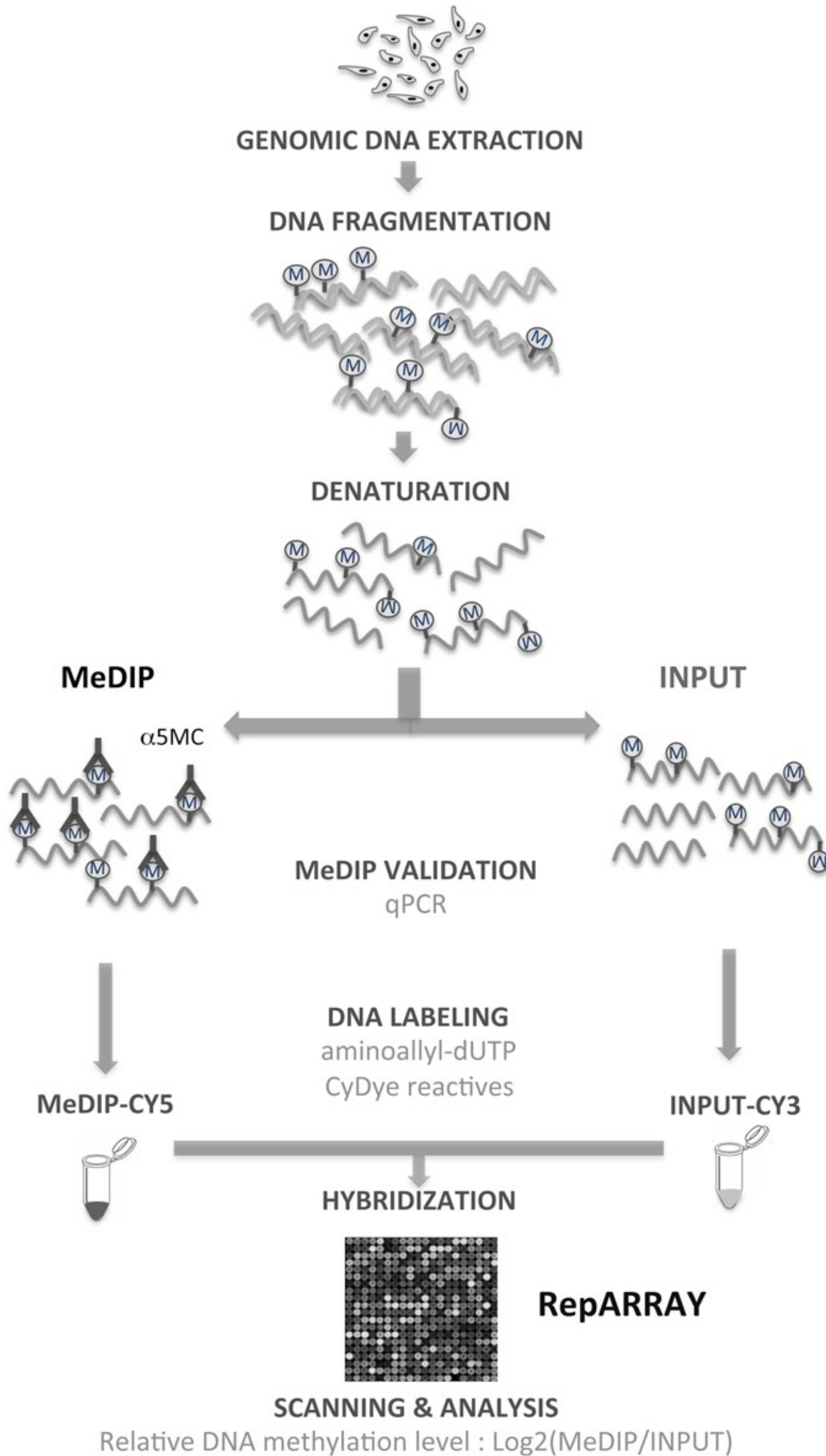

Fig. 1. Overview of the MeDIP-on-RepArray assay. Schematic diagram showing the steps involved in MeDIP from genomic DNA isolation, fragmentation by sonication, immunoprecipitation using anti-5 methylcytosine (α5mC) antibodies, real-time PCR validation of MeDIP reaction, labeling of MeDIP and Input DNA by random priming using aminoallyl-dUTP and coupling with CyDyes and hybridization to the repeat-specific oligo-array RepArray.

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244 245 ***3.2. Fragmentation of Genomic DNA***

244 DNA fragmentation is a critical step since it defines the resolution
245 of MeDIP. The sheared DNA fragments should ideally range
246 from 200 bp to 1,000 bp. Large DNA fragments should be elimi-
247 nated for a better resolution and small DNA fragments below a
248 size of 200 bp will be difficult to detect by PCR or microarray.
249 The sonication protocol reported here is suitable for various cell
250 lines (HCT116, HeLa, BJ, U2OS, NT2, human B lymphocytes),
251 nevertheless optimization may be required for other cell lines,
252 different amount of starting material or sonicator devices. Performing
253 a sonication time course and products analysis on a 1% agarose gel
254 should optimize sonication efficiency.

