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Multiplexed Elimination of Wild-Type DNA and High-Resolution Melting Prior to Targeted Resequencing of Liquid Biopsies

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BACKGROUND: The use of clinical samples and circulating cell-free DNA (cfDNA) collected from liquid biopsies for diagnostic and prognostic applications in cancer is burgeoning, and improved methods that reduce the influence of excess wild-type (WT) portion of the sample are desirable. Here we present enrichment of mutation-containing sequences using enzymatic degradation of WT DNA. Mutation enrichment is combined with high-resolution melting (HRM) performed in multiplexed closed-tube reactions as a rapid, cost-effective screening tool before targeted resequencing.

METHODS: We developed a homogeneous, closed-tube approach to use a double-stranded DNA-specific nuclease for degradation of WT DNA at multiple targets simultaneously. The No Denaturation Nuclease-assisted Minor Allele Enrichment with Probe Overlap (ND-NaME-PrO) uses WT oligonucleotides overlapping both strands on putative DNA targets. Under conditions of partial denaturation (DNA breathing), the oligonucleotide probes enhance double-stranded DNA-specific nuclease digestion at the selected targets, with high preference toward WT over mutant DNA. To validate ND-NaME-PrO, we used multiplexed HRM, digital PCR, and MiSeq targeted resequencing of mutated genomic DNA and cfDNA.

RESULTS: Serial dilution of KRAS mutation-containing DNA shows mutation enrichment by 10- to 120-fold and detection of allelic fractions down to 0.01%. Multiplexed ND-NaME-PrO combined with multiplexed PCR-HRM showed mutation scanning of 10–20 DNA amplicons simultaneously. ND-NaME-PrO applied on cfDNA from clinical samples enables mutation enrichment and HRM scanning over 10 DNA targets. cfDNA mutations were enriched up to approximately 100-fold (average approximately 25-fold) and identified via targeted resequencing.

CONCLUSIONS: Closed-tube homogeneous ND-NaME-PrO combined with multiplexed HRM is a convenient approach to efficiently enrich for mutations on multiple DNA targets and to enable prescreening before targeted resequencing.

There is mounting evidence that tumor mutations identified in cell-free DNA (cfDNA)[4] can potentially act as a powerful liquid biopsy-based diagnostic tool *(1–5)*. Clinical studies indicate the use of cfDNA to complement *(6)* or replace *(2)* tissue biopsies. Rare mutations identified in cfDNA via digital droplet PCR or massively parallel sequencing can lead to changes in clinical practice *(7)*. Despite its promise, technical hurdles persist. The limited amount of cfDNA obtained from a standard blood draw and the excess amount of circulating wild-type (WT) DNA are persistent issues that often compromise the diagnostic results. Several approaches have been described to reduce excess WT DNA *(8)*, thereby facilitating detection of mutated DNA, via the use of PCR *(9–19)*. Use of PCR-based mutation enrichment on multiple DNA targets simultaneously has also been reported *(20, 21)*. However, the approach is technically demanding and requires extensive optimization, which becomes more difficult as the number of multiplexed targets increases *(22)*. Enzymatic approaches using endonucleases to degrade WT DNA, on the other hand, can be highly parallel, but these are restricted to sequences

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[4] Nonstandard abbreviations: cfDNA, cell-free DNA; WT, wild type; NaME-PrO, Nuclease-assisted Minor Allele Enrichment using Overlapping Probes; DSN, duplex-specific nuclease; dsDNA, double-stranded DNA; ND-NaME-PrO, No Denaturation Nuclease-assisted Minor Allele Enrichment with Probe Overlap; HRM, high-resolution melting.

