Methylation-sensitive enrichment of minor DNA alleles using a double-strand DNA-specific nuclease

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ABSTRACT

Aberrant methylation changes, often present in a minor allelic fraction in clinical samples such as plasma-circulating DNA (cfDNA), are potentially powerful prognostic and predictive biomarkers in human disease including cancer. We report on a novel, highly-multiplexed approach to facilitate analysis of clinically useful methylation changes in minor DNA populations. Methylation Specific Nuclease-assisted Minor-allele Enrichment (MS-NaME) employs a double-strand-specific DNA nuclease (DSN) to remove excess DNA with normal methylation patterns. The technique utilizes oligonucleotide-probes that direct DSN activity to multiple targets in bisulfite-treated DNA, simultaneously. Oligonucleotide probes targeting unmethylated sequences generate local double stranded regions resulting to digestion of unmethylated targets, and leaving methylated targets intact; and vice versa. Subsequent amplification of the targeted regions results in enrichment of the targeted methylated or unmethylated minority-epigenetic-alleles. We validate MS-NaME by demonstrating enrichment of *RARb2*, *ATM*, *MGMT* and *GSTP1* promoters in multiplexed MS-NaME reactions (177-plex) using dilutions of methylated/unmethylated DNA and in DNA from clinical lung cancer samples and matched normal tissue. MS-NaME is a highly scalable single-step approach performed at the genomic DNA level in solution that combines with most downstream detection technologies including Sanger sequencing, methylation-sensitive-high-resolution melting (MS-HRM) and methylation-specific-Taqman-based-digital-PCR (digital Methylight) to boost detection of low-level aberrant methylation-changes.

INTRODUCTION

Aberrant methylation has been linked to a wide variety of human conditions including cancer and is associated with transcriptional repression (1). Methylation changes are potentially powerful prognostic and predictive biomarkers in cancer diagnosis and treatment (2–10). Such aberrant methylation changes that provide clinically useful information are often present in a minor 'epigenetic-alleles' ('alleles') fraction in clinical samples such as plasma circulating DNA (cfDNA), and excess wild-type alleles may mask the biomarkers of interest. For example, detection of aberrantly methylated promoters in sputum (11), tissue of origin-related epigenetic marks in cfDNA (12–14), or detection of cfDNA copy number variation (15) rely on the ability to distinguish small amounts of methylated or unmethylated DNA in one or more regions within excess cfDNA originating from hematopoietic cells or other normal tissues.

Methylation-specific PCR-based techniques that utilize primers specific for amplification of methylated or unmethylated epigenetic alleles in mixed samples have been widely employed for detecting minority epigenetic alleles (16–19). COLD-PCR based enrichment of mutations (20–22) has been adapted for selective enrichment of unmethylated epigenetic alleles (23). These techniques are highly selective for methylation states of choice but they have limited throughput as they are usually applied for one target per reaction. Approaches that enrich methylomic regions in large genomic fractions prior to sequencing have been developed (24). Methylation sensitive restriction enzymes which can differentiate between methylated and unmethylated DNA are used to generate fragments for further epigenetic analysis (25,26). One drawback is that they only provide limited methylation profile analysis since a restriction enzyme cleaves only at specific sequences. Methylated-CpG binding domain (MBD) proteins (27,28) or anti-5-methylcytosine antibodies (Methylated DNA immunoprecipitation, MeDIP) (29,30) are also used to enrich for methylated DNA fragments following immobilization on beads or other solid support, and the captured DNA can then be examined via massively parallel se-

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quencing. Methods using molecular inversion probes (31) or 'bait' oligonucleotides (32) have also been reported. Target capture-based approaches require separation after binding the target DNA which may lead to substantial loss of starting DNA material. This requirement necessitates relatively large amounts of genomic DNA, near-perfect bisulfite conversion and deep sequencing coverage, especially when minority, low-levels alleles are targeted.

Here, we describe a novel one-step technique Methylation Specific Nuclease-assisted Minor-allele Enrichment (MS-NaME) which is performed in solution, can be applied on multiple DNA targets chosen, is simpler and faster than capture methods. MS-NaME employs a double strand specific DNA nuclease (DSN) which has minimal activity against single stranded DNA or DNA that bears mismatches near the enzyme binding sites (33,34). The method (Figure 1) utilizes oligonucleotides ('probes') that direct DSN activity to multiple targets simultaneously. Following sodium bisulfite DNA treatment the resulting single stranded DNA is hybridized to probes that match either the methylated or unmethylated versions of the interrogated sequences at positions containing CpG. Probes targeting unmethylated sequences (U probes) generate local double stranded regions resulting to digestion of unmethylated targets; and vice versa, probes targeting methylated sequences (M probes) result to digestion of methylated targets. Subsequent amplification of the targeted regions results to enrichment of the targeted methylated or unmethylated minority alleles (Figure 1). MS-NaME is applied at the genomic DNA level prior to amplification, hence it can increase the sensitivity of most existing downstream approach, such as methylation-sensitive high resolution melting (MS-HRM), bisulfite Sanger sequencing or methylation-specific Taqman real time PCR (Methylight). We provide validation for MS-NaME in serially diluted mixtures of methylated and unmethylated DNA and in clinical lung cancer samples, while targeting multiple biologically relevant targets simultaneously.

MATERIALS AND METHODS

Genomic DNA and circulating DNA samples

EpiTect PCR Control DNA Set (Qiagen GmbH, Hilden, Germany) which included both methylated and unmethylated bisulfite converted human control DNA, 10 ng/μl, was used as standard reference methylated and unmethylated DNA. Clinical lung tumor and matched normal lung tissue specimens were provided by the Massachusetts General Hospital Tumor Bank and used following approval by the Internal Review Board of the Dana Farber Cancer Institute. Genomic DNA was isolated using the DNAeasy™ Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. The concentration of extracted DNA was measured on an Invitrogen™ Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Eugene, OR, USA) using Qubit® dsDNA HS Assay Kits (Thermo Fisher Scientific, Eugene, OR, USA).