255 1. In a 1.5 ml microtube, dilute 8 μg of RNA-free genomic DNA
256 in 300 μl of 10 mM NaP04 buffer pH 7.0, (see Note 11).

257 2. Sonicate for seven cycles 25 s ON/30 s OFF at the highest
258 output level while cooling the samples to 4°C in the Bioruptor
259 waterbath.

260 3. Resolve 5–10 μl by 1% agarose gel electrophoresis to check
261 DNA fragmentation. A smear should be observed between 200
262 and 1,000 bp (Fig. 2). If necessary, sonicate for one or two addi-
263 tional pulses until the size of the DNA is 200–1,000 bp.

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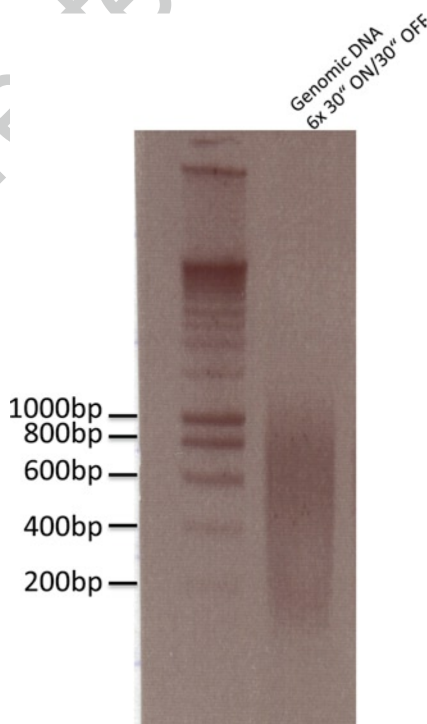

Fig. 2. DNA fragmentation by sonication. Example of gel electrophoresis analysis of sonicated genomic DNA. Typically, the sheared DNA fragments range from 200 bp to 1,000 bp.

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4. Denature DNA by heating the solution 10 min at 94°C and immediately cool on ice.
5. Remove an Input sample: save 20 μl (≈ 500 ng) of the denatured DNA solution and keep at 4°C until step 18.
6. Divide the denatured DNA solution into 2× 140 μl aliquots (≈ 4 μg of DNA) in 1.5 ml microtubes, one that serves as the methylcytosine IP sample (5mC-IP) and the other as negative control (IgG-IP), (see Note 11).
7. Add 50 μl of 10× IP buffer and 300 μl of water to each DNA aliquot.
8. Add 10 μl of anti-5mC antibody (5mC-IP) or purified IgG (IgG-IP) and incubate O/N at 4°C with continuous gentle rotation. This step can be carried out in shorter time period (2–6 h) if more convenient.
9. Prepare a 50% slurry of preblocked Protein A-Sepharose beads. Into a 14 ml screw cap tube, wash the required amount of Sepharose beads in 10 volumes of 1× PBS so it swells up, then centrifuge at 700 × *g* (do not exceed 2,500 rpm), remove the supernatant and discard. Add 10 volumes of 1× PBS supplemented with 0.1% BSA and incubate O/N at 4°C with gentle overhead shaking. Remove the supernatant and make a 50% slurry (v/v) with 1× PBS-0.1% BSA. The Protein A-Sepharose slurry is now ready to use (see Note 12). [AU6]
10. Into 1.5 ml microtubes, aliquot 80 μl of Protein A-Sepharose slurry, centrifuge at 2,500 rpm using a 24-place microcentrifuge. Discard supernatant and continue with the pellet (beads), (see Note 13).
11. Capture the immune-complex by adding the 500 μl of immunoprecipitation mix from step 8 on the Protein A-Sepharose pellet (step 10) and incubate 2 h at 4°C with continuous gentle rotation.
12. Pellet the beads by centrifugation for 2 min at 2,500 rpm using a 24-place refrigerated microcentrifuge. Transfer supernatant (unbound fraction) to a new tube without disturbing the pellet.
13. Wash the beads with 1 ml of cold 1× IP buffer for 5 min on rotating wheel at 4°C.
14. Repeat wash step 13 once. Recover the beads between washes by centrifugation for 2–3 min at 2,500 rpm at 4°C using a 24-place refrigerated microcentrifuge and remove the supernatant nearly completely without disturbing the pellet.
15. Wash the beads with 1 ml of cold 1× IP buffer supplemented with 160 mM NaCl to give 1× IP buffer-300 mM NaCl for 5 min at 4°C with gentle rotation.

