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Chapter 26

Quantitative Region-Specific DNA Methylation Analysis by the EpiTYPER™ Technology

Sonja Kunze

Abstract

DNA methylation plays a profound role in development and health as well as development and progression of disease. High-throughput quantitative DNA methylation analysis is therefore crucial for the study of the normal physiology of the epigenome and its dysregulation in disease. Many target areas are identified by a range of emerging genome-wide cytosine methylation techniques, but these whole genome scans usually only provide methylation data for a few individual CpG sites (CpGs) within a region. The EpiTYPER™ assay is a region-specific method for the detection and quantitative analysis of DNA methylation that allows performing a high-resolution scan of selected regions. It thus enables a more detailed analysis of single CpGs and the surrounding area and can, in addition to candidate gene methylation analysis, be used to validate CpGs detected by genome wide techniques. The EpiTYPER™ assay allows a fast and reproducible targeted quantification of individual CpGs in a high throughput manner and is based on base-specific cleavage of bisulfite-converted genomic DNA and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Up to 85% of the CpGs within a target region can be analyzed and the detection precision allows quantifying methylation differences as low as 5–7%.

Key words EpiTYPER, Base-specific cleavage, MALDI-TOF MS, DNA methylation pattern, Quantitative, Bisulfite

1 Introduction

The identification, quantification, and mapping of DNA methylation within the genome can be conducted by a wide range of available methods and approaches [1]. The method of choice depends on the intended application and required level of information. Since the methods for DNA methylation analysis differ in their coverage and sensitivity, they enable global profiling of DNA methylation as well as the measurement of the degree of methylation at specific target regions or the creation of genome-wide methylation maps.

For region-specific analysis numerous methods have been reported. While the earlier ones relied exclusively on restriction enzymes, utilization of bisulfite conversion has revolutionized the

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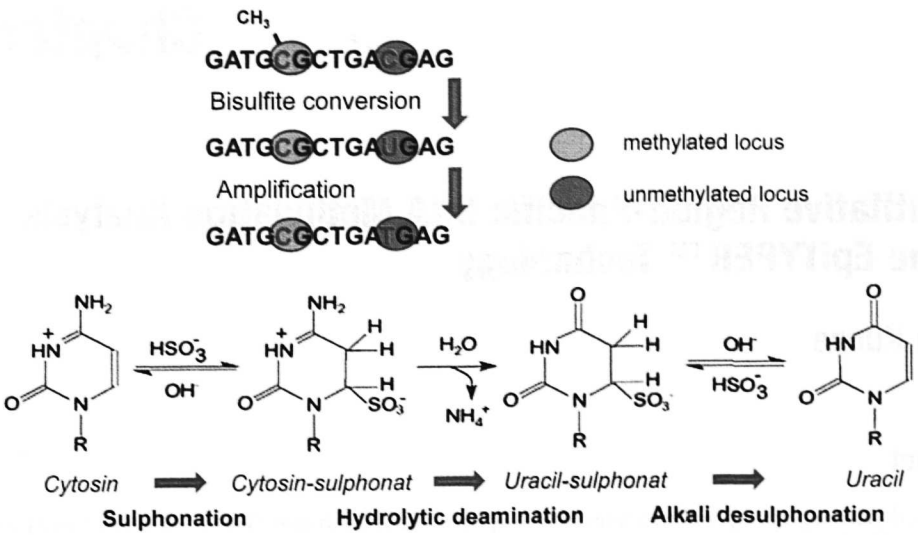

Fig. 1 Principle of the bisulfite conversion and chemical background. The chemistry of cytosine deamination by sodium bisulfite involves three steps: sulfonation (the addition of bisulfite to the 5–6 double bond of cytosine), hydrolic deamination of the resulting cytosine-bisulfite derivative to give a uracil-bisulfite derivative and alkli desulfonation (the removal of the sulfonate group by an alkali treatment) to give uracil

field. Treatment of denatured single-stranded genomic DNA with sodium bisulfite converts unmethylated cytosines to uracils, whereas methylated cytosine bases are protected from conversion and remain unchanged. The uracils are amplified in subsequent polymerase chain reactions (PCR) as thymines, producing methylation-dependent sequence variations of C to T for the unmethylated locus (Fig. 1). One method based on the bisulfite treatment of genomic DNA is, besides for example pyrosequencing (*see* Chapter 22), the EpiTYPER assay from Agena Biosciences formerly Sequenom. It is used to carry out region-specific high-throughput DNA methylation analysis for the targeted quantitation of individual CpGs. In short, the assay detects and quantitatively analyzes DNA methylation using base-specific cleavage of bisulfite-converted genomic DNA and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [2]. An overview of the concept of the EpiTYPER method is given in Fig. 2. The bisulfite treatment of genomic DNA is followed by PCR amplification in which a T7- promoter tag is introduced by a T7-promoter tagged reverse primer. Also, a 10mer tag sequence is added to the forward primer to balance the PCR primer length. The PCR primers are independent of the methylation state of the genomic DNA, i.e., they bind to both methylated and nonmethylated template, and should be designed to yield a product within a 200–600 base pair (bp) range (henceforth referred to as amplicon). After an incubation with Shrimp Alkaline Phosphatase (SAP) reaction, that removes unincorporated dNTPs, in vitro RNA