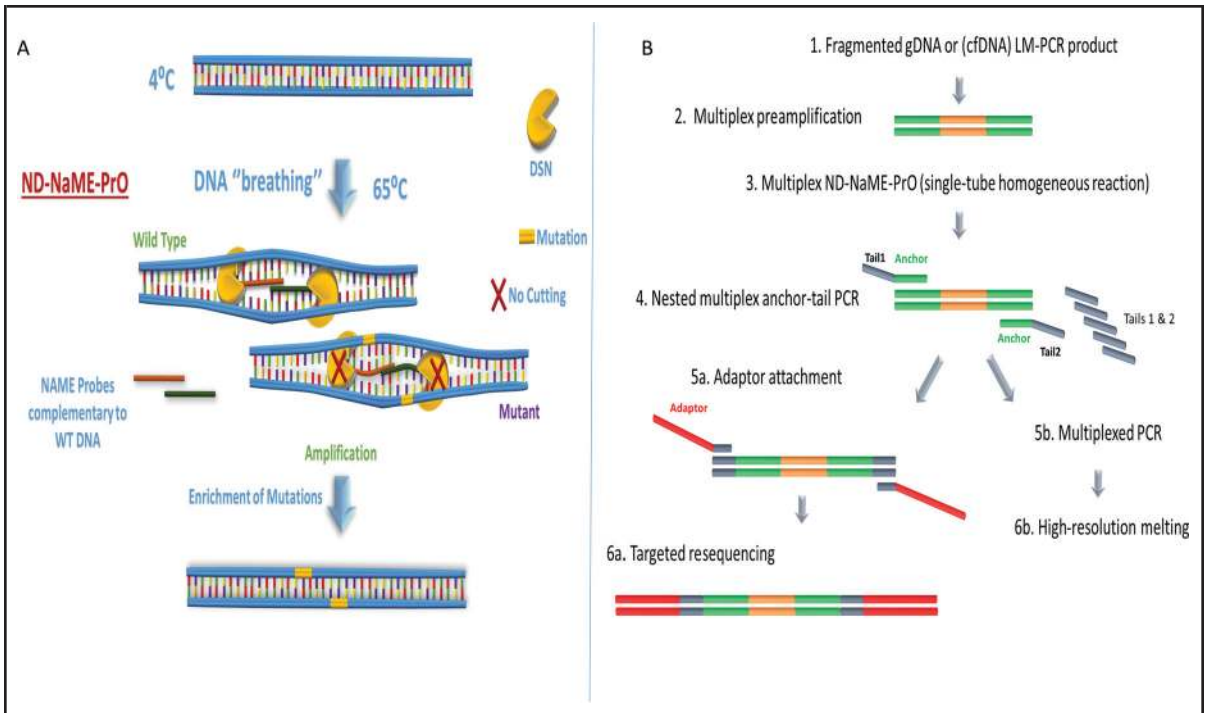

Fig. 1. Concept and workflow for ND-NaME-PrO.

ND-NaME-PrO and the spontaneous partial denaturation of dsDNA (DNA "breathing") at increased temperatures remaining below DNA melting temperatures, allowing overlapping probes to bind to complementary target DNA strands (A). DSN is then digesting fully matched templates, whereas mismatched sequences remain substantially undigested, thereby resulting in mutation enrichment upon subsequent amplification. It should be noted that dsDNA not targeted by probes also becomes digested but at a much lower rate than targeted DNA (vide infra).Sample preparation workflow for multiplexed ND-NaME-PrO followed by targeted resequencing (B). The workflow includes an initial multiplex amplification from genomic DNA or cfDNA followed by nested multiplex anchor-tail PCR for a target panel. The anchor-tail PCR product is then enriched for mutations at multiple positions by multiplexed ND-NaME-PrO, followed by multiplexed HRM scanning and MiSeq sequencing of HRM-positive samples.

recognized by the enzyme used *(23–27)*. Accordingly, mutation enrichment before highly parallel processes like targeted resequencing remains difficult to implement.