Blood samples were obtained from healthy donors after informed consent and Dana Farber-Cancer Institute Institutional Review Board approval. Blood samples were centrifuged at 1600 g for 20 min at room temperature within 3h

from collection. Plasma was carefully separated from the lymphocyte layer and re-centrifuged at 1600 g for another 15 min at 4°C. Plasma was carefully separated and stored at −80°C. On thawing a third and final centrifugation at 16 000 g for 5 min at room temperature was performed and plasma was carefully transfered into a separate tube away from any residual debris before extraction of circulating DNA. Cell-free circulating DNA (cfDNA) was isolated from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen GmbH, Hilden, Germany), and quantified on an Invitrogen™ Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Eugene, OR, USA) using Qubit® dsDNA HS Assay Kits (Thermo Fisher Scientific, Eugene, OR, USA).

Bisulfite conversion of DNA samples

Bisulfite conversion of DNA samples (20–100 ng input per reaction) was performed using MethylEdge Bisulfite Conversion System (Promega Corporation, Madison, WI, USA) as described by the manufacturer. The concentration of collected bisulfite converted DNA was measured on an Invitrogen™ Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Eugene, OR, USA) using Qubit® ssDNA Assay Kits (Thermo Fisher Scientific, Eugene, OR, USA).

Methylation-specific Nuclease-assisted Minor-allele enrichment (MS-NaME) reaction

Twenty nanogram bisulfite converted DNA and probes (50 nM final concentration for each probe, sequences listed in Supplementary Tables S4–S6) were added to 1× duplex specific nuclease (DSN, Evrogen, Moscow, Russia) buffer to a final 10 μl volume. The sample was denatured at 98°C for 30 s in a PCR machine (Mastercycler EP Gradient S, Eppendorf, Hamburg, Germany). Then the temperature was reduced to 63°C and 0.2 units of DSN was added followed by 20 min of incubation at 63°C and 2 min of inactivation at 95°C. Identical samples with DSN enzyme omitted ('No DSN control') were run in parallel as enrichment-negative controls. MS-NaME on bisulfite converted, WGA-amplified DNA was applied using probes addressing both strands of double-stranded DNA, and using the same protocol except that the first denaturation step at 98°C was extended to 2 min.

Methylation-sensitive high-resolution melting (MS-HRM)

MS-HRM was performed on a CFX Connect™ real-time PCR (Bio-Rad, Hercules, CA, USA) following protocols reported by Wojdacz et al. (35). HRM primers corresponding to methylation-neutral regions are listed in Supplementary Table S1 and were synthesized by Integrated DNA Technologies Inc., Coralville, IA, USA. Two micro liter of DSN treated samples or No-DSN control samples were added into 18 μl of PCR master mix containing 1× LightCycler® 480 High Resolution Melting Master Mix (Roche Diagnostics Corporation, Indianapolis, IN, USA), 250 nM of each primer and 3 mM of $MgSO_4$. For PCR amplification, an initial denaturation step was performed for 10 min at 95°C, followed by 45 cycles of 5 s denaturation at 95°C, 5 s annealing at customized annealing temperature and 5 s elongation
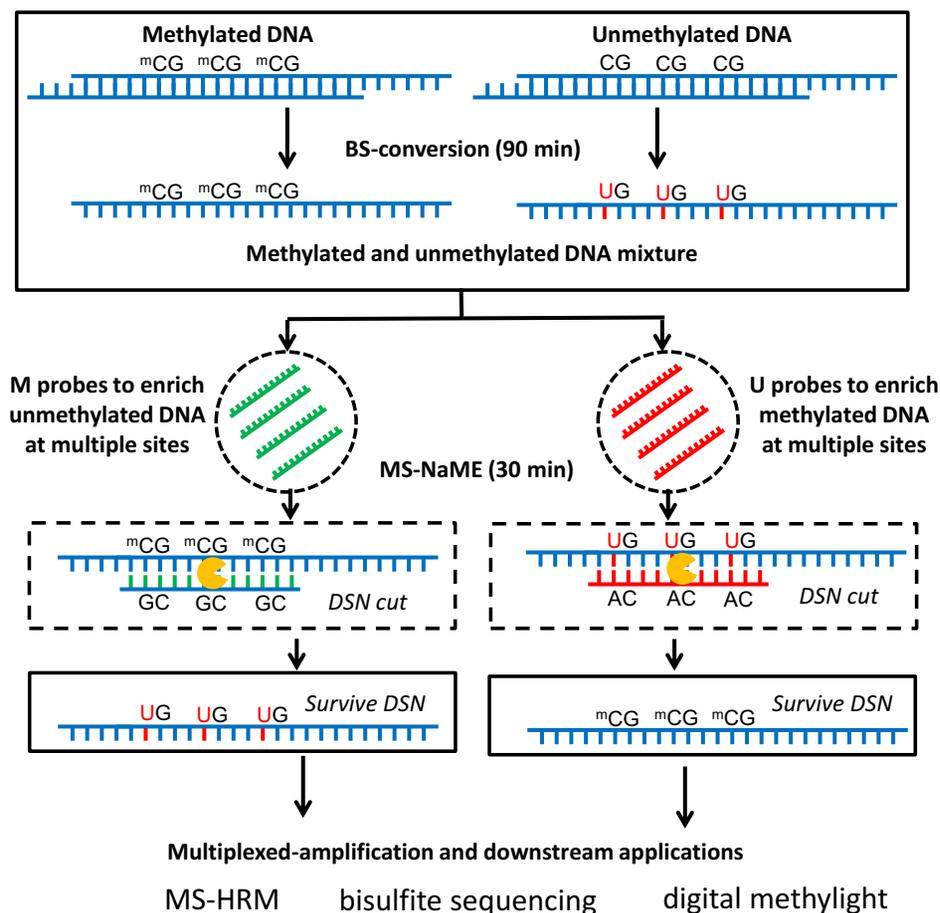
Figure 1. Schematic workflow of Methylation-Sensitive Nuclease-assisted Minor-allele Enrichment, MS-NaME.

at 72°C. The final step included 1 min at 95°C, 1 min at 70°C and a melting curve (0.2°C step increments, 5 s hold before each acquisition) from 65°C to 95°C. The primer annealing temperature was optimized for individual genes, such that when using 50:50 methylated and unmethylated DNA template mix it yields approximately equal melting peaks, indicating equal amounts of the methylated.