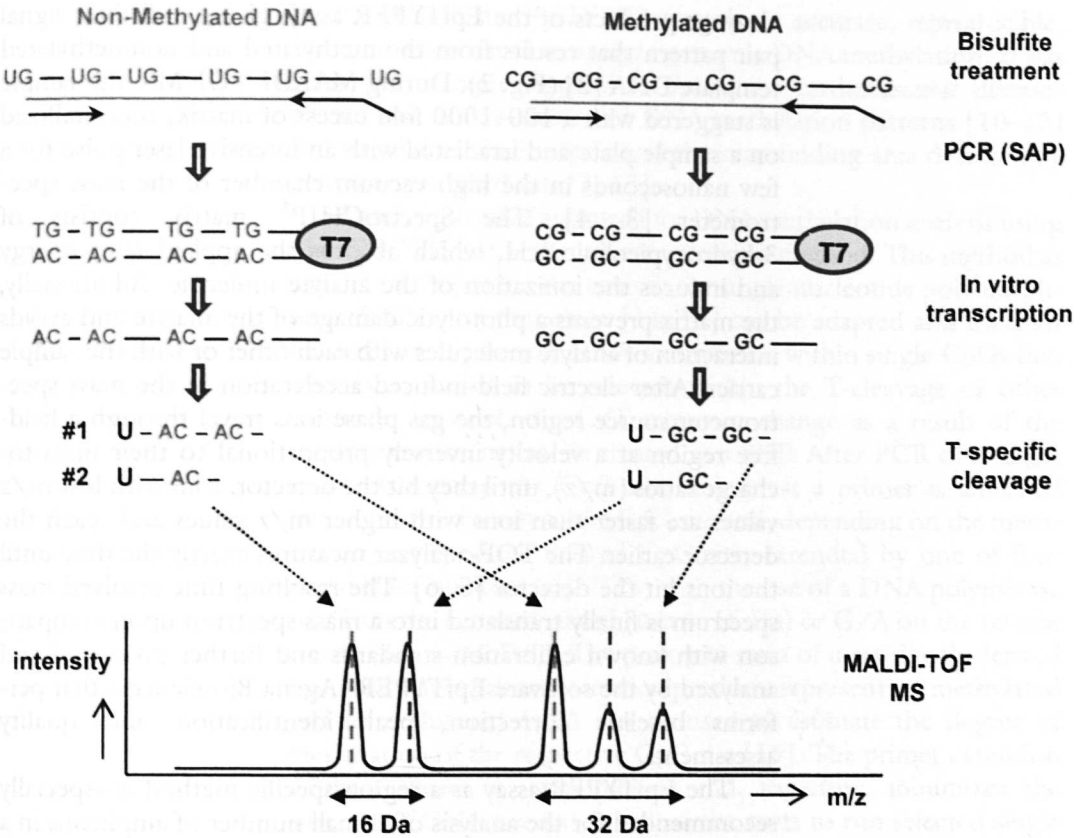

Fig. 2 Scheme of the EpiTYPER technique. Bisulfite-treated DNA is amplified by PCR using primers located around the CpG site of interest, one of them being tagged with a T7-promoter sequence. The PCR product is transcribed into a RNA transcript and cleaved in a base-specific manner after each T (thymin). The cleavage products are then analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and a characteristic mass signal pattern can be obtained. The *green* signal represents nonmethylated DNA and the *blue* one represents methylated DNA. This figure was modified after Ehrich et al. [2]

transcription is performed on the reverse strand, followed by base-specific T-cleavage using RNase A. Within this step, cleavage products are generated for the reverse transcription reaction for U (thymidine (T)). Since T is found in DNA it is used from this point forward, however, cleavage actually happens at U in an RNA molecule. Both methylated and nonmethylated regions are cleaved at every T to produce fragments of different length and mass. For a specific CpG site the cleavage products resulting from methylated and nonmethylated DNA respectively have the same length and differ only in their nucleotide composition. The methylation dependent C/T sequence changes introduced by bisulfite treatment are presented as G/A changes on the reverse strand and therefore result in a mass difference of 16 Da (Dalton) for each CpG site enclosed in the cleavage products generated from the RNA transcript. MALDI-TOF MS allows measurement of the

cleavage products of the EpiTYPER assay, giving a distinct signal pair pattern that results from the methylated and nonmethylated template DNA [2] (Fig. 2). During MALDI-TOF MS, the sample is staggered with a 100–1000 fold excess of matrix, cocrystallized on a sample plate and irradiated with an intensive laser pulse for a few nanoseconds in the high vacuum chamber of the mass spectrometer [3, 4]. The SpectroCHIP® matrix consists of 3-hydroxypicolinic acid, which absorbs the applied laser energy and induces the ionization of the analyte molecule. Additionally, the matrix prevents a photolytic damage of the analyte and avoids interaction of analyte molecules with each other or with the sample carrier. After electric field-induced acceleration in the mass spectrometer source region, the gas phase ions travel through a field-free region at a velocity inversely proportional to their mass-to-charge ratios (m/z), until they hit the detector. Ions with low m/z values are faster than ions with higher m/z values and reach the detector earlier. The TOF-analyzer measures exactly the time until the ions hit the detector [5, 6]. The resulting time-resolved mass spectrum is finally translated into a mass spectrum upon comparison with known calibration standards and further processed and analyzed by the software EpiTYPER (Agena Biosciences) that performs baseline correction, peak identification and quality assessments.

The EpiTYPER assay as a region-specific method is especially recommended for the analysis of a small number of amplicons in a large sample cohort. It is commonly used in candidate gene methylation analysis and is a great tool to validate single CpGs and the surrounding area detected by, for example, genome wide methods (e.g., Illumina 450K or EPIC, *see* also Chapter 16) [7]. The advantages of the EpiTYPER assay are the fast, targeted quantification of individual CpGs in a high-throughput manner. The time needed for bisulfite-treated DNA to data is approx. 8 h and the detection precision allows to quantify methylation differences as low as 5–7% within a methylation range of 10–90% [2]. Disadvantages of the EpiTYPER assay are for example difficulties to measure DNA methylation across CpG islands and that only the average methylation data per cleaved fragment is examined. Ideally, a fragment contains a single isolated CpG site and has a unique molecular weight. However, a fragment may also contain multiple CpGs or may have a molecular weight that overlaps with another CpG-containing fragment and then the constituent CpGs have to be measured in aggregates. Or the fragment molecular weight is too small or too large and lies outside the testable mass window. For these reasons, depended on the sequence content and the distribution of CpGs, up to 85% of the CpGs within a target region are analyzed. Another point is that polymorphisms in the DNA sequence analyzed can be a problem for the MassCLEAVE assay and obscure the analysis of the degree of DNA methylation

[8, 9]. Overall the EpiTYPER assay is an accurate, reproducible, and quantitative tool to analyze genomic DNA methylation. It has been, for example, applied to determine cardiovascular disease-related and cancer-associated DNA methylation patterns [10–12] and to validate single CpGs and the surrounding area detected by genome-wide methods [7, 13].

Another possible approach for DNA methylation analysis using MALDI-TOF MS is the primer extension method. This method as usually employed for the analysis for single nucleotide polymorphisms (SNPs) and mutations [14, 15] can be adapted and used for the analysis of genomic DNA methylation within single CpGs that have been found to be relevant within the T-cleavage or other methods. In brief, the C/T sequence change as a result of the bisulfite treatment can be treated as a SNP. After PCR of a target region harboring the CpG site of interest a primer is annealed immediately adjacent to the CpG site and, depending on the methylation status of the desired CpG site, extended by one of four possible terminators (ddNTPs) with the use of a DNA polymerase (C/T on the forward strand ($\Delta m = 15$ Da) or G/A on the reverse strand ($\Delta m = 16$ Da)). The peak area ratio of mass signals derived from the single primer extension products representing methylated and unmethylated DNA can be used to estimate the degree of methylation of the respective CpG site [16]. The primer extension method allows for multiplexing and, therefore, minimizes the amount of DNA needed as well as the costs to run selected single CpGs in a large sample size.