To circumvent these hurdles, we recently developed Nuclease-assisted Minor Allele Enrichment using Overlapping Probes (NaME-PrO) *(28)*, an enzymatic approach to remove WT DNA from multiple DNA targets selected at will, before DNA amplification, after which current genomic analysis processes remain substantially unchanged. After DNA denaturation, the temperature is reduced to allow addition of a thermostable double-stranded DNA Duplex specific nuclease (DSN) and mutation-overlapping oligonucleotide probes that guide nuclease digestion to the selected WT DNA sequences *(28)*. NaME-PrO can be applied to numerous DNA targets in parallel and provides mutation enrichment up to several hundred-fold depending on conditions applied, resulting in mutation detection of allelic frequencies of ≤0.01%. Despite these advantages, the requirement of adding DSN enzyme after an initial denaturation step prevents application in a homogeneous format that can be performed on multiple clinical samples in parallel using commonly available laboratory equipment, e.g., a 96-sample PCR machine. Indeed, manual tube opening and addition of reagents in the pre-PCR setting introduce a major risk for cross-contamination, a critical concern for the reliable detection of rare mutations.

Here we introduce a modified approach by which NaME-PrO is applied in a homogeneous closed-tube format using partial denaturation at 65 °C and without a denaturation step at 98 °C (No Denaturation NaME-PrO [ND-NaME-PrO]). The modified technique (Fig. 1A) uses the strong preference of a thermostable duplex-specific nuclease (DSN) toward digestion of dsDNA over single-stranded DNA *(29)*. Local DNA mismatches considerably reduce DSN digestion *(30)*, thereby enabling oligonucleotides matching WT DNA on the top and bottom strands to direct DSN digestion and discriminate

between mutation and WT sequences *(28)*. ND-NaME-PrO uses a natural property of the dsDNA to spontaneously locally and partially denature below DNA melting temperatures Tm (DNA "breathing") *(31)*. Our data indicate that, under appropriate conditions, DNA breathing allows the overlapping oligonucleotide probes to transiently invade the double-stranded structure and bind the partially denatured DNA strands (Fig. 1A), thereby guiding and enhancing DSN digestion of WT sequences despite absence of complete denaturation of the parent strands. ND-NaME-PrO provides a key practical advantage over the originally described NaME-PrO, as it can be performed using a common 96-well thermocycler at high throughput and with reduced risk for contamination.

We further show the use of single-tube, multiplexed nested PCR followed by high-resolution melting (HRM) scanning *(32, 33)* of 10–20 DNA targets combined with mutation enrichment. This process can be adopted as a rapid prescreening tool to identify the presence or absence of mutations on multiple targets simultaneously, before conducting demanding targeted resequencing. By combining ND-NaME-PrO with multiplexed PCR-HRM, we amplify mutation-enriched targets and interrogate them for mutations before library formation for targeted resequencing. This novel process is applied in mutations containing genomic DNA and circulating DNA from clinical cancer samples.

Materials and Methods

CELL LINES AND CLINICAL SAMPLES

Human genomic DNA was extracted from the SW480 (ATCC CCL-228™, KRAS mutation p.G12V, c.35G>T) commercial cell line. DNA extraction was performed with the DNeasy™ Blood and Tissue kit (Qiagen) following the manufacturer's protocol. A standard reference DNA (Horizon Discovery HD728) was used to provide genomic DNA containing multiple mutations. Human genomic DNA (Promega) was used as a WT control DNA. Serial dilution DNA mixtures of WT and mutant DNA were prepared to obtain 5%, 1%, 0.3%, 0.1%, 0.03%, and 0.01% mutation abundance. cfDNA samples obtained under Institutional Review Board approval from patients with breast cancer were provided by the Broad Institute. Tumor biopsies and matched blood samples were collected from patients with metastatic breast cancer who consented to Dana-Farber Cancer Institute Institutional Review Board protocol 05–246.