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16. Wash the beads with 1 ml of 2× TE for 3 min at room temperature.
17. From that step, continue the procedure also with the Input sample (saved at step 5).
18. Elute DNA from the beads by adding 250 μl of proteinase K digestion buffer plus 7 μl of proteinase K (10 mg/ml stock) and incubate 3 h at 55°C while vigorously shaking (see Note 14). Add 230 μl of proteinase K digestion buffer to the Input sample.
19. Recover DNA by adding one volume (250 μl) phenol/chloroform/isoamyl alcohol, vortex vigorously, and centrifuge at 13,000 rpm for 10 min in order to separate the phases. Carefully transfer the upper aqueous phase to a fresh microtube.
20. Precipitate DNA by adding 1/10 volume of 3 M Na-acetate pH 5.2, 1 μl of glycogen and 2.5 volume of ice-cold 100% ethanol. Mix and incubate for at least 2 h at −20°C. This step can be carried out O/N if more convenient.
21. Centrifuge at 13,000 rpm for 20 min at 4°C to pellet the precipitated DNA. Wash pellet in 500 μl of ice-cold 70% ethanol. Centrifuge at 13,000 rpm at 4°C to collect DNA.
22. Dissolve DNA in 40 μl of DNA resuspension buffer (10 mM Tris pH 7.4).
23. Measure DNA concentration with a NanoDrop spectrophotometer. The sample can be stored at −20°C.

3.3. Analysis by PCR

It is highly recommended to verify the MeDIP specificity by real-time PCR analysis on immunoprecipitated DNA using positive and negative control primers before starting labeling/hybridization procedure. Positive control primers typically amplify fragments spanning a region of the genome though to be methylated while negative control primers must be specific for a region expected to be unmethylated which is typically the promoter region from a housekeeping gene. Table 2 contains a set of control primers suitable for several human cell lines. qPCR using these primers is standardly carried out on MeDIP, IgG-IP, and Input samples. For repetitive sequences analysis, primers were designed to a consensus sequence for each repetitive element family and thus amplify a global pool of repetitive element rather than a single element or genomic locus.

1. Dilute part of the DNA samples in milliQ water to 8–10 ng/μl.
2. Prepare a master mix of A and B primers by mixing each primer at a final concentration of 5 mM in nuclease-free water.

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Table 2
Control primers. For qPCR primers design we utilized Primer 3 (v.0.4.0) software (http://frodo.wi.mit.edu/primer3/) using the following specified constraints:

Product size range	100–300 bp		
Primer size	Min: 18 bp	Opt: 20 bp	Max: 25 bp
Primer Tm	Min: 57°C	Opt: 60°C	Max: 63°C

Locus		Sequences 5′–3′	Annealing	References
H4 promoter	Neg	AAATGGTGGGATCACAGACG CGAGCTTCTTGTTTCCGTGT	57°C	(9)
Gapdh promoter	Neg	CGTGCCCAGTTGAACCAG CGCCCGTAAAACCGCTAGTA	57°C	(9)
Beta Actin promoter	Neg	CCTGACTCCCCCAACACCAC ~~TGAAGTCGGCAAGGGGGAGT~~ ACTCCCCCTTGCCGACTTCA	57°C	
TSH2B	Tissue-specific	CAGACATCTCCTCGCATCAA GGAGGATGAAAGATGCGGTA	57°C	(11)
MLH1	Cell-specific	CGAACCAATAGGAAGAGCGGA GGACAGCTTGAATGCCAGTCA	57°C	(10, 11)
RASSF1	Cell-specific	CCCAAGTGAAGGCTCGAGACT TGTTTCTGGAGGCCAGCTTTA	57°C	(10, 11)
RARB2	Cell-specific	GAGCAAACGAGTGCAGTCAA CTCTGTGCGCCTTTCTGTCT	57°C	(22)
H19-ICR	Pos	GAGCCGCACCAGATCTTCAG TTGGTGGAACACACTGTGATCA	57°C	(10, 11)
Satellite 2	Pos	TCGCATAGAATCGAAGGAA GCATTCGAGTCCGTGGA	57°C	(20)
Alu-all	Pos	TGAAACCCCGTCTCTACTAAAAA GTCTCGCTCTGTCGCCCA	57°C	(9)
D4Z4 megasatellite	Pos	CTCAGCGAGGAAGAATACCG ACCGGGCCTTAGACCTAGAAG	57°C	(9)

Primers were optimized for real-time PCR by performing a standard curve with sheared genomic DNA. Amplification efficiency should be ≥95% (rate of amplification ≥1.9) and dissociation peak need to be checked

[AU7]

3. Combine the following in an optically clear 96-well PCR plates (see Note 15):

2× QuantiTec SYBRGreen PCR master mix	12.5 µl	1× final (contains 2.5 mM $MgCl_2$)
$MgCl_2$ 25 mM	1.5 µl	Provides a final concentration of 4 mM
Primers A + B Master Mix	1.5 µl	0.3 µM final each
UltraPure Water DNA sample (diluted)	7.5 µl 2 µl	

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Each qPCR reaction for all samples is to be done in duplicate. Process also a "no DNA" control reaction without sample DNA to monitor possible contamination problems with foreign DNA (we recommend the use of filter-tips)

4. Set up and run the following PCR program (Mx300OP™, see Note 15):

Initial activation step	95°C	15 min
Denaturation	94°C	15 s
Annealing	57°C	30 s
Extension	72°C	30 s
	40 cycles	

5. To evaluate the enrichment in the MeDIP fraction we calculate the difference in cycles between the immunoprecipitated sample (MeDIP or IgG-IP) and the Input DNA for primer pair of interest and for negative control pair. The methylation enrichment of a particular sequence is then expressed as fold over the background methylation level at negative control locus (see Note 16).