Methylation-sensitive COLD-PCR

For comparison of preferential amplification (enrichment) of un-methylated alleles using MS-NaME versus MS-COLD-PCR, we performed a fast-COLD-PCR protocol on 1% mixtures of un-methylated to methylated DNA. In contrast to two other forms of COLD-PCR, ice-COLD-PCR (36) or full-COLD-PCR (37), MS-COLD-PCR amplifies preferentially T_m-lowering alleles (23) hence un-methylated sequences that contain Tm-lowering G>T transversions are amplified preferentially. The primers and reagents described above for PCR–MS–HRM reactions were also adopted for MS-COLD-PCR. The critical denaturation temperature (T_c) and cycling applied during MS-COLD-PCR distinguishes between methylated and un-methylated alleles, as we described (23). Specifically, the PCR cycling applied for *ATM* and *GSTP1*, respectively, were: initial denaturation at 95°C for 10 min, then 5 cycles of conventional PCR (5 s denaturation at 95°C, 5 s annealing at customized annealing temperature and 5 s elongation at 72°C), followed by 50 cycles at T_c of 80.5°C (*ATM*) or 78.5°C (*GSTP1*) for 5 s, annealing at 59°C (*ATM*) or 57°C (*GSTP1*) for 5 s and extension at 72°C for 5 s. COLD-PCR was followed by melting curve analysis as described above.

Two-color digital Methylight

Real time PCR (38–40) and Taqman-based approaches have been adapted successfully for detection of methylation (17,18). We developed a two-color digital droplet Methylight reaction to evaluate the methylation/unmethylation ratio before and after MS-NaME assay. Primers are listed in Supplementary Table S1 and probes (synthesized by Integrated DNA Technologies Inc., Coralville, IA, USA) are listed in Supplementary Table S2. Amplifications were performed in a 20 μl volume containing 1× ddPCR Supermix for probes (Bio-Rad, Hercules, CA, USA), 900 nM forward and reverse primers, 250 nM FAM and HEX probes and MS-HRM products (1–1 000 000 final dilution). Droplets were then generated using the DG8™ droplet generator cartridges (Bio-Rad, Hercules, CA, USA) which contained 20 μl aqueous phase with 70 μl of droplet generation (DG) oil (Bio-Rad, Hercules, CA, USA). Prepared samples were transferred to a 96-well reaction plate and then sealed using

the PX1 PCR plate sealer (Bio-Rad, Hercules, CA, USA) for 10 s at 180°C prior to thermal cycling. The thermal cycling program was performed on an Eppendorf Mastercycler ep Gradient S (Eppendorf, Hamburg, Germany) with an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 30 s denaturation at 94°C, 60 s annealing at 58°C, and with a final step holding at 98°C for 10 min. Then the plate was transferred to QX100 droplet reader (Bio-Rad, Hercules, CA, USA) for endpoint reading. Calculation of absolute number of positive events for a given channel (FAM or HEX), the ratio and the fractional abundance of methylation for each sample were performed by the Quantasoft Software (Bio-Rad, Hercules, CA, USA). The determination of the number of target copies per droplet (number of copies of target molecule) was adjusted by the software to fit a Poisson distribution model with 95% confidence level.

Sanger sequencing of bisulfite converted products

PCR products of MS-NaME treated samples were digested by Exonuclease I and Shrimp Alkaline Phosphatase (New England Biolabs, Ipswich, MA, USA) and processed for Sanger sequencing (Eton Bioscience Inc., Boston, MA, USA). To enable sequencing of short PCR amplicons, a 30-T tail was added to the 5′-end of the forward primer (listed in Supplementary Table S3).

Whole genome amplification of bisulfite converted DNA

To examine application of MS-NaME on double-stranded DNA generated via whole genome amplification (WGA) of bisulfite converted DNA, 20 ng of bisulfite converted DNA was used. The single-stranded bisulfite converted DNA was converted to double-stranded DNA using a ds-DNA Conversion Kit based on random priming (Epigentek Group Inc., Farmingdale, NY, USA). After random primer cleanup with QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany), the bisulfite-converted ds-DNA was amplified using the NEBNext Ultra II DNA Library Prep kit for Illumina (New England Biolabs, Ipswich, MA, USA). Amplification was performed according to the manufacturer's instructions with the following modifications: (i) NEBNext Adaptor for Illumina was treated with USER enzyme (New England Biolabs) and then reannealed before adaptor ligation; (ii) KAPA HiFi HotStart Uracil+ ReadyMix PCR Kit (KAPA Biosystems, Boston, MA, USA) was used instead of NEBNext Ultra II Q5 Master Mix for PCR amplification. After 15 cycles of PCR and cleanup with QIAquick PCR Purification Kit, 0.5–1 µg of WGA products were typically recovered.

RESULTS

MS-NaME for a single target

In preliminary experiments we investigated the influence of the number of CpG sites addressed by each MS-NaME probe on the enrichment obtained for target sequences. Probes for the promoter of the Ataxia Telangiectasia Mutated gene (*ATM*) which is often hypermethylated in breast cancer (7) were employed. We designed oligonucleotide probes containing 1, 2 or 3 CpG sites targeting the bisulfite-converted methylated sequence (*ATM*-M-1 CpG, *ATM*-M-2 CpG and *ATM*-M-3 CpG) or alternatively the unmethylated sequence (*ATM*-U-1 CpG, *ATM*-U-2 CpG and *ATM*-U-3 CpG). Probe sequences are listed in Supplementary Table S4. As shown in Supplementary Figure S1, probes containing two CpG sites and three CpG sites have similar enrichment while probes containing one CpG site have lower enrichment compared to the other two. We repeated this experiment on another gene retinoic acid receptor β2 (*RARb2*), and similar results were obtained. Accordingly, for the remainder of this work which involves regions of high CpG density we retained probes addressing three CpG sites although additional designs fitting different situations can be also pursued. In terms of general design, as shown in our recent work with single point mutations (34) probes of 20–25 bp enable the nuclease to recognize the double stranded structure and retain selectivity for double stranded DNA vs single stranded DNA or mismatched DNA. Furthermore probes may optionally contain a polymerase block on their 3′ end to prevent polymerase extension in subsequent amplification reactions. When enough starting material is available a fraction of the nuclease reaction can be used for subsequent PCR and this dilutes the probes, hence 3′end blockage may not be unnecessary. MS-NaME probes in the present work were designed to have a melting temperature (T_m) of about 63°C and length of 20–25 bp.