SHEARING OF GENOMIC DNA

Genomic DNA shearing was performed with the use of dsDNA Shearase Plus (Zymo Research) in a total 10-μL reaction (1× dsDNA Shearase Plus Reaction Buffer, 100 ng of genomic DNA and 1 U of dsDNA Shearase Plus enzyme). Genomic DNA was quantified with Qubit 3.0 fluorometer (Life Technologies).

PREAMPLIFICATION PCR

PCR reactions targeting KRAS exon 2 were prepared in a final volume of 25 μL [10 ng of genomic DNA, 1× GoTaq buffer (Promega), 400 nmol/L of each primer, 200 μmol/L of each of the 4 deoxynucleotide triphosphates (BioLine), 1.25 U of GoTaq Polymerase (Promega), and 10× LC Green]. The primer sequences (KRAS-2-F1 and KRAS-2-R1) are depicted in Table 1 in the Data Supplement that accompanies the online version of this (article, letter) at http://www.clinchem.org/content/vol63/issue10. The reaction was performed on a SmartCycler real-time PCR system (Cepheid). PCR protocol included an initial denaturation step at 98 °C for 2 min followed by 40 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 20 s, and elongation at 72 °C for 10 s.

ND-NaME-PrO

The single-plex ND-NaME-PrO was performed on the preamplified PCR products for KRAS exon 2. The 10-μL reaction contained 1 μL of 1000-fold diluted KRAS PCR products, 0.75× DSN buffer, 0.375× GoTaq buffer, and 200 nm of each overlapping probes (KRAS-sense-1 and KRAS-antisense-2 in Table 2 in the online Data Supplement). The reaction setup was prepared on ice and mixed well. Then 1 μL of 1 U/μL of DSN enzyme (Evrogen) was added in each reaction tube followed by brief vortex-mix and centrifugation. Also, a No-DSN control sample (No-DSN enzyme) was included and assessed in parallel with the rest of the samples. Next, the PCR tubes were put onto a preheated thermocycler at 65 °C. The incubation program was 65 °C for 20 min, followed by an enzyme deactivation step at 95 °C for 2 min. ND-NaME-PrO was followed by a nested PCR using KRAS-2-F2 and KRAS-2-R2 primers (sequences in Table 1 in the online Data Supplement), following the same condition as the preamplification PCR described above (1 μL of ND-NaME-PrO or No-DSN control product was used as DNA input for this reaction). In selected experiments, the activity of DSN enzyme on dsDNA was quantified by performing real-time PCR before and after digestion, using nested KRAS primers *(34)*.

DROPLET DIGITAL PCR FOR MUTATION ABUNDANCE VALIDATION

Droplet digital PCR reaction was prepared as previously described *(28)* to quantify the mutation abundance of DNA samples before and after ND-NaME-PrO mutation enrichment. Primer and probe sequences are depicted in Table 1 in the online Data Supplement. PCR was performed using an Eppendorf Mastercycler EP gra-

dient thermocycler (Eppendorf), and the reaction plate was transferred to a QX100 droplet reader (Bio-Rad) for endpoint reading. Quantification analysis and calculation of the ratios of positive events for a given channel (FAM or HEX) were performed with QuantaSoft™ software (Bio-Rad) to calculate the mutation abundance.