Although this chapter will only address the protocol for the EpiTYPER assay, the primer extension method is a great tool on top of the EpiTYPER assay. The EpiTYPER assay allows the simultaneous analysis of multiple CpGs within a specific region, whereas the primer extension method allows the analysis of selected CpGs with proven functional relevance of different regions, both within a single reaction.

2 Materials

2.1 Software

1. Primer Design software: www.epidesigner.com (Agena Biosciences) or MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi).

2.2 Sample Preparation and Bisulfite Treatment

1. NanoDrop spectrophotometer.
2. EZ-96 DNA Methylation™ Kit (Zymo Research).
3. Nanopure Water (ddH_2O).
4. 100% ethanol.
5. Centrifuge for 96-well microplates.

6. Standard thermocycler, for cycling of 96-well microplates.
7. Plate-sealing film.
8. Methylation standards (e.g., EpigeneDX).

2.3 PCR, SAP and MassCLEAVE™ Protocol

1. EpiTYPER T Reagent Set—Complete PCR Reagent Set (includes: HotStart Buffer (10×; containing 15 mM $MgCl_2$), HotStart Taq DNA Polymerase (5 U/μL) and dNTP Mix (25 mM each); Agena Biosciences formerly Sequenom).
2. Nanopure Water (ddH_2O).
3. PCR primers (designed during Assay Design step, *see* Subheading 3.2 of this chapter).
4. EpiTYPER T Reagent Set—MassCLEAVE T7 Kit (includes: RNase-free ddH_2O, SAP (Shrimp Alkaline Phosphatase; 1.7 U/μL), T7 polymerase buffer (5×), T-cleavage Mix, DTT (dithiothreitol, 100 mM), T7 RNA & DNA Polymerase (50 U/μL) and RNase A (10 mg/mL); Agena Biosciences).
5. 384-well microplates.
6. Centrifuge for 384-well microplates.
7. Standard thermocycler, for cycling of 384-well microplates (with temperature gradient functionality if possible).
8. Plate-sealing film.

2.4 Conditioning, Nanodispensing and MALDI-TOF MS Measurements

1. EpiTYPER T Reagent Set—SpectroCHIP II Arrays & Clean Resin Kit (includes: CLEAN Resin and SpectroCHIP® II G384; Agena Biosciences).
2. Rotator.
3. 4-Pt Calibrant (included in the MassCLEAVE T7 Kit; Agena Biosciences).
4. MassARRAY™ Nanodispenser (Agena Biosciences).
5. MassARRAY™ Compact MALDI-TOF MS (Agena Biosciences).
6. MassARRAY™ EpiTyper software v1.2 (Agena Biosciences).

3 Methods

3.1 Bisulfite Treatment

Protocols are performed according to the manufacturer's instructions (Zymo Research EZ DNA methylation kit) with slight modifications as recommended by Agena Biosciences.

The bisulfite conversion can be carried out in a 96-well plate format or in single tubes, depending on the sample size (*see* **Note 1**). The protocol differs only slightly; in the following protocol the steps for the conversion in 96-well plate format are listed.

1. Prepare a 45 µL DNA mix in a Conversion Plate (96-well format) for each of the DNA samples by adding a certain amount of genomic DNA to the respective volume of nanopure water, depending on the DNA concentration. For one reaction a minimum amount of 10 ng of bisulfite-treated DNA is needed. Therefore the amount of genomic DNA added depends on the number of planned reactions. Usually a range between 500–1000 ng is recommended, however if DNA is limited, the input can be reduced to 200 ng (*see* **Note 2**).
2. Add 5 µL M-Dilution Buffer to each DNA mix, mix by pipetting up and down, seal the plate and incubate the samples at 37 °C for 15 min.
3. During the incubation time of the samples, prepare the CT Conversion Reagent by adding 7.5 mL of nanopure water and 2.1 mL of M-Dilution Buffer to one bottle of CT Conversion Reagent. Mix the solution with frequent vortexing for at least 10 min at room temperature (fix the tube to the vortexer by adhesive tape; prolong the vortex step if the solution is not clear of crystals) (*see* **Note 3**).
4. After incubation, add 100 µL of the CT Conversion Reagent to each sample, mix by pipetting up and down.
5. Incubate the Conversion Plate on a thermal cycler using the following program: 20 cycles of 95 °C for 30 s and 50 °C for 15 min, hold at 4 °C.
6. After incubation, keep the samples on ice for 10 min, prepare the M-Wash Buffer by adding 144 mL of 100% ethanol to the 36 mL M-Wash Buffer concentrate and set the centrifuge to a temperature of 25 °C.
7. Stack a Silicon-ATM Binding Plate on top of a Collection Plate and add 400 µL of M-Binding Buffer to each well.
8. Centrifuge the samples which have been on ice for 10 min, load them into the wells of the Silicon-ATM Binding Plate containing the M-Binding Buffer, and mix by pipetting up and down as long as smear is observable.
9. Centrifuge the plate for 3 + 3 min at $\geq 3000 \times g$ (RCF 4500) (*see* **Note 4**) and discard the flow-through to prevent contamination of the column contents.
10. Add 500 µL of M-Wash-Buffer to each well and centrifuge for 3 + 3 min at $\geq 3000 \times g$. Discard flow-through.
11. Add 200 µL of M-Desulfonation Buffer to each well and incubate the samples at room temperature (20–30 °C) for 15–20 min.
12. Centrifuge for 3 + 3 min at $\geq 3000 \times g$. Discard flow-through.

13. Add 500 µL of M-Wash Buffer to each well and centrifuge for 3 + 3 min at ≥3000 × *g*. Discard flow-through.
14. Add another 500 µL of M-Wash Buffer and centrifuge for 6 + 6 min at ≥3000 × *g*. Discard flow-through.
15. Place the Silicon-ATM Binding Plate onto an Elution Plate and pipette an appropriate volume of nanopure water (*see* **Note 5**) directly to the binding matrix in each well.
16. Incubate the plate for about 2–5 min and centrifuge for 3 + 3 min at ≥3000 × *g*.
17. The bisulfite-converted DNA can be used immediately stored at 4 °C for 1 week or at or below −20 °C for later use (*see* **Note 6**).
18. Since the DNA is single stranded due to the conversion process with limited nonspecific base-pairing at room temperature, an absorption coefficient of 260 nm, which resembles that of RNA, has to be used to determine the concentration of the recovered bisulfite-treated DNA (value of 40 µg/mL for Abs260 = 1.0), if desired.