To examine single target MS-NaME, probes matching the bisulfite-converted methylated sequence (*ATM-M-antisense probe*, sequence listed in Supplementary Table S5) or alternatively the unmethylated sequence (*ATM-U-antisense probe*, sequence listed in Supplementary Table S5) were used. Methylated and unmethylated bisulfite converted human control DNA were mixed to obtain 1%, 10% and 40% methylation. *ATM-U-antisense probe* was added to hybridize to unmethylated *ATM* sequences, and the fully complementary double-stranded DNA regions were digested by DSN. The MS-NaME product was PCR-amplified and tested via MS-HRM (Supplementary Figure S2A, left panel). The increased methylated peak height as compared to No-DSN control indicates the enrichment of methylated sequence via MS-NaME. Similarly, we prepared samples containing 1%, 10% and 40% unmethylated DNA. *ATM-M-antisense probe* was added to enable preferential digestion of methylated *ATM* sequences by DSN, as indicated by an increased unmethylated peak height (Supplementary Figure S2A, right panel). To repeat the test for a different target, another gene glutathione-S-transferase P1 (*GSTP1*) an epigenetic biomarker for prostate cancer was used (41). Similar to *ATM*, the *GSTP1* data indicated the preferential enrichment of methylated or unmethylated sequences via MS-NaME (Supplementary Figure S2B, left or right panels, respectively). Comparison to the enrichment obtained with another technique, methylation-sensitive co-amplification at lower denaturation temperature MS-COLD-PCR (23) followed by HRM (42) is presented in Supplementary Figure S2C. MS-COLD-PCR-HRM enriches very effectively un-methylated amplicons that have lower denaturation temperature, but cannot en-

rich methylated sequences that have higher denaturation temperature.

MS-NaME for multiple targets

For a first multiplexed application of MS-NaME we applied a four-plex assay targeting *ATM*, *GSTP1*, retinoic acid receptor β2 (*RARb2*), and DNA repair gene O^6-methylguanine-DNA methyltransferase (*MGMT*). Aberrant *RARb2* promoter methylation is a biomarker for lung cancer ([43](),[44]()) while *MGMT* is frequently methylated in colorectal and brain cancers ([45](),[46]()). 50:50 methylated and unmethylated DNA mix was prepared with methylated and unmethylated bisulfite converted human control DNA, and then treated with four-plex *M-antisense probes* or *U-antisense probes*, respectively (listed in Supplementary Table S5). As depicted in Figure [2]()A, the methylated and unmethylated melting peaks in the absence of MS-NaME (No DSN control samples) were almost equal in height for all four genes. After MS-NaME treatment, the unmethylated peaks increased when *M-antisense probes* were applied. Similarly, the methylated peaks increased when *U-antisense probes* were applied. The bisulfite Sanger sequencing results of those PCR products confirmed that the four-plex MS-NaME assay worked simultaneously for all four genes (Figure [2]()B).

Although MS-HRM and bisulfite Sanger sequencing are convenient, labor and cost-efficient, they lack in quantification and sensitivity. For this reason, we developed a two-color Methylight assay for *ATM* and *RARb2* to investigate multiplex MS-NaME at lower abundance levels using digital droplet PCR. In the digital Methylight assay, we retained the primers used in MS-HRM and designed hydrolysis probes (Supplementary Table S2) corresponding to methylated (5′-HEX labeled M-Taqman probe) and unmethylated (5′-FAM labeled U-Taqman probe) sequences. When these hydrolysis probes were used in a single tube, the methylation levels could be determined directly via ddPCR. Samples of 10%, 1%, and 0.1% methylation (or alternatively, unmethylation) were then treated by four-plex MS-NaME. Digital Methylight for both *ATM* and *RARb2* genes were tested and indicated similar enrichment of methylated, or unmethylated sequences respectively, Figure [3]()A and B. For example, for the 0.1% methylation or unmethylation samples the four-plex MS-NaME reaction led to a ∼200-fold enrichment to a final 20% methylation or unmethylation abundance, respectively. When the original methylation abundance of the samples increased, the resulting methylation abundance after MS-NaME enrichment also increased. When U probes were applied to 100% unmethylated DNA, or when M probes were applied to 100% methylated DNA, no methylation change was observed, indicating no false-negatives. Furthermore, 0.1% methylation and unmethylation samples were tested with bisulfite Sanger sequencing to confirm the digital Methylight results (Figure [3]()C and D). 0.1% is far below the sensitivity of Sanger sequencing. However, following MS-NaME, traces of minor methylated or unmethylated alleles could be clearly identified in the sequencing chromatographs.

Next, we expanded the number of target genes in our probe pool by adding an additional 173 targets correspond-ing to promoters that can be potentially methylated in cancer stem cells ([47]()). We thus designed MS-NaME probes corresponding to the unmethylated sequences in these regions, to obtain a MS-NaME assay for a total of 177 targets (Supplementary Table S6). The 177-plex MS-NaME was applied to serially diluted samples with a 10%, 1% and 0.1% methylation. Similar enrichment of the methylated alleles to the one obtained during four-plex MS-NaME was achieved for the four genes *ATM*, *RARb2*, *MGMT* and *GSTP1* for MS-HRM (Supplementary Figure S3) and digital Methylight (Supplementary Figure S4). These data indicate that the 173 additional probes did not affect the enrichment obtained via MS-NaME. We also examined whether methylation enrichment was obtained for additional, randomly chosen genes within the group of 177 DNA targets. For example, adenomatous polyposis coli (*APC*), cyclin D2 (*CCND2*), methyl-CpG binding domain 4 (*MBD4*) and myogenic differentiation 1 (*MYOD1*) genes were tested after 177-plex MS-NaME. Both MS-HRM and bisulfite Sanger sequencing results indicated that all targets examined displayed enrichment of methylated alleles (Supplementary Figure S5 and S6).