TARGETED RESEQUENCING (MiSeq) SAMPLE PREPARATION USING NESTED MULTIPLEXED ANCHOR-TAIL PCR REACTIONS

In total, 30 ng of fragmented genomic DNA samples with serially diluted mutated DNA or cfDNA were initially amplified for 10 cycles by ligation-mediated PCR using common linkers (adaptor and primer sequences shown in Table 3 in the online Data Supplement) as per instructions of the NEBNext® Ultra™ II DNA Library Prep kit (New England Biolabs). Then 30 ng of ligation-mediated PCR product was transferred into a multiplex preamplification reaction using Ion AmpliSeq™ Cancer Hotspot Panel v2 (Thermo Fisher Scientific) or customized targets panel (primer sequences in Table 3 in the online Data Supplement). Multiplex preamplification products were purified with Agencourt® AMPure XP (Beckman Coulter), and 1.5 ng of product input was included in 10-μL ND-NaME-PrO reactions with 200 nmol/L for each probe in the multiplex probe pool and 1 U of DSN. The incubation was at 65 °C for 20 min followed by 95 °C for 2 min. A no-treatment control sample with the same DNA input but without probes or DSN was run in parallel. Then 5 μL of the ND-NaME-Pro or no-treatment product was added into 25-μL multiplex "anchor-tail" PCR reactions to further amplify the intended target sequences (primer sequences and PCR protocols are in Tables 4 and 5 in the online Data Supplement). The anchor-tail PCR reactions include a low concentration (1–10 nmol/L) of primers that are nested to the first set of primers and contain a gene-specific portion and common oligonucleotide "tails." In addition, a high concentration (200 nmol/L) of the forward and reverse oligonucleotide tails is added to the same reaction. The anchor-tail PCR product was purified with Agencourt AMPure XP, and 5 μL of product was added into a final adaptor PCR for library preparation using NEBNext Ultra II Q5® Master Mix and NEBNext Multiplex Oligos for Illumina (New England Biolabs). The PCR protocol followed is depicted in Table 5 in the online Data Supplement. The libraries were then purified with Agencourt AMPure XP and delivered to the Center for Cancer Computational Biology at the Dana-Farber Cancer Institute to perform Illumina MiSeq sequencing. Libraries with ligated Illumina adapters were assessed for DNA quality and quantity using an Agilent bioanalyzer and then pooled together into a single tube before MiSeq sequencing. Data analysis was conducted using MiSeq Reporter software, and the alignment sequencing data were loaded into Integrative Genome Viewer 2.3 (Broad Institute) using human genome hg19 as reference.

WHOLE-EXOME SEQUENCING AND ANALYSIS

Whole-exome sequencing of tumor, germline, and cfDNA samples was performed using the Nextera Rapid Capture Exome Kit, with the exception that cfDNA libraries were first constructed using the KAPA Hyper Prep Kit (Kapa Biosystems). MuTect *(35)* was used to identify the somatic mutations in the whole-exome sequencing data.

MULTIPLEX HRM ANALYSIS

The multiplex anchor-tail PCR products were diluted 100-fold into H_2O, and 1 μL was transferred into 25 μL-multiplex-anchor-tail-PCR reactions by using the targets selected for each sample. Each sample was run in duplicate, and WT samples were run in quadruplicate. The PCR protocol is depicted in Table 5 in the online Data Supplement. Further, 10 μL of the 25-μL multiplex anchor-tail PCR products was then transferred to a 96-well plate, and 20 μL of mineral oil was added to each well. HRM was performed on a 96-well LightScanner® system (Idaho Technology). All experiments were independently replicated at least 3 times for assessing the reproducibility of results.

Results

SINGLE DNA TARGET ND-NaME-PrO

To examine ND-NaME-PrO efficiency in removing WT DNA and enriching mutations, a serial dilution experiment with decreasing KRAS exon 2 mutation abundance into WT DNA was assessed (1%, 0.3%, 0.1%, 0.03%, and 0.01%), shown in Fig. 2A. Preamplification of KRAS was first conducted on approximately 100-ng fragmented genomic DNA containing various KRAS mutation abundances, and then ND-NaME-PrO was conducted to diluted PCR products. The mutation abundance, before and after ND-NaME-PrO, was derived with the use of droplet digital PCR. Mutation enrichment ranging from 10- to >120-fold enrichment was obtained. Although it is understood that, at mutation abundances as low as 0.01%, Poisson statistics can lead to significant variability in the number of mutant copies, the data indicate a trend for more pronounced mutation enrichment for lower initial mutation abundance (Fig. 2B). The dependence of mutation enrichment on probe concentration is depicted in Fig. 1 in the online Data Supplement. Mutation enrichment increases with probe concentration with a maximum reached at about 200 nmol/L. In the absence of probes, dsDNA digestion proceeds, but there is no mutation enrichment (see Fig. 1 in the online Data Supplement). Mutation enrichment was then examined at a single mutation abundance of 0.3%