A complete bisulfite conversion is essential for the DNA methylation analysis, as incomplete conversion of cytosines to uracils can result in false-positive methylation signals. Incomplete bisulfite conversion can arise from incomplete denaturation before bisulfite treatment or reannealing during the bisulfite conversion, as the conversion only takes place within single stranded DNA. Thus repeated denaturation cycles, as stated in **step 5**, are part of the bisulfite conversion process. On the other hand overtreatment with bisulfite degrades DNA and may lead to an increased incidence of methylated cytosines converting to thymine residues, which results in under-reporting of DNA methylation (*see* **Note 7**).

3.2 Assay Design

The design of the assays is the most crucial step for a successful outcome of the methylation analysis. In our laboratory primers are designed using Agena Biosciences's Primer Design software as we usually have good experiences with this tool. But also other commercial and freely available software's can handle primer design on bisulfite-converted DNA such as MethPrimer.

The genomic sequence is pasted into the EpiDesigner, the PCR parameters adjusted or left as default (*see* recommended settings in Table 1). The optimal size range for PCR amplicons is 200–600 bp, depending on the quality of bisulfite-treated DNA. A good DNA quality allows successful amplification of sequences exceeding 600 bp, whereas highly degraded DNA will only yield stable results for shorter sequences. The strand can be selected (or both) as well as the reaction type (T-cleavage). The primers of the amplicons are designed to contain at least four non-CpG cytosines within the 1st Primer that will be replaced by Ts, or guanines within the 2nd Primer that will be replaced by adenines, as a consequence, these

Table 1
Settings for Primer design using the Agena Biosciences Primer Design software

Setting	Minimum	Optimum	Maximum
Primer T_m (°C)	56	62	64
Primer size (bp)	20	25	30
Product size (bp)	100	300	600
Primer non-CpG 'C's	4	–	–

primers will only bind to fully converted templates and should yield a defined, single band on an agarose gel. Also an assay name can be added and the run can be saved for a later time in "My Primers" (*see* **Note 8**).

The advantage of the EpiDesigner is that one can review primer locations and sequences in the results window. By moving the mouse pointer over the potential amplicons displayed in the results chart one can also verify which CpGs within the sequence will be covered by each corresponding amplicon and which ones will be not (e.g., due to low or high mass). If the detection of a certain CpG site is required (e.g., to validate the methylation difference of a CpG detected with the Illumina 450K) the amplicon pairs can be selected accordingly to certainly cover the desired CpG (*see* **Note 9**).

Also, the T7 promoter tag including an 8 bp insert ("cagtaatacgactcactatagggagaaggct") and the 10mer tag ("aggaagagag") are added automatically by the software upon export of the designed PCR primers (*see* **Note 10**). The T7-promoter tagged reverse primer is incorporated into the amplification product for in vitro transcription during PCR amplification and the 10mer-tag sequence to the forward primer is added to balance the PCR primer length.

3.3 Polymerase Chain Reaction (PCR)

To obtain a defined, single band on the agarose gel (without any additional nonspecific bands) and thus the ideal annealing temperature of the primers, PCR incubation conditions can be optimized by test PCR.

3.3.1 Optimizing Polymerase Chain Reaction (PCR)

1. Prepare 1 μM PCR primer mixes in 1.5 mL tubes by adding 4 μL of both forward and reverse primers to 392 μL of nanopure water for each assay.
2. Prepare PCR cocktails for 20 reactions in 1.5 mL tubes according to Table 2 including the Primermix (the 20 reactions include 8 bisulfite-treated DNA samples for the different temperatures, 8 negative controls without adding bisulfite-treated DNA for the different temperatures and overhang).

Table 2
PCR cocktail for EpiTYPER methylation analysis

Reagent	Volume for single reaction in µL	Volume for test PCR in µL	Volume for 384-well plate[a] in µL
ddH_2O	1.42	28.4	654.34
10× PCR-buffer (containing 15 mM $MgCl_2$)	0.50	10	230.40
dNTP (25 mM each)	0.04	0.8	18.43
PCR enzyme (Agena biosciences, 5 U/µL)	0.04	0.8	18.43
Mastermix volume:	2.00	40	921.60
Primermix (1 µM) (T7Reverse & 10mer forward tags)	2.00	40	–
Bisulfite-treated DNA (10 ng/µL)	1.00	–	–
Total volume	5.00	–	–

[a]Volumes for a 384-well plate include 20% overhang to account for possible pipetting loss

3. Dispense 4 µL of the PCR—Primermix cocktail into the wells of a 384-well plate in a way that allows running one sample and one negative control each with the different annealing temperatures, seal and centrifuge the plate.
4. Dispense 1 µL of the bisulfite-converted DNA (10 ng/µL) to the PCR—Primermix cocktail (*see* **Note 11**), but leave out the negative controls and add 1 µL of nanopure water instead.
5. Run a Gradient-PCR (56–64 °C) to determine the optimum annealing temperature using the following program: 94 °C for 4 min, 45 cycles of 94 °C for 20 s, 56–64 °C for 30 s and 72 °C for 1 min. Terminate the program at 72 °C for 3 min and hold at 4 °C (*see* **Note 12**).
6. After the incubation step, run all samples on a 3% agarose gel.
7. Choose the annealing temperature that shows the best result with a defined, single band on the gel. If primers do not show good PCR products, it is necessary to design new assays and test again (*see* **Note 13**).

3.3.2 Preparing Polymerase Chain Reaction (PCR)

1. Design a Plate Layout to simplify the pipetting of the PCR plate. In addition to the bisulfite-converted DNA samples, 0%, 25%, 50%, 75%, and 100% methylation standards should be included within each run as an indicator of the intra-run variation in the degree of bisulfite-related bias (if there are not enough free wells available on the plate to include all methylation standards at least the 25% and 75% ones should be

a **Amplicons**

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
B	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
C	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
D	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
E	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
F	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
G	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
H	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
I	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
J	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
K	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
L	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
M	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
N	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
O	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
P	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5