3.4. Labeling of Immunoprecipitated DNA with Aminoallyl-dUTP

For our hybridization experiments, we compare the enrichment of the MeDIP sample relative to the Input DNA. For this, we label MeDIP sample and Input genomic DNA with different fluorescent dyes and co-hybridize them to the RepArray. We then generate a ratio of methylated versus Input signal for each sequence spotted on the array and use this as readout for the methylation level: a positive log2 ratio indicates hypermethylation, and a negative log2 ratio indicates hypomethylation. For labeling our DNA samples, we favor an indirect procedure. Indirect means of labeling incorporates a nonfluorescent nucleotide analogue, such as aminoallyl-dUTP (aa-dUTP), followed by cyanine dye coupling to the incorporate aminoallyl group. Because the small conjugate is incorporated in both the MeDIP and the Input samples equally, this method helps eliminating the incorporation biases that occur when using direct labeling methods.

1. In a microtube, bring 0.2–0.5 µg of MeDIP or Input DNA to 36.4 µl with milliQ water and mix with 4 µl of 10× random prime reaction buffer and 3 µl of 0.1 U/µl random primers pd(N)6 solution (see Note 17).
2. To denature DNA, incubate 10 min at 94°C and immediately place on ice afterward.
3. Add 2 µl of 20× dNTPs mix, 0.6 µl of 10 mM aa-dUTP, and 1 µl of 50 U/µl Klenow (exo-) polymerase. Incubate overnight at 37°C with gentle shaking. [AU8]
4. Stop the reaction by adding 2 µl of 0.5 M EDTA pH 8.0.

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5. Recover the labeled DNA using QiaQuick PCR Purification kit. We follow the Qiagen protocol except that we used column washing buffer (15 mM K-phosphate buffer pH = 8.0, EtOH 80%) for washing the silica column and then DNA is eluted using 2 × 40 μl of freshly prepared DNA elution buffer (15 mM K-phosphate buffer pH = 8.0) as the subsequent dye-coupling step is sensitive to amine buffer. It is very important to remove as much as possible of the unincorporated aminoallyl-dUTP as it will bind the NHS-ester cyanine dyes and thus reduce the labeling efficiency.
6. Check the quality of DNA after labeling step on a 1% agarose gel (5% of the final product). Determine DNA concentration and purity with a NanoDrop spectrophotometer using a DNA spectrum from 230 to 320 nm (see Note 18).
7. Spin-dry the prepared aminoallyl-DNA to ≈5 μl using a speed-vac centrifuge. Be careful not to overdry the DNA as it may become difficult to resuspend.

3.5. Coupling Monofunctional Reactive Cyanine Dyes to the Modified DNA

As Cy3 and Cy5 reactive dyes are easily photobleached, it is best to protect Cy dyes and all labeled products from light as much as possible during the procedure.

1. Bring the volume of aminoallyl-labeled DNA (from Subheading 3.4 step 7) to 40 μl by adding appropriate volume of 0.1 M sodium bicarbonate pH 9.0. Pipettes several times to perfectly mix the sample.
2. Transfer DNA samples to a freshly open vial of Cy3 or Cy5 reactive dye (see Note 19). For a given array, the MeDIP sample is coupled to one dye and the total Input to the other. Typically, we coupled MeDIP sample to the Cy5 dye and the Input reference to the Cy3 dye, but the reverse works as well.
3. Pipette up and down several times to resuspend completely the reactive dye/aminoallyl-labeled DNA mix. Bring all the contents at the bottom by briefly spinning.
4. Incubate at room temperature for 90 min in the dark to couple the NHS-ester cyanine dye to the DNA.
5. While coupling is going on, rehydrate NucAway™ Spin Column according to manufacturer's instructions. We have found that removal of the unincorporated dye is quite efficient
6. Add 15 μl of 4 M hydroxylamine and incubate at room temperature in the dark for 15 min. This will inactivate unreacted Cy dyes NHS-ester molecules
7. Bring the volume of labeling reaction to 85 μl by adding nuclease-free water.
8. Place the rehydrated NucAway™ Spin Column on top of a new collecting microtube, centrifuge 2 min at 750 × *g*. Discard the

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flow-through. Slowly and carefully apply the labeled DNA directly to the center of the gel bed at the top of the NucAway™ Spin Column (avoid disturbing the gel surface).

9. Centrifuge at 750 × *g* for 2 min to collect the purified labeled DNA. The eluted dye-coupled product should be slightly colored (see Note 20).
10. Determine DNA yield and total dye incorporation by measuring the absorbance at A260, A550, and 650 nm using a NanoDrop spectrophotometer. The pmol incorporation of dye is calculated by using the formula: A550/0.15 (extinction coefficient for Cy3) or A650/0.25 (extinction coefficient for Cy5). Dye labeling density might be roughly estimated according to manufacturer's instructions manual (see Note 21).
11. Combine the respective Cy3- (MeDIP) and Cy5- (Input) labeled DNA and spin dry with heat the purple sample to ≈30 μl using a speed-vac centrifuge protected from light (be careful, this might take time). This is the probe solution ready for immediate hybridization (see Note 22).