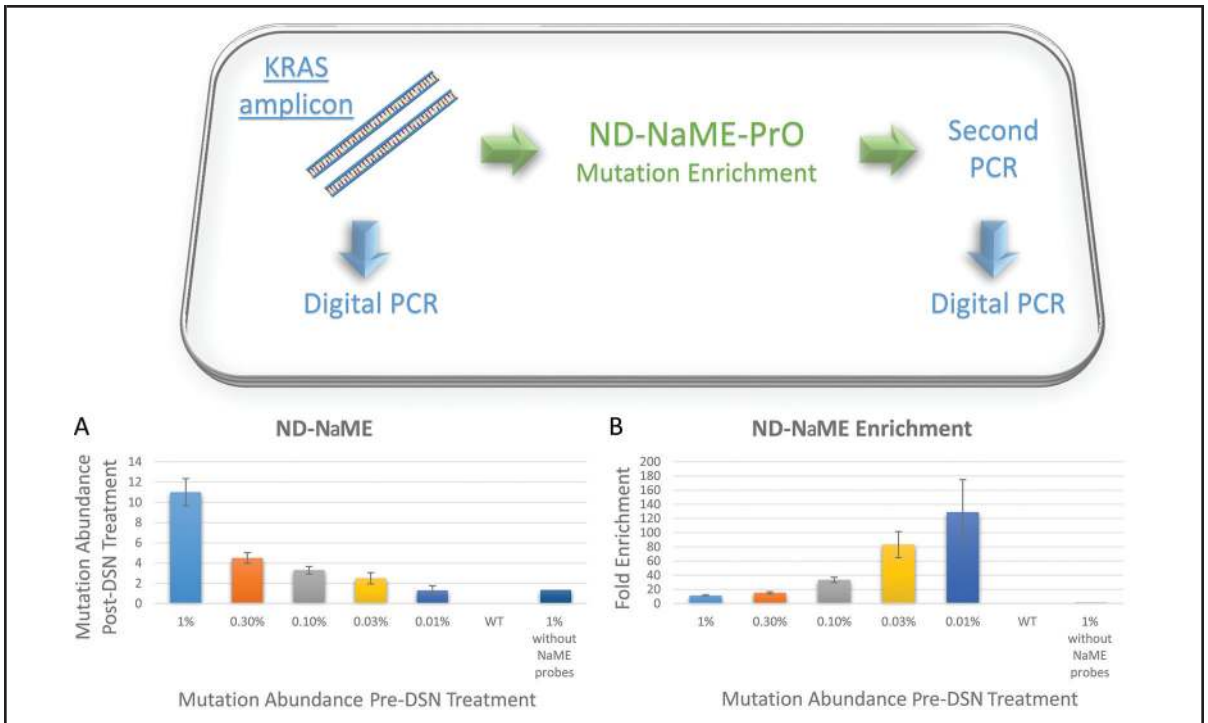

Fig. 2. Single-plex ND-NaME-PrO from PCR products.