Amplicon 1: cg05575921 Amplicon 2: cg21566642 Amplicon 3: cg03636183 Amplicon 4: cg06126421 Amplicon 5: cg19572487

b **Samples**

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	1	17	33	1	17	33	1	17	33	1	17	33	1	17	33
B	2	18	34	2	18	34	2	18	34	2	18	34	2	18	34
C	3	19	35	3	19	35	3	19	35	3	19	35	3	19	35
D	4	20	36	4	20	36	4	20	36	4	20	36	4	20	36
E	5	21	37	5	21	37	5	21	37	5	21	37	5	21	37
F	6	22	38	6	22	38	6	22	38	6	22	38	6	22	38
G	7	23	39	7	23	39	7	23	39	7	23	39	7	23	39
H	8	24	40	8	24	40	8	24	40	8	24	40	8	24	40
I	9	25	41	9	25	41	9	25	41	9	25	41	9	25	41
J	10	26	42	10	26	42	10	26	42	10	26	42	10	26	42
K	11	27	0%	11	27	0%	11	27	0%	11	27	0%	11	27	0%
L	12	28	25%	12	28	25%	12	28	25%	12	28	25%	12	28	25%
M	13	29	50%	13	29	50%	13	29	50%	13	29	50%	13	29	50%
N	14	30	75%	14	30	75%	14	30	75%	14	30	75%	14	30	75%
O	15	31	100%	15	31	100%	15	31	100%	15	31	100%	15	31	100%
P	16	32	NTC	16	32	NTC	16	32	NTC	16	32	NTC	16	32	NTC

0%: unmethylated DNA; 25 - 100%: methylated DNA; NTC: Negative control

Fig. 3 Example of a (**a**) amplicon layout and (**b**) sample layout for the preparation of the PCR plate. Only the first 15 columns are shown. The amplicon layout shows where the amplicons analyzed within the plate (1–5) should be located and the sample layout shows the location of the corresponding samples including the bisulfite-converted DNA samples (1–42), methylation standards (0–100%) and negative controls (NTC)

included). The DNA methylation standards are only included to show if the experiment worked in principle and if the trend given for each CpG site is correct. They should not be used as a standard curve for adjustment of the methylation data. It is important to also include negative controls (NTC's, by adding nanopure water instead of bisulfite-treated DNA) to verify the occurrence of PCR contamination and formation of primer dimers (Fig. 3) (*see* **Note 14**).

2. Prepare PCR cocktails as displayed in Table 2 without adding the bisulfite-treated DNA.
3. Dispense 2 μL of the PCR cocktail into the wells of a 384-well plate; seal and centrifuge the plate (*see* **Note 15**).
4. Dispense 2 μL of the PCR primer mixes prepared in Subheading 3.3.1 of this chapter to each well as per the layout in Fig. 3a, seal and centrifuge the plate.
5. Dispense 1 μL of the bisulfite-converted DNA (10 ng/μL) to each well as per layout in Fig. 3b.

6. Properly seal the plate with plate sealing film, vortex and centrifuge at 540 × *g* for 1 min.
7. Incubate the 384-well plate on a thermal cycler using the following program: 94 °C for 4 min, 45 cycles of 94 °C for 20 s, 57 °C* for 30 s and 72 °C for 1 min. Terminate the program at 72 °C for 3 min and hold at 4 °C (*Annealing temperature adjusted according to test PCR).
8. After the incubation step, run all 384 PCR products on a 3% agarose gel. As primers will only bind to fully converted templates a defined, single band on the agarose gel for each PCR product suggests that the corresponding sample is fully converted (*see* **Note 7**).

3.4 SAP Reaction

To reduce the presence of by-products, the amplification products have to be treated with shrimp alkaline phosphatase (SAP), which cleaves phosphates of unincorporated dNTPs, rendering them unavailable for future polymerase reactions.

1. Prepare the SAP enzyme solution in a 1.5 mL tube according to Table 3.
2. Thoroughly mix the SAP enzyme solution by vortexing and centrifuge.
3. Centrifuge the 384-well PCR plate after completion of the PCR.
4. Add 2 μL SAP enzyme solution to each well of the 384-well PCR plate using a 12-channel pipettor or liquid handling robot. The SAP enzyme solution is moderately viscous, so this has to be done with care to minimize loss of solution due to adhesion to the pipette tips.
5. Properly seal the 384-well PCR plate with plate sealing film, vortex and centrifuge at 3000 × *g* for 1 min.
6. Incubate the plate on a thermal cycler at 37 °C for 20 min and inactivate the enzyme at 85 °C for 5 min, hold at 4 °C.

Table 3
SAP enzyme solution

Reagent	Volume for single reaction in μL	Volume for 384-well plate[a] in μL
RNase-free ddH_2O	1.70	783.36
Shrimp alkaline phosphatase (SAP)	0.30	138.24
Total volume	2.00	921.60

[a]Volumes for a 384-well microtiter plate include 20% overhang to account for possible pipetting loss (*see* **Note 16**)

Table 4
T-cleavage transcription/RNase cocktail

Reagent	Volume for single reaction in μL	Volume for 384-well plate[a] in μL
RNase-free ddH_2O	3.21	1479.16
5× T7-polymerase buffer	0.89	410.11
T-cleavage mix	0.22	101.38
DTT, 100 mM	0.22	101.38
T7-RNA & DNA polymerase	0.40	184.32
RNase A (10 mg/mL)	0.06	27.65
Total volume	5.00	2304.00

[a]Volumes for a 384-well microtiter plate include 20% overhang to account for possible pipetting loss (*see* **Note 16**)

3.5 MassCLEAVE Reaction

In this step, in vitro RNA transcription is performed on the reverse strand followed by base-specific T-cleavage using RNase A (*see* **Note 17**). This yields to fragmented RNA molecules that can be analyzed by MALDI-TOF MS.

1. Prepare the T-cleavage transcription/RNase A cocktail according to Table 4.
2. After SAP treatment, transfer 2 μL of each PCR product of the 384-well PCR/SAP plate "one to one" into a new 384-well T-cleavage plate using a 12-channel pipettor or liquid handling robot. Leftovers of the PCR/SAP products can be stored at −20 °C or discarded.
3. Add 5 μL of the T-cleavage transcription/RNase cocktail per well.
4. Properly seal the plate with plate sealing film, vortex and centrifuge at 540 × *g* for 1 min.
5. Incubate the plate on a thermal cycler at 37 °C for 3 h.
6. Upon completion, condition the sample plate immediately (next step) or store it at −20 °C. Do not leave the plate on 4 °C or room temperature.

3.6 Conditioning

After the MassCLEAVE reaction a cation exchange resin (Clean Resin) has to be added to the products to remove residual salt that could interfere with mass spectrometry analysis.

1. Spread out 6 mg/well Clean Resin on a 384-well Clean Resin plate (also called dimple plate) with the help of the spoon and scraper.