3.6. Prehybridization/ Hybridization of the Array

Prehybridization should be done immediately preceding the application of labeled DNA to the target. Typically, we carried out the prehybridization of the microarrays while carrying concentration of probe solution (see Subheading 3.5 step 11). As general precautions, work in a clean and dust free environment and do not use powdered gloves when working with microarrays.

1. RepArray slides are placed for 1 h at 42°C in 50 ml of freshly prepared Prehybridization solution: 5× SSC, 0.1% SDS (w/v), 1% BSA (w/v). It is convenient to use 50 ml conical tubes to treat each slide individually.
2. Rinse briefly the slides five times in 50 ml milliQ water, then once in propanol-2. Place the slides in a new conical tube and dry by centrifugation for 4 min at 500 × *g* in a tabletop centrifuge at room temperature. Help the drainage of liquid from the slides by placing a piece of folded KimWipes paper at the bottom of the tube. The dried prehybridized slides are kept in dust-free container while completing the preparation of the hybridization solution.
3. Prepare the hybridization solution by mixing the concentrated probes (≈30 μl from 4.5 step 11) with one volume of 2× hybridization stock solution (see Note 10). Tap gently to mix well and spin down. [AU9]
4. Denature hybridization mixture at 95°C for 4 min. Briefly centrifuge to collect condensed water droplets and allow it to cool at room temperature for 2–3 min. Do not place the mixture on ice as this would cause SDS precipitation.

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5. The denatured mixture is applied to the array by carefully adding the solution to one end of the slides and then a clean coverslip is carefully lower onto the array using forceps (see Note 23). Care must be taken to exclude formation of air bubbles and not to put pressure on the coverslip when handling the array.
6. Quickly place the microarray-cover assembly into the Corning hybridization chamber, coverslip side up, attach and seal the lid. Keep the chamber right side up and in a horizontal position all along the procedure.
7. Submerge the hybridization chamber in a humidified chamber consisting of a closed plastic box containing milliQ water prewarmed to 42°C (see Note 24).
8. Hybridizations are carried out in the dark for up to 16 h at 42°C in a water bath.
9. On day 2, prepare the post-hybridization wash solutions 1–3. Dispense all the necessary washing solutions in multiple 50 ml conical tube before starting the procedure. The post-hybridization wash solution 1 should be warmed at 42°C before use.
10. Remove array slides from humidified chamber and remove coverslip. The coverslip will slide off after a brief immersion in 50 ml of preheated post-hybridization wash solution 1. Do not agitate to avoid scratching the array with the loose coverslip. Gently move up the array using forceps and quickly transfer it into a new conical tube containing preheated post-hybridization wash solution 1
11. Wash arrays for 4 min at room temperature with gentle agitation on a platform shaker.
12. Transfer arrays in 50 ml of post-hybridization wash solution 2 for 4 min on a platform shaker.
13. Finally, to remove residual SDS wash arrays twice in 50 ml post-hybridization wash solution 3, each time for 4 min with gentle shaking.
14. Dry arrays by centrifugation for 4 min at $500\times g$ in a 50 ml conical tube as described in step 2 (see Note 25). Store the arrays in a slide holder protected from light until ready to scan. It is recommended to proceed to scan as soon as possible, as the fluorescence will decay over time.

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3.7. Scanning and Data Acquisition

The slides are scanned using an Axon 4000B scanner according to manufacturer's instructions.

1. Before scanning, warm up the 532 nm and 635 nm solid-state lasers for 30 min.
2. Load the slide into slide holder (for RepArray, the slide should be inserted with the printed side face down and label positioned

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close to the user). Quickly scan the slides at low resolution to give a preview picture.

3. Set data acquisition area by selecting the area encompassing the spots. Set the laser intensity to 100%, adjust "pixel resolution" to 10 μm, "line to average" to 1, focus position 0 μm and optimize signal intensity by manually adjusting photomultiplicator (PMT) gain values (see Note 26). [AU10]

4. Full scan the selected area and save the image as a multiimage TIFF files.

5. Acquired images are analyzed using the GenePix Pro6.0 Microarray Analysis Software. For this, load the ".gal" file and adjust Alignment Setting (find circular features, do not resize the features during alignment, flag as "unfound" features that fail background threshold criteria). Find and align blocks manually so that the circular features of the .gal file are over the appropriate spots. Inspect all spots individually and flag as "bad" any feature affected by microarray imperfection, such as scratches or dust.