Mutation abundance obtained after single-plex ND-NaME-PrO for KRAS (c.35G>T, p.G12V) on serially decreasing mutated KRAS-containing DNA diluted in WT KRAS-containing DNA (A). Mutation abundance was assessed via droplet digital PCR following ND-NaME-PrO. Mutation enrichment obtained for KRAS mutations after ND-NaME-PrO ranged from 10-fold to 120-fold for 1% and 0.01% mutation abundances, respectively (B).

for different DSN enzyme incubation temperatures during ND-NaME-PrO (see Fig. 2, A and B in the online Data Supplement). The mutation enrichment is pronounced for temperatures ranging from 60 °C to 70 °C, whereas there is little enrichment outside this range of temperatures. As shown in Fig. 2C in the online Data Supplement by using real-time PCR to examine threshold differences after DSN digestion, the lack of enrichment outside the 60–70 °C temperature range is not due to DSN inactivity. In addition, the DSN digestion efficiency in the presence or absence of NaME probes is shown in Fig. 2, D and E in the online Data Supplement. The data are consistent with the scheme proposed in Fig. 1, in which transient DNA denaturation ("breathing") allows probe binding to fully matched sequence positions and site-specific DNA digestion by DSN. As temperature decreases, DNA breathing is also expected to decrease along with site-specific probe binding, whereas at temperatures >70 °C, probe Tm is exceeded and DNA binding is reduced. It should be appreciated that because DNA remains double-stranded during ND-NaME-PrO, all DNA molecules are digested to some extent. However, the data indicate that at temperatures at which DNA is expected to undergo "breathing," WT DNA targets addressed by NaME-PrO probes become digested faster than mutated DNA or DNA not targeted by probes. Fig. 2D in the online Data Supplement indicates that, when probes are present, WT KRAS is almost completely eliminated, as assessed by the threshold of a real-time PCR reaction applied to the ND-NaME-PrO product (the threshold is equal to that of a no-template control reaction run in parallel). In contrast, when probes are omitted, DNA undergoes only modest DSN digestion as assessed by the PCR threshold compared with no-DSN control. Further, in the presence of mutated KRAS plus probes, the digestion is also modest, and the PCR reaches threshold earlier because there are mismatches at the probe-binding positions. Accordingly, the data are consistent with ND-NaME-PrO causing modest general degradation of the DNA sample, with significantly increased degradation at WT DNA sites addressed by probes (see Fig. 2E).

Finally, ND-NaME-PrO was also performed directly from sheared genomic DNA without previous amplification. Mutation enrichment was observed (see Fig. 3, A and B in the online Data Supplement); however, the