In summary, we used MS-HRM, bisulfite Sanger sequencing and digital Methylight to demonstrate that MS-NaME could enrich minor methylated or unmethylated alleles on numerous targets simultaneously.

MS-NaME applied to double-stranded WGA product following bisulfite-conversion

Although bisulfite conversion is regarded as a gold standard method in DNA methylation analysis, this approach has limitations such as considerable DNA degradation and instability of the single-stranded bisulfite converted DNA under storage conditions ([48]()). This can be a major limitation when limited starting DNA is available. Whole-genome amplification of bisulfite-treated DNA (WGA) overcomes these limitations by amplifying DNA and converting it to double-stranded DNA ([49]()). Additionally, WGA is routinely applied for preparation of libraries for whole-genome bisulfite sequencing (WGBS) which enables genome-wide identification of cytosine DNA methylation states at single-base resolution ([50](),[51]()). Accordingly, it is of interest to adapt MS-NaME for application with double stranded DNA. To this end, we designed *sense probes* for the complementary bottom strand of the target genes, to be used together with the *antisense probes* described in previous sections (Supplementary Figure S7A). WGA was applied using an approach where ligation of adapters is performed after bisulfite conversion and random-primed second strand synthesis combined with amplification using KAPA HiFi (U+) as recommended by Ji *et al.* ([52]()). This approach in our experience leads to less 'bias' between methylated and unmethylated alleles as compared to ligation of bisulfite-resistant adaptors prior to bisulfite treatment (data not shown). We prepared WGA products from a 50:50 methylated: unmethylated DNA mix and then applied four-plex MS-NaME assay with (i) only *antisense probes*; (ii) only *sense probes*; (iii) both *antisense* and *sense probes*, then MS-HRM and *RARb2* digital Methylight was used to measure the methylation change. We designed *sense probes* for both methylated
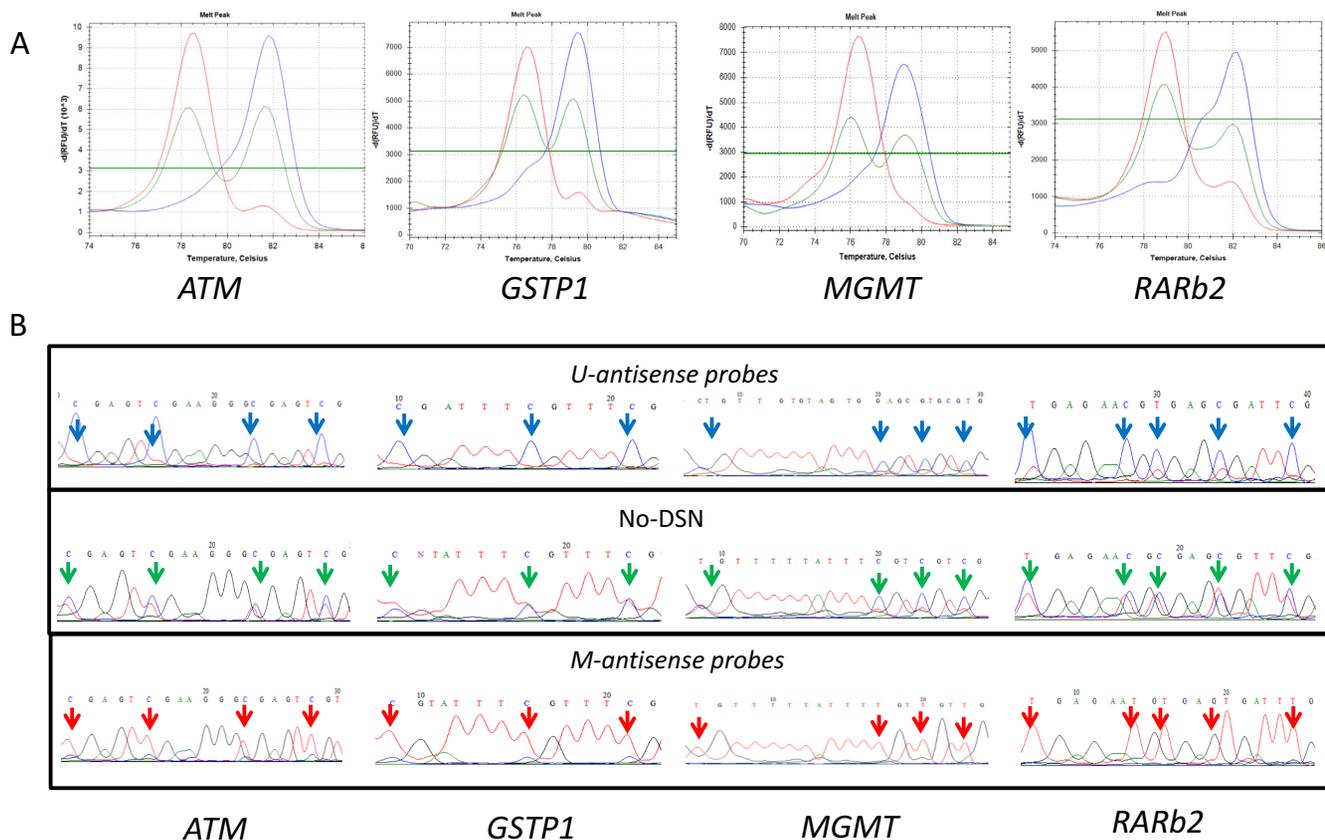
Figure 2. Application of four-plex MS-NaME (*ATM*, *RARb2*, *MGMT* and *GSTP1* gene promoters) on bisulfite-treated DNA to enrich methylated or unmethylated DNA. A 50:50 methylated: unmethylated DNA mix was used and results were assessed via MS-HRM (**A**) or sequencing (**B**). The MS-HRM curves are as follows: No-DSN control-green, *U-antisense probes*-blue, and *M-antisense probes*-red.

and unmethylated sequences of the four target genes (listed in Supplementary Table S5). The *antisense probe* and *sense probe* are designed to target different CpG sites within each gene. If there is an overlap between corresponding *sense* and *antisense probes*, this is less than 10 bp to avoid forming probe-probe dimers. Four-plex MS-NaME was conducted with either U probes or M probes. As shown in Supplementary Figure S7B, for WGA products of bisulfite converted DNA both the *antisense probes* and *sense probes* are needed to enable complete digestion of both strands in WGA products, and the absence of either probe results in reduced or no enrichment. The changes in MS-HRM curves with either U probes (Supplementary Figure S7C) or M probes (Supplementary Figure S7D) further confirmed this result.