6. GenePix Pro 6.0 extracts numerical values and reports them in a table. Save the result file (.gpr file).

7. Preprocessing of the raw data is performed in R programming environment (http://cran.r-project.org/). Download the following tools: limma (http://www.bioconductor.org/packages/2.2/Software.html), marray (http://www.bioconductor.org/packages/2.2/Software.html), ade4 (http://pbil.univ-lyon1.fr/ade4html/00Index.html), gplots (http://cran.r-project.org/web/packages/gplots/index.html). Using the predictive model, a synthetic value for each spot on each individual array is computed and a data frame containing a set of nominal and numerical values ("Exp" "Lame" "Block" "Column" "Row" "Name""F635.Mean" "F635.SD" "B635.Mean" "B635.SD" "F532.Mean" "F532.SD" "B532.Mean" "B532.SD" "Flags" "Sens") is generated. Slides are kept in the analysis according to quality criteria, including signal-to-noise ratio inspection, control of the deviance from linear model. Features flagged "bad" or "unfound" and features that have not yielded data in minimum number of hybridizations (<75%) are excluded. Filtering for robust data may reduce the usable fraction but will ultimately result in greater confidence in data interpretation. Raw features intensities are used to determine $\log_2$ ratios MeDIP/Input (routinely $\log_2$ (F635/F532), see Subheading 3.5 step 2). Relative quantification of methylation level of individual sequence is inferred from the averaged log2 ratios (MeDIP/Input) of replicated features (see Note 27). Keep in mind that MeDIP enrichment at any target sequence depends both on the degree of methylation and on the density of CpG (10).

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A modest enrichment can thus reflect an unmethylated state or the scarcity of CpG in the target region. Nevertheless, demethylation can affect repetitive sequences heavily or modestly methylated (9). 568 569 570 571

8. Validation of results: real-time PCR is a straightforward method for validation of methylation profiles at specific loci (see Subheading 3.3). Deeper analysis of methylation level can then be performed on individual identified repeat-class using bisulfite-PCR pyrosequencing for example (8). 572 573 574 575 576

[AU11]

4. Notes 577

1. Other sonication devices using direct sonication horns can be used, but conditions need to be adjusted for each apparatus. The Bioruptor™ sonicator allows the simultaneous processing of several samples (up to 6 × 1.5 ml tubes) in sealed tubes (no risk of contamination). 578 579 580 581 582
2. We mainly used the polyclonal 5-methylcytosine antibody from Megabase Research (#CP 51000), but mouse monoclonal 5-methylcytosine antibody from Eurogentec (#BI-MECY-0500) (20 μg per IP experiment) works well (as originally described by Weber et al. (11)). Since polyclonal antibodies are prone to batch-to-batch variability, each new immune-sera batch it is recommended to systematically check immunoprecipitation efficiency. Today, several companies (EUROMEDEX, DIAGENODE, etc…) propose similar poly- or monoclonal 5mC antibodies but DNA immunoprecipitation conditions must be optimized for each antibody. MeDIP performed with an unproven antibody must include real-time PCR validation step using appropriate controls. Antibodies must be aliquoted upon receipt and stored at −20°C to avoid freeze-thaw cycles. 583 584 585 586 587 588 589 590 591 592 593 594 595 596
3. Sepharose is a registered trademark of GE Healthcare for a cross-linked, beaded form of agarose. Several protocols have been developed using magnetic beads Dynabeads M-280 Sheep anti-mouse IgG (Dynal Biotech #112 01), for details see refs. (12, 13). 597 598 599 600 601
4. Methylation enrichment can be evaluated either by real-time PCR or by classical PCR (12). 602 603
5. Do not store aa-dUTP (or aminoallyl-labeled DNA) in a frost-free freezer. When stored properly, the reagent is stable for several months. 604 605 606
6. Do not store fluorescent components in a frost-free freezer. In addition, reactive dyes are sensitive to light and should be stored and used in dark as much as possible. 607 608 609

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610 7. The CyDye reactive dye coupling to aminoallyl-containing DNA only occurs under alkali conditions between pH 8.5 and 9.5 which is set using 0.1 M sodium bicarbonate buffers. When stored properly, the reagent is stable for several months at −20°C or make the buffer fresh prior to setting up the labeling reactions.

616 8. (a) A full description of the probes selection procedure was previously described (9). Briefly, a conserved region specific for each repeat subfamily was inferred from multiple alignments of the most 50 similar DNA sequences identified using FASTA similarity search program in RepBase Update or GenBank databases. This region was used to design oligonucleotide sense probes using OligoArray 2.0 software (14). The probe search parameters were length range from 50 to 52 nucleotides, 25–65% CG percentage and melting temperature between 76 and 96°C. Oligonucleotides containing five consecutive A, C, G, or T were discarded. OligoArray 2.0 selects probes with the lowest cross-hybridization; the absence of secondary structure and the final oligonucleotide set is balanced in term of melting temperature. To limit overlapping between probes, an FASTA similarity search was conduct against RepBase and GenBank databases using the designed oligonucleotides. An antisense probe was then deduced from each designed sense probe. A full description of the oligonucleotides is hosted at: http://www.landesbioscience.com/supplement/HorardEPI4-5-Sup.pdf (see Supplemental Table 3).

637 (b) Only oligonucleotides of the highest quality should be used for microarraying. Functionalization if the oligonucleotide with an amine or other reactive group is not necessary when using the UltraGAPS™ substrate.