2. Scrape excess Clean Resin off the plate and return it to its container.
3. Allow the 384-well Clean Resin plate to stand for about 15–25 min (*see* **Note 18**).
4. Meanwhile, centrifuge the 384-well T-cleavage plate at 540 × *g* for 1 min after completion of the MassCLEAVE reaction.
5. Dilute the samples with 20 µL of nanopure water, best prepared in a reservoir, using a 12-channel pipettor.
6. Seal the plate and centrifuge at 540 × *g* for 1 min.
7. To add the Clean Resin to the products gently place the 384-well T-cleavage plate upside-down onto the Clean Resin plate directly after the centrifugation step.
8. Hold the two plates together and flip them over so that the Clean Resin plate is on top and the Clean Resin falls into the T-cleavage plate.
9. Make sure all the Clean Resin fell into the T-cleavage plate and tap the Clean Resin plate to help the Clean Resin fall into the T-cleavage plate if necessary.
10. Properly seal the T-cleavage plate and rotate it 360° perpendicular to its long axis for about 30–60 min at room temperature using a rotator at lowest speed.
11. Centrifuge the plate at 3200 × *g* for 5 min.

The reaction products are now ready for transfer to a SpectroCHIP® (*see* **Note 19**).

3.7 Nanodispensing

The reaction products need to be transferred from the 384-well plate format to a 384-element chip array (SpectroCHIP®) using a Nanodispenser.

1. Perform a volume check on the Nanodispenser to determine, which dispense speed is required to spot an optimal product volume of 15–25 nL. This step is necessary, since the volume deposited on the chip is affected by the low viscosity of the reactions.
2. Dispense the 4-point calibrant, a mix of four oligonucleotides with known masses, to the SpectroCHIP®.
3. Dispense the reaction products from the T-cleavage plate to the SpectroCHIP®.

3.8 MALDI-TOF MS Measurements

MALDI-TOF MS analyzes the mixture of cleavage products differing in length and mass. A distinct signal pair pattern results from the mass signals representing methylated and nonmethylated template DNA, which is representative for the CpGs within the analyzed sequence substring.

1. Set up the plate in the "Plate Editor" of the EpiTYPER software by defining the three constituents (T-cleavage reaction, amplicons and samples) for each well according to the Plate Layout designed for pipetting of the PCR plate (*see* Subheading 3.3.2 of this chapter, Fig. 3) and export it to the RT Workstation (check the "Duplicate Wells Report" under "View" before the export as plates with incomplete or duplicate wells cannot be exported. Correct any wells if necessary and resave the experiment).
2. Load the SpectroCHIP® into a MassARRAY mass spectrometer.
3. Set the MassARRAY Workstation Compact to MassCLEAVE (MassCleave.par) for both FlexControl and SpectroAcquire.
4. Load the plate into the MassARRAY Workstation.
5. Before starting the run, control the 4-point calibrant manually on stage position "F0" (alternatively G0 or H0). The solution has four standardized spectra mass signals for calibrating the analysis system (in Da) at 1479.0, 3004.0, 5044.4, and 8486.6, which should not be shifted for more than 2–3 Da.
6. Run the SpectroCHIP®.

3.9 Data Analysis

The EpiTYPER software automatically calculates the amount of methylated DNA for each CpG position by the ratio of the intensities of the distinct signal pairs resulting from the mass signals representing methylated and nonmethylated template DNA. Quantitative results can be obtained for each sequence-defined CpG unit, which contains either one individual CpG site or an aggregate of subsequent CpG sites (CpG Unit). The resulting mass spectra, the nucleotide sequences that produced the spectrum and the amplicon-specific methylation data can be viewed in the EpiTYPER Analyzer program. The so-called EpiGram tab allows a graphical representation for better visualization then given by the result table alone (Fig. 4). The EpiTYPER methylation data can then be exported as desired and analyzed with various statistical packages. One example of how the EpiTYPER as a region-specific method can be used as a validation technique for single CpGs and the surrounding area detected by genome-wide methods (e.g., Illumina 450K) is given in Table 5.

3.9.1 Test for Complete Bisulfite Conversion and SNP Prediction Analysis

It is important to test for complete bisulfite conversion, as incomplete conversion of cytosine to uracil can result in false-positive methylation signals. The R package "MassArray" offers a bisulfite conversion efficiency calculation by measuring levels of unconverted non-CpG cytosines in a given sample [8]. As cytosine methylation in mammals usually occurs in the context of CpG, unmethylated cytosines outside this context can be used to measure