In conclusion, MS-NaME can be applied to enrich methylated or unmethylated targets within WGA products of bisulfite-converted DNA by including both *sense* and *antisense probes* for each DNA target.

MS-NaME applied to clinical samples and to spike-in controls in plasma circulating DNA

To apply MS-NaME on clinical specimens, we applied four-plex MS-NaME to bisulfite converted DNA exacted from eight clinical lung tumors (TLs). For six of these samples, there were available matched normal lung tissue (NL) samples which were screened in parallel. The *U-antisense probes*

were used for MS-NaME, and the products were tested via PCR-MS-HRM, digital Methylight and PCR—bisulfite Sanger sequencing. No aberrant methylation was found before and after MS-NaME treatment for *ATM*, *GSTP1* and *MGMT* (data not shown). In contrast, we found low level methylation, ranging from 1% to 10%, for *RARb2* for 8/8 lung tumor samples according to digital Methylight (Figure 4A). *RARb2* was previously identified as a candidate biomarker for lung cancer (43). After applying to these tumor samples four-plex MS-NaME, the methylation levels were enriched to 28–53% which enabled detection of methylation by the less sensitive MS-HRM and bisulfite Sanger sequencing. The digital Methylight result was further supported by bisulfite Sanger sequencing (Supplementary Figure S8). The methylation signals in the 6 normal lung samples were equal to noise levels ('limit of blank') both before and after MS-NaME (Supplementary Table S7), in agreement also to bisulfite Sanger sequencing (Supplementary Figure S9). The data, MS-HRM, digital Methylight and Sanger sequencing, are summarized in Table 1. The results of all three methods, MS-HRM, bisulfite Sanger sequencing and digital Methylight came to a satisfactory agreement following MS-NaME. The data indicate that MS-NaME can enhance the sensitivity of MS-HRM or bisulfite Sanger sequencing which are used routinely for methylation analysis such that their accuracy approaches or equals that of
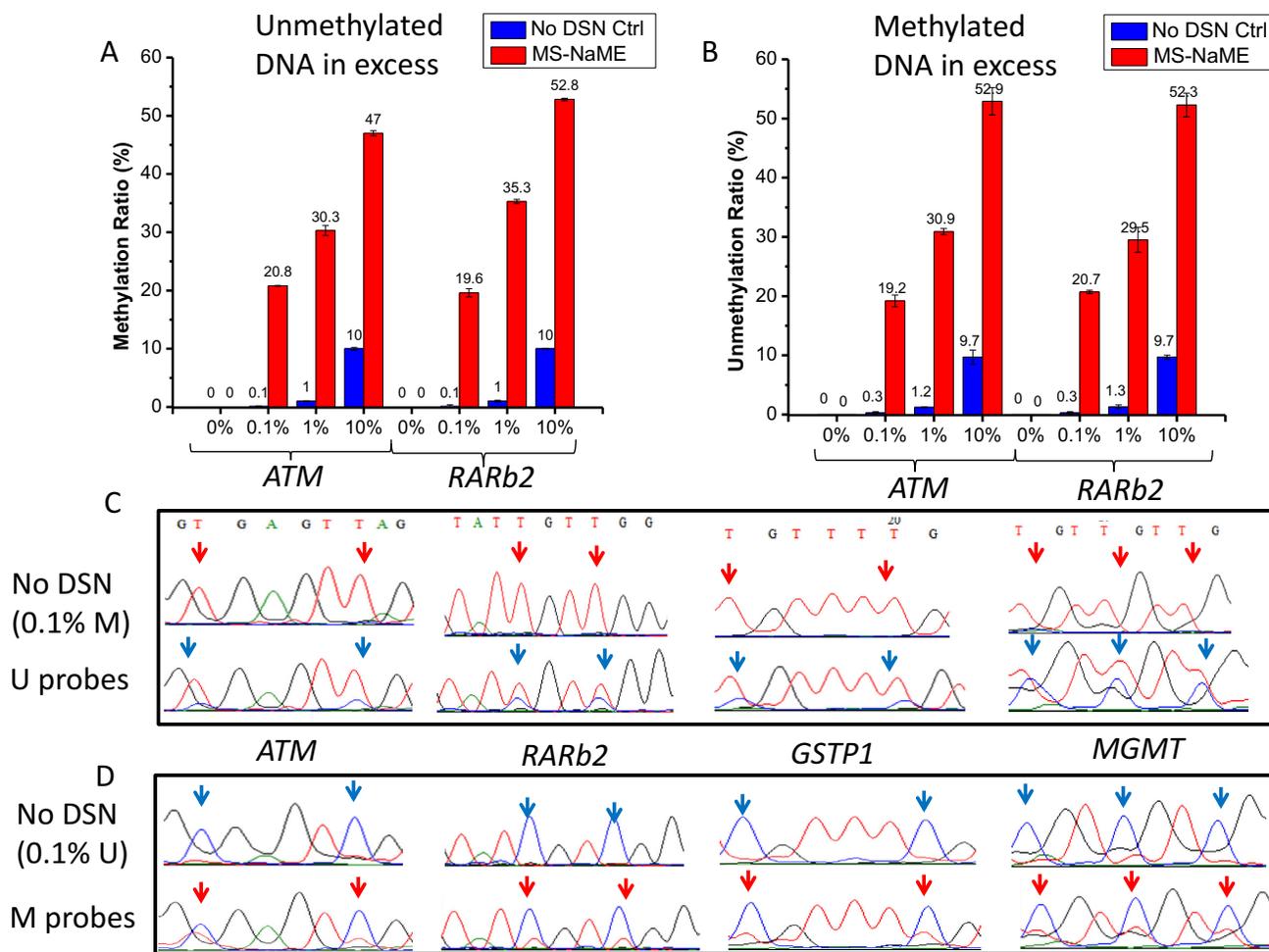
Figure 3. Application of four-plex MS-NaME (*ATM*, *RARb2*, *MGMT* and *GSTP1* gene promoters) on bisulfite-treated DNA to enrich serially diluted levels of methylated or unmethylated DNA. 0.1–10% methylated DNA or 0.1–10% unmethylated DNA were treated with U probes and M probes, respectively. (**A** and **B**) *ATM* and *RARb2* were assessed via digital Methylight to determine changes in methylation and unmethylation levels as compared to no treatment. Error bars represent the standard error of the mean from three independent experiments. (**C** and **D**): Bisulfite Sanger sequencing for the four genes was conducted with 0.1% methylated DNA or 0.1% unmethylated DNA following MS-NaME treatment with U probes and M probes, respectively.