641 9. RepArray slides were printed at The Transcriptome Plateform from Biology Department Genomic Service at ENS, Paris—FRANCE and information concerning microarray printing are hosted at: http://www.transcriptome.ens.fr/sgdb/. Briefly, dry oligonucleotides from were rehydrated in Pronto!™ Universal Spotting Solution (Corning # 40027) at a concentration of 0.5 ng/μl and printed on commercially prepared silinazed slides using a Microgrid TAS arrayer and pins from Biorobotics. Probes were arrayed as duplicates in a random order to minimize the risk of misinterpretation due to a geometric artifact during array hybridization. The printed arrays were kept at room temperature in a dessicator.

653 10. Prepare 2× hybridization stock solution, sterile filtrate, and make aliquots for storage at −20°C. Before use, 2× hybridization aliquot should be thawed completely and prewarmed at

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68°C for few minutes to ensure complete dissolution of SDS crystals. 656 657

11. We recommended the use of 4 μg as starting DNA material per immunoprecipitation point. Smaller amounts down to 0.5 μg have been used successfully when combined with real-time PCR evaluation of enrichments. However, the yields of MeDIP DNA starting from small amount of material will be lower and thus not be adequate for immediate labeling and hybridization to microarray. Typical negative control reactions either use normal immunoglobulin (IgG) from the same species of the 5mC antibody or omit the antibody. 658 659 660 661 662 663 664 665 666

12. Protein A-coupled Sepharose beads are usually suitable when using polyclonal antibodies. If using a mouse monoclonal antibody combine (v/v) Protein A- and G-coupled matrix since some mouse isotypes do not bind to Protein A-Agarose. Lyophilized Sepharose should be swollen in 1× PBS (10 volumes) for at least 30 min at room temperature with gentle overhead shaking (1 g of powder typically swells to 3–4 ml of hydrated gel). Pre-swollen beads as slurry ready for use are also available from various manufacturers. Swollen Sepharose can be store at 4°C for few days in PBS 1×. To block nonspecific interactions with Protein A-Sepharose, it is important to use BSA 0.1% rather than salmon sperm DNA to avoid contaminations with unrelated repetitive DNA. 667 668 669 670 671 672 673 674 675 676 677 678 679

13. It is recommended to cut off the tip of the pipette when manipulating Protein A-Sepharose to avoid disruption of the beads. Beads will tend to stick to the sides of the tip so try to minimize the movement in the pipette. 680 681 682 683

14. We recommend using a shaking heat block at 750–900 rpm (Eppendorf Thermomixer R) to prevent sedimentation of the beads. 684 685 686

15. PCR program and cycling conditions might need to be adjusted according to qPCR reagents and machine used. It is best to premix all PCR components with the exception of the DNA template in a microtube (always prepare for one reaction more than you needed), aliquot the premix in the 96-well PCR plates and add the template. 687 688 689 690 691 692

16. The Ct values for each reaction within the duplicate are averaged if the duplicate does not fluctuate greater than or less than 0.5 cycles. It is recommended to repeat the PCR for samples where Ct ranges are >0.5. The rate of amplification is calculated for each PCR reaction. The theoretical optimum rate of amplification (R) is 2.0, but to make accurate and meaningful comparisons between primers pairs, the differences in amplification rates should be calculated. Amplification rate is determined by performing standard curves using serial dilutions 693 694 695 696 697 698 699 700 701

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702 (0.01–30 ng) of sheared genomic DNA (=Input). The fold
703 enrichment of a specific target sequence in the MeDIP sample
704 relative to the Input sample is then calculated using the
705 following equation: $R^{(\mathrm{CtInput}-\mathrm{CtMeDIP})}$.Finally, the methylation
706 enrichment at a particular sequence relative to the back-
707 ground level is calculated according the following equation:
708 $R^{(\mathrm{CtInput}-\mathrm{CtMeDIP})\mathrm{Target}} / R^{(\mathrm{CtInput}-\mathrm{CtMeDIP})\mathrm{Neg.cont}}$. The IgG-IP samples
709 allow demonstrating that enrichment is specific for methylated
710 DNA.

711 17. With the described conditions, the MeDIP procedure generally
712 yields sufficient amount of material to allow direct labeling by
713 Klenow random priming, thus bypassing the need for a PCR
714 amplification step that might introduce bias in sequences
715 representation. We confirm using qPCR that specific enrich-
716 ments of several control loci detected in MeDIP samples versus
717 Input samples are the same before (DNA from Subheading 3.2
718 step 21) and after the random priming reaction (DNA from
719 Subheading 3.4 step 5). Typically, the larger the amount of
720 DNA template used, the greater the yield of probe. Start labeling
721 procedure from the same amount of MeDIP and Input DNA
722 samples.

723 18. Successful labeling results in a smear from 100 to 1,000 bp
724 with an average of size of 300–400 bp (i.e. slightly shorter than
725 the smear detected after sonication). This is due to random
726 incorporation of pd(N)6 oligonucleotide into DNA. For pure
727 DNA, optimal absorbance ratios are A260/280 ≈ 1.8 and
728 A260/230 ≈ 1.8. Typically, Klenow (exo-) reactions produce
729 between 4 and 6 μg of aminoallyl-labeled DNA.