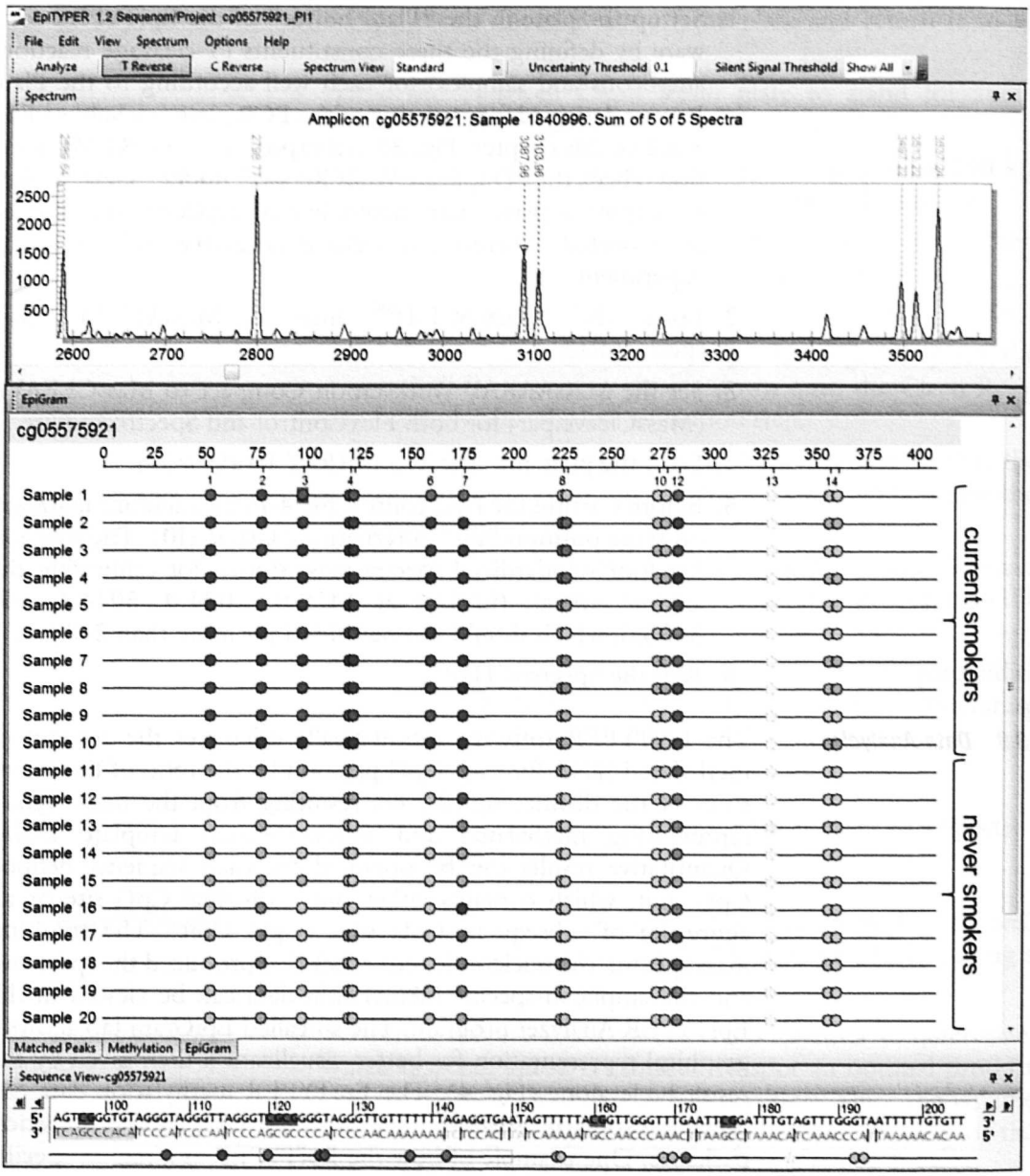

Fig. 4 EpiTYPER methylation data of genomic DNA samples from human whole blood for the smoking-associated candidate gene *AHRR*. The figure shows the varying DNA methylation levels of 10 current compared to 10 never smokers in a 375 bp long amplicon located within the *AHRR* gene as it can be viewed in the EpiGram tab of the EpiTYPER™ Analyzer program. The EpiGram tab is a graphical representation of methylation ratio, each circle displays a CpG site and the color code within the circles denotes the level of methylation found at this particular site in the selected sample. The default color spectrum ranges from *red* (0% methylated) to *yellow* (100% methylated). Above the set of samples the nucleotide position and the CpG site number are given. The amplicon includes an Illumina 450K site (cg05575921; CpG site number 3 in the EpiGram) repeatedly identified to be differentially methylated between never and current smokers [7]. The Spectrum panel displays the mass spectrum for all analyzed amplicons and the Sequence View panel shows the forward and reverse nucleotide sequence for the selected amplicon. This screenshot was made from the EpiTYPER™ Analyzer program of the EpiTYPER Version 1.2 software (Agena Biosciences)

Table 5
Technical validation of Illumina 450K BeadChip methylation intensities by the EpiTYPER assay

Gene CpG	450K: Median ß-value methylation difference	Median ß-value methylation difference in %	*p*-value	Pearson r^2	ß-value as median (first quartile–third quartile)	
					Never smokers	Current smokers
AHRR						
CpG1*	CpG3	−42.50	8.52E−07		0.820 (0.798–0.823)	0.395 (0.295–0.565)
CpG2*	cg05575921:	−24.50	3.53E−04		0.640 (0.610–0.710)	0.395 (0.308–0.488)
CpG3*	−24.40%	−38.00	3.46E−07	0.976456	0.820 (0.800–0.833)	0.440 (0.305–0.560)
CpG4.5*		−40.00	6.11E−06		0.835 (0.783–0.850)	0.435 (0.305–0.555)
CpG6*		−40.00	2.41E−08		0.885 (0.878–0.893)	0.485 (0.388–0.613)
CpG7		−26.50	4.11E−02		0.650 (0.420–0.713)	0.385 (0.260–0.485)
CpG8.9		−12.50	1.63E−02		0.935 (0.855–0.960)	0.810 (0.675–0.900)
CpG10.11*		−8.00	2.05E−04		0.930 (0.920–0.940)	0.850 (0.808–0.870)
CpG12		−5.50	1.24E−01		0.615 (0.565–0.670)	0.560 (0.443–0.608)
CpG14.15		−7.00	6.71E−03		0.930 (0.920–0.940)	0.860 (0.815–0.890)

Displayed is the median ß-value methylation difference between 41 current and 41 never smokers measured by the EpiTYPER, and the corresponding result of the 450K analysis based on 749 never and 262 current smokers. The calculation was done with a linear model adjusted for age, sex, BMI, alcohol, and white blood cell count. For the EpiTYPER validation, the correlation coefficient (Pearson r^2) between the Illumina array derived *b*-value and the EpiTYPER methylation is reported for the associated CpG site cg05575921. *Significant after Bonferroni $p \leq 0.05/28 = 0.0018$ [7]

bisulfite treatment efficiency, acting as "conversion controls". It is essential to measure the extent of bisulfite conversion, because even though the primers are designed to enrich for completely converted sequences (they contain at least 4 non-CpG "C"s), some proportion of amplicons may contain remnant unconverted cytosines, which may cause a molecular weight shift in the subsequent fragmentation profile. The disadvantage of this approach is that it can only be implemented after the EpiTYPER methylation analysis has been carried out, as an output file of the methylation data for each amplicon has to be generated in order to run the pipeline.

Also SNPs within the target sequence of an amplicon could have an effect on the DNA methylation analysis, whether in the form of a fragment mass shift or a different fragmentation pattern, which can lead to misinterpretation of the results [9]. Each novel peak among the MassArray spectrum can be explained by any number of potential SNPs. By using an exhaustive string substitution approach as implemented in the R package "MassArray" [8], putative SNPs can be identified by comparing expected and observed data (*see* **Note 20**).

4 Notes

1. Since the 96-well plate format compared to the tube format is much easier to handle in the lab and therefore less prone to handling errors as well as more cost effective, it is

recommended to favor the plate format when exceeding a certain sample size.