the highly sensitive digital Methylight while also providing sequence information (Sanger) or convenience (MS-HRM). Finally, the 177-plex MS-NaME was also applied to the same lung tumor and matched normal lung samples screened by four-plex MS-NaME. The enrichment and relationship between original and enriched samples remained essentially unchanged, indicating that including additional probes for multiplexing higher numbers of target genes does not affect the performance of the MS-NaME enrichment (Figure 4B) and that MS-NaME can be applied to enrich for complete epigenetic signatures such as those of stem cells, from a mixed sample.

The application of MS-NaME to circulating DNA was performed using plasma collected from heathy individuals. All these sample were unmethylated in the *ATM* gene promoter. To simulate low levels of methylation, we performed 'spike-in' experiments using reference methylated DNA at 10% or 1% levels as compared to plasma-circulating DNA. Then 177-plex MS-NaME was applied to enrich the 'spiked-in' methylated DNA. MS-HRM (Figure 4C) and

ATM digital Methylight (Figure 4D) were employed to detect the methylation ratio with and without application of MS-NaME. MS-HRM on it own could detect 10% spike-in methylated DNA but failed to detect 1% spike-in methylated DNA. MS-NaME followed by MS-HRM enabled detection of 1% methylation (Figure 4C). Although *ATM* digital Methylight was sensitive enough to detect both 10% and 1% spike-in, the presence of 1% methylation became more evident using MS-NaME (Figure 4D). Thus, MS-NaME enriched 1% methylation to ∼30% methylation.

DISCUSSION

Traces of epigenetically altered DNA in clinical samples provide vital clues regarding disease states and preferred treatments and detecting these minor alleles is important in several fields of biology, biotechnology and medicine including cancer, prenatal diagnosis, infectious diseases, organ transplantation and forensics (12,15,47,53–55). Here, we introduced and experimentally validated MS-NaME, a highly parallel approach for enriching minor differen-
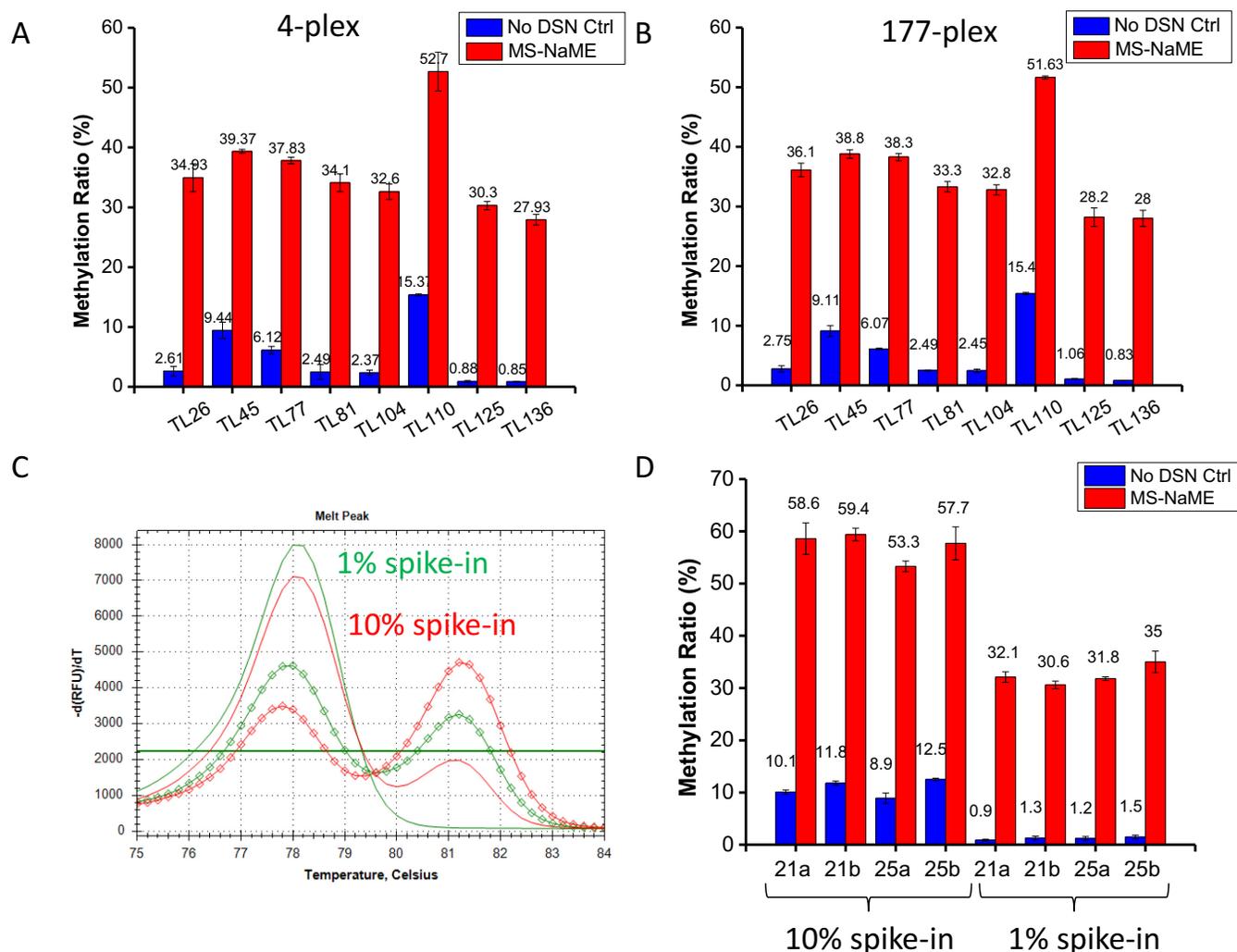
Figure 4. Application of MS-NaME in clinical tumor and plasma samples. Lung tumor samples were treated with (**A**) four-plex and (**B**) 177-plex MS-NaME treatment and measured with *RARb2* digital Methylight to quantify the methylation ratio change. Plasma circulating-DNA samples from two healthy donors (#21 and #25) which were unmethylated in *ATM* promoter were spiked with 1% or 10% methylated DNA (final ratio), and then treated by 177-plex MS-NaME. Error bars represent the standard error of the mean from two independent experiments. (**C**) *ATM* MS-HRM was applied to sample 21a. The melting curves are as follows: 1% spike-in-No DSN-green, 1% spike-in-MS-NaME-green with square symbol, 10% spike-in-No DSN-red, 10% spike-in-MS-NaME-red with square symbol. (**D**) *ATM* digital Methylight was applied to plasma DNA samples 21a, 21b, 25a and 25b. a and b denote samples taken from the same healthy donor but spiked with different amounts of methylated DNA. Error bars represent the standard error of the mean from two independent experiments.