730 19. Place reactive dye pouch at room temperature for 15 min
731 before opening the vials.

732 20. The color of the purified probe is a good indicator for the pro-
733 cedure success. If the eluted probe is colorless, the labeling was
734 probably not efficient. If eluted product has a strong pink
735 (Cy3) or blue color (Cy5), the dye removal procedure most
736 likely failed.

737 21. According to manufacturer's instructions, the labeling density
738 of one dye per X unlabeled nucleotide can be roughly esti-
739 mated by using the formula:

740
$$\text{Dye ratio } (X) = ((\text{total DNA yield } (\text{ng}) \times 1{,}000) / 324.5) / \text{pmol incorporation of dye}$$

741 where: Total DNA yield (ng) = A260 × 37 μg/μl × total volume,
742 dye yield (pmol) = A550 (or 650)/dye coefficient × total volume,
743 324.5 is the average molecular weight of nucleotide in DNA.
744 1,000 is the DNA length average assumed. Best hybridization
745 results are obtained when one dye per every 30–60 unlabeled
746 nucleotides is targeted.

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22. Because cyanine dyes are extremely sensitive to degradation (by air humidity, ambient light, ozone), we recommend to proceed immediately to hybridization and not to store the labeled DNA for further hybridization.
23. Rinse cover glass in milliQ water then store them in 95% ethanol. Before use, allow cover glass to air dry.
24. We prefer that hybridization chambers do not directly contact the bottom of the water bath since it might cause nonhomogeneous heat transfer.
25. Transferring the slides to fresh conical tubes between wash steps helps to minimize SDS (which is autofluorescent) carryover. As liquid evaporating slowly from the slides can lead to wash artifacts, proceed quickly with the final centrifugation step. Transfer the arrays from wash solution to conical tube placed in the centrifuge and immediately start the centrifuge.
26. Because some repeats account for 50 copies in the human genome while other can account for 5×10^7 copies, large amplitude of signal intensity is observed on a scan. Therefore, each array is scanned at three different photomultiplier tubes (PMTs) settings (low, median, and high). In order to correlate the measurements over a wide range of incidental light intensities, we developed a linear predictive model that generates a synthetic value of intensities. A full description of the predictive model is available at: http://www.landesbioscience.com/supplement/HorardEPI4-5-Sup.pdf (see Supplemental Method).
27. At least three independent MeDIP reactions are performed and hybridized to total genomic DNA (Input). The hybridized slides are then grouped together accordingly for the analysis. Friedman's analysis of variance can be performed on the whole dataset to check the coherence between the different microarrays hybridized. Both sense and antisense features populations might display similar $\log_2$ ratios in methylation experiments (Supplementary figure 1 at: http://www.landesbioscience.com/supplement/HorardEPI4-5-Sup.pdf), analysis can be performed on mean $\log_2$ ratios from one strand (sense or antisense) or on averaged $\log_2$ ratios from both strands (sense + antisense). Once DNA methylation status of each element on the array has been determined, methylation pattern can be investigated across samples. Principal component analysis and unpaired test analysis might facilitate methylome comparisons (9). To identify aberrant DNA methylation, it is imperative to have a "reference" map for comparison and thus experimental strategies should include control and test samples. For example, comparison a comparison of DNA methylation from tumor and nonmalignant samples is a common strategy in cancer research.

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Uncorrected Proof

Author's Proof

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Uncorrected Proof

Author's Proof

Author Queries

Chapter No.: 16   0001482303

Queries	Details Required	Author's Response
AU1	Please check if the term "methyl-DNA immunoprecipitaton" here in the sentence "Combined to a ..." can be changed to "methylated DNA immunoprecipitation" for consistency.	
AU2	Please check layout and footnotes of Tables 1 and 2.	
AU3	Should "10 mM Tris pH 7.4" be "10 mM Tris-HCl pH 7.4" in the sentence "DNA resuspension..."? Please check susequent occurrences in chapter.	
AU4	Should "O.1% SDS" be "0.1% SDS" in the sentence "Prehybridization solution..."? Please check.	
AU5	Figure 1 was not cited in the text. An in text citation has been introduced at the end of sentence "The complete protocol...". Please check.	
AU6	Please provide 'g-force value' for '2,500 rpm' in the sentence 'Into a 14 mL screw cap tube...' and subsequent occurrences in chapter.	
AU7	Reference (22) is cited in the text but not listed in the references list. Please check.	
AU8	"exo" has been changed to "exo-" all the occurrences in chapter. Please check if appropriate.	
AU9	Please check the representation 30 l from 4.5 step 11 in the sentence "Prepare the hybridization solution " and amend the changes accordingly.	
AU10	Please check if the term "photomultiplicator (PMT)" is correct in the sentence "Set the laser intensity..."	
AU11	In the sentence "Validation of results..." "3.3 chapter" has been changed to "Subheading 3.3". Please check if appropriate.	

Uncorrected Proof