2. It is strongly recommended to convert enough DNA to be able to repeat the PCR step or the entire procedure for all amplicons if necessary (e.g., calculate for three times as many reactions as originally planned). Leftover bisulfite-treated DNA can be stored at −20 °C/−80 °C for a couple of months, depending on the manufacturer of the kit, and used as a test DNA to optimize amplicon annealing temperatures for other EpiTYPER projects (*see* **Note 11**).
3. As the CT Conversion reagent is light sensitive, the exposure to light has to be minimized by darkening the lab room. For best results the prepared CT Conversion Reagent should be used immediately after preparation for all experiments. Alternatively, the CT Conversion Reagent solution can be stored overnight at room temperature, 1 week at 4 °C or up to 1 month at −20 °C. Stored CT Conversion Reagent solution must be warmed to 37 °C, then vortexed well prior to use.
4. The following is relevant for all centrifugation steps within the cleanup of the bisulfite-treated DNA: After each step all wells have to be completely dry, but in our lab usually a few are not even after an extended centrifugation time, which can be seen by swaying the plate under the bench light. This problem can be solved by rotating the plate 180° in between the centrifugation step (thus 3 + 3 min), probably due to a slight change of the centrifugal force. This might not be necessary for all users, if all wells are dry after the usual 5 min centrifugation step.
5. The volume of nanopure water used for the elution depends on the amount of DNA added. If 500 ng DNA is added, the elution step can be carried out with 40 µL of nanopure water as the following PCR step should be carried out with at least 10 ng of bisulfite-treated DNA and a loss of about 20% of the input DNA has to be expected during the bisulfite conversion process. The elution volume should not be too small to assure sufficient recovery of the bisulfite-treated DNA. If the amount of input DNA is small, it might be therefore necessary to add more than 1 µL of the bisulfite-treated DNA to the PCR-Mix to gain the 10 ng needed for the PCR reaction.
6. It is best to use the bisulfite-treated DNA fresh and to avoid freezing/thawing. If it is not possible to do all PCRs within a week (the period within the bisulfite-treated DNA can be kept at 4 °C) it is recommended to make aliquots and only thaw them once.
7. Some working groups carry out a test PCR with one primer pair specific for bisulfite-converted DNA that covers a short sequence that contains many non-CpG cytosines, which are

converted to thymines (Ts) during the bisulfite treatment. But unfortunately, even though resulting DNA bands are as expected and seem to confirm a complete bisulfite conversion, we observed that incomplete conversion can still be detected within the Bisulfite Treatment Control Probes of the Illumina 450K BeadChip in some cases. For that reason we think that a single test PCR covering only a small region of the genome is not really trustworthy, as even though the test PCR shows good results, other parts of the genome than the one covered by the test primers, might still lack complete conversion. We therefore check completion of the bisulfite treatment for each sample within the actual amplicons designed for the corresponding project (Subheading 3.2 of this chapter) after PCR by running an agarose gel with the PCR products (*see* Subheading 3.3.2 of this chapter). Also, the bisulfite conversion efficiency can be tested with the generated data of the MassArray platform by using the corresponding analytical tool of the MassArray package as addressed in Subheading 3.9.1 of this chapter.

8. For primers saved in "My Primers": Because of a bug in the EpiDesigner software the "detail file" that has to be exported for the EpiTYPER process is incorrect concerning the target sequences with an "R"-letter in the "Direction" column. These sequences should be displayed as reverse complement but are incorrectly displayed as forward sequences. Therefore it is important to change these target sequences from forward to reverse complement in order to correct the file. For this purpose the freely available web-based program "Reverse Complement" (http://www.bioinformatics.org/sms/rev_comp.html) can be used for example.

9. Nevertheless, with the EpiDesigner it is not possible to see if the covered CpGs within the desired amplicon will be analyzed as single or in units and if for example silent peaks will overlap the methylated peak. It is possible to reutilize a SpectroChip in order to carry out a "Ghost Run" in the laboratory and get more information about the expected mass spectrum. In principle, an already used SpectroCHIP® has to be loaded into the MassARRAY mass spectrometer. A plate has to be set up by defining the three constituents (T-cleavage reaction, the desired amplicons and any samples) in the "Plate Editor", exported to the RT Workstation and run as described in Subheading 3.8. Also, it can be tricky to figure out which CpG site within the amplicon is the actual desired one. Keep in mind that you look at a forward or revers sequence depending on the direction of the sequence loaded into the EpiDesigner and the direction of the selected amplicon.

10. If alternative software's such as MethPrimer are used for the design of the primers, it is important to keep in mind that the T7 promoter tag ("cagtaatacgactcactataggg") including an 8 bp insert ("agaaggct") for reducing abortive cycling during transcription (5′–cagtaatacgactcactatagggagaaggct + gene-specific sequence–3′) and the 10mer tag (5′–aggaagagag + gene-specific sequence–3′) have to be added manually.
11. Since it is best to use fresh bisulfite-treated DNA for the actual later experiments, stored bisulfite-treated DNA of former experiments can be used for this test process (*see* **Note 2**).
12. If no thermal cycler with temperature gradient functionality is available, the primers can also be tested with selected annealing temperatures. In our hands most primers have an optimum annealing temperature of 57–60 °C, so one test run with 57 °C and another one with 60 °C might already give enough information on which annealing temperature might be best to yield good PCR products.
13. For time-critical projects we recommend to order two different sets of primers for each amplicon and run both with the test PCR. Usually at least one of them works properly, which may avoid the need of redesigning, ordering and testing new primers and therefore save time.
14. If only a small number is analyzed it is recommended to run assays in duplicate or even triplicate for all samples and to take the mean of methylation value for each sample/CpG site. It is also possible to pool genomic DNA [17] in order to use less amount of DNA and minimize costs and then to only run differentially methylated amplicons with single samples in a second run.
15. Depending on the number of amplicons and samples within one plate, the pipetting process can be quite time-consuming. The preparation of the 384-well plate for PCR should then be done on ice.
16. If a liquid handling robot is used it might be necessary to increase the excess volume to over 30%.
17. The C-specific cleavage reaction theoretically provides specific results for selected CpGs but is to our knowledge practically not used by most working groups. Its usability is limited by low CpG coverage and at least within our projects we could not find a use-case for it that would have brought additional value in comparison to the T-cleavage reaction. Moreover, Agena Biosciences has discontinued this kit recently.
18. Do not let the Clean Resin overdry, only wait as long as necessary to be able to transfer it into the T-cleavage plate (*see* Subheading 3.6, **steps 8** and **9**). The color should not

appear too light (yellowish) and the Clean Resin should remain as soggy as possible. The time therefore depends on the relative room temperature and humidity.

19. If the reaction products cannot be immediately transferred to a SpectroCHIP®, the conditioned T-cleavage plate can be tightly sealed by an adhesive sealing foil and stored at −20 °C for up to 2 weeks. If so, thaw and centrifuge the plate at 540 × *g* for 3 min before the transfer.
20. It is of note that these analyses cannot directly confirm or deny the presence or absence of a SNP. However, if samples show a putative high-confidence SNP that maps to a fragment containing one or more CpGs, methylation data from that site should be interpreted with caution.

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