tially methylated allele populations within an excess of alleles of opposite methylation status. As such, MS-NaME has the potential to be combined with massively parallel or targeted bisulfite sequencing to enable detection of low-levels of aberrant methylation with potentially broad genomic coverage without the need for deep sequencing, e.g. following a ~200-fold MS-NaME enrichment of a methylated allele with 0.1% original abundance to 20% abundance as in Figure 3, low-pass sequencing would be sufficient. Indeed, the main requirement for highly parallel application of MS-NaME is the hybridization of appropriately designed probes to the bisulfite-converted targets in the starting DNA (Figure 1). This step is already established as the initial step in 'bait-based' capture approaches for thousands of targets (32). Accordingly, it is possible that MS-NaME can be used for simultaneous enrichment of minor methyla-

tion levels within large genomic fractions involving numerous targets. Optionally, in cases of limited starting material such as when cfDNA is used, application of WGA prior to MS-NaME (Supplementary Figure S7) can be employed.

MS-NaME was able to enrich minor epigenetic allele populations such that 0.1% abundance can be detected relatively easily (Figure 3). If adequate amounts of starting material is available and Ms-NaME is combined with digital methylight, and assuming a complete bisulfite conversion rate, even lower levels might be detectable. Detection of rare differentially alleles down to 0.1% can also be achieved by single-target PCR techniques (19,23). However, the clinical advantages of screening multiple biomarkers in samples that contain limited amounts of DNA as opposed to a single biomarker are well-established. For example, Chiu *et al.* (56) showed that analyzing numerous loci for aneuploidy

Table 1. Application of MS-NaME to screen for *RARb2* methylation in tumor and matched normal lung samples

Sample	MS-HRM		Sanger sequencing		Digital Methylight	
	No DSN	MS-NaME	No DSN	MS-NaME	No DSN	MS-NaME
TL26	−	+	−	+	+	+
TL45	−	+	+	+	+	+
TL77	−	+	+	+	+	+
TL81	−	-	−	+	+	+
TL104	−	−	−	+	+	+
TL110	+	+	+	+	+	+
TL125	−	+	−	+	+	+
TL136	−	−	−	+	+	+
NL77	−	−	−	−	−	−
NL81	−	−	−	−	−	−
NL104	−	−	−	−	−	−
NL110	−	−	−	−	−	−
NL125	−	−	−	−	−	−
NL136	−	−	−	−	−	−

'+' stands for 'methylation-positive' and '−' stands for 'methylation-negative'. Three different methods, MS-HRM, bisulfite Sanger sequencing and digital methylight, with and without MS-NaME, were compared in the ability to detect low level *RARb2* methylation.

in cfDNA provides a higher degree of confidence in prenatal diagnosis than single target assessment via quantitative PCR. Similarly Zhang *et al.* (57) demonstrated that a panel including multiple epigenetic biomarkers improves the sensitivity and specificity compared to any single biomarker in NSCLC. While hybridization and bait-capture-based approaches provide highly parallel target selection, these require separation steps. Accordingly, advantages of MS-NaME are its simplicity and convenience as it entails a brief incubation in solution and no separation steps while also providing major enrichment of desired methylation states. Potentially, desired combination of U probes and M probes can be applied simultaneously to eliminate, for example, excess unmethylated promoters of tumor suppressor genes or methylated promoters of oncogenes, and help identify low-levels of tumor-initiated epigenetic changes. A potential limitation of MS-NaME, as well as certain capture-based methods, is that the current probe design assumes complete methylation or unmethylation of the targeted sequences, i.e. all three CpGs targeted by the probes are ^{5m}C or C, respectively. Thus in relative rare partial methylation cases where only one to two out of three targeted CpGs are methylated the current probes would not result to satisfactory enrichment since single base mismatches abolish DSN action (33,34). This limitation can be overcome by designing and including probes containing additional combinations of methylated/unmethylated CpGs, so that there are probes providing a full-match to partial or fully methylated high abundance templates in all cases. Indeed, Supplementary Figure S1 and recent work (34) demonstrate enrichment via use of probes covering one or two CpGs, or single base changes. Enriching for minor CpG variations using MS-NaME would require increasing the number of probes used for a given number of DNA targets. Notably, the ratio of the underlying configurations would be only known in a semiquantitative manner since, in its current format, MS-NaME cannot provide strictly quantitative information of the original methylation changes.

Since MS-NaME enrichment is applied at the genomic DNA level, right after bisulfite conversion and prior to other treatments, it is applicable with most downstream sample preparation amplification or endpoint detection methods, with almost no change in the existing workflows. As the comparison of techniques demonstrates, increasing the detection sensitivity, while retaining specificity, can reduce false negatives (Table 1). Accordingly, MS-NaME enables established but less sensitive techniques to be applied more effectively. Further envisioned applications for MS-NaME would be enrichment for relatively rare hydroxy-methylated sequences, by incorporating TET-treatment (58,59) in the protocols.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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