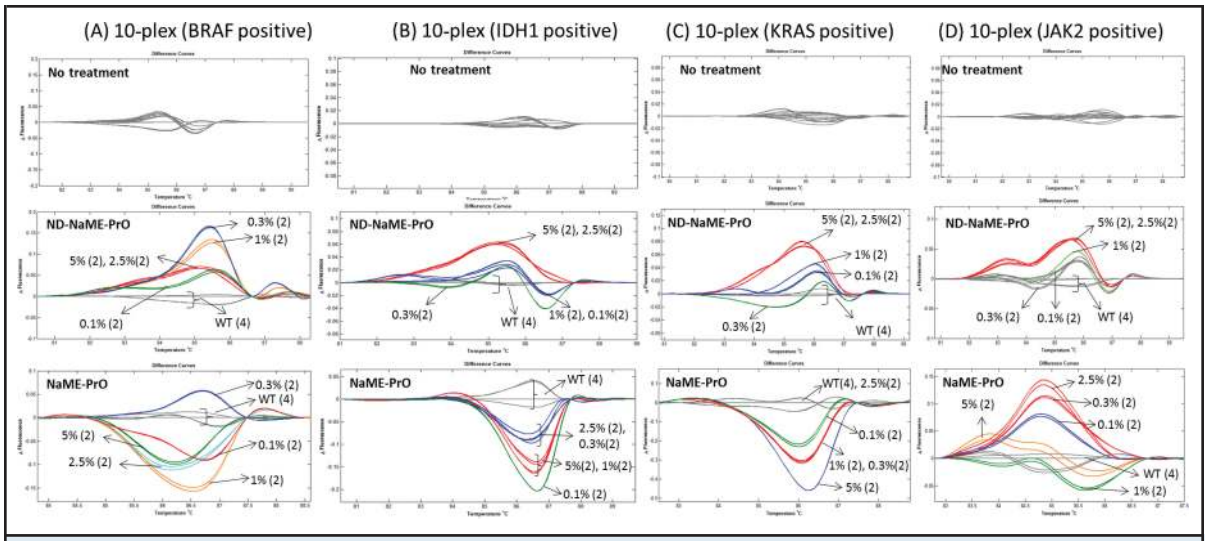

Fig. 3. Multiplexed ND-NaME-PrO followed by 10-plex PCR and multiplexed HRM scanning of amplicons containing a single mutated target at decreasing dilutions.

BRAF (A), *IDH1* (B), KRAS (C), and *JAK2* (D). In the top 4 panels, samples run in parallel while omitting DSN treatment. In the middle 4 panels, samples treated with ND-NaME-PrO (homogeneous, closed-tube process). In bottom 4 panels, samples treated with NaME-PrO (nonhomogenous process). All mutation samples were assessed in duplicate *(2)* and WT controls in quadruplicate *(4)*.

enrichment was lower than that obtained from a PCR product. In addition, the lowest mutation abundance detectable was about 0.3%. Further, 100 ng of genomic DNA was used as the starting material in this experiment, corresponding to 10 000–30 000 amplifiable genomic DNA copies. Because during ND-NaME-PrO a proportion of mutated DNA molecules is also digested, 1 potential explanation for the difference between the results obtained when starting from genomic DNA vs a PCR product is the limited number of mutated molecules in the former case. Accordingly, in all subsequent experiments, ND-NaME-PrO was performed following an initial PCR amplification step.

MULTIPLEXED ND-NaME-PrO FOLLOWED BY MULTIPLEX HRM

To perform multiplex ND-NaME-PrO followed by single-tube multiplexed HRM and MiSeq sequencing, we followed the sample preparation workflow depicted in Fig. 1B. Fragmented genomic DNA samples with serially decreasing mutation abundances (5%, 2.5%, 1%, 0.3%, and 0.1%) were first amplified by ligation-mediated PCR, targeted multiplexed PCR, and multiplexed anchor-tail PCR to obtain common tails at each amplicon matching the MiSeq adaptors used for targeted resequencing (Illumina). The anchor-tail PCR products were then diluted and processed by ND-NaME-PrO performed in a multiplexed single-tube reaction using probes targeting multiple DNA targets. The ND-NaME-PrO products were then added to adaptor PCR reactions including Illumina adaptors and sample indexes, to yield sequencing libraries. No-treatment control samples were also run in parallel throughout the process, by omitting ND-NaME-PrO. Before conducting MiSeq targeted resequencing, multiplexed single-tube HRM was performed for rapid prescreening before time- and expense-demanding sequencing.

To validate the use of multiplexed HRM for mutation scanning before conducting targeted resequencing, 10-plex PCR reactions in which just 1 of 10 DNA targets was mutated were first tested by HRM, using serially decreasing mutation dilutions. In addition, 4 different genes, namely, BRAF, IDH1, KRAS, and JAK2, were tested in this approach, in separate experiments (Fig. 3). Although it was not possible to discriminate the mutations from WT samples in the no-treatment samples, in the presence of ND-NaME-PrO, the presence of a mutated target was clearly evident, and the detection limit was 0.1%–1%, depending on the target gene. When ND-NaME-PrO was replaced with NaME-PrO, which includes a denaturation step before initiating DSN digestion *(28)*, similar results were observed, indicating the equivalence of the 2 approaches. In another test, a 20-plex HRM was conducted, in which 8 targets contained serially decreasing mutation abundances (5%, 2.5%, 1%, 0.3%, and 0.1%). HRM was not able to distinguish mutant DNA from WT DNA in the no-treatment samples (see Fig. 4 in the online Data Supplement). When ND-NaME-PrO was conducted, the mutant DNA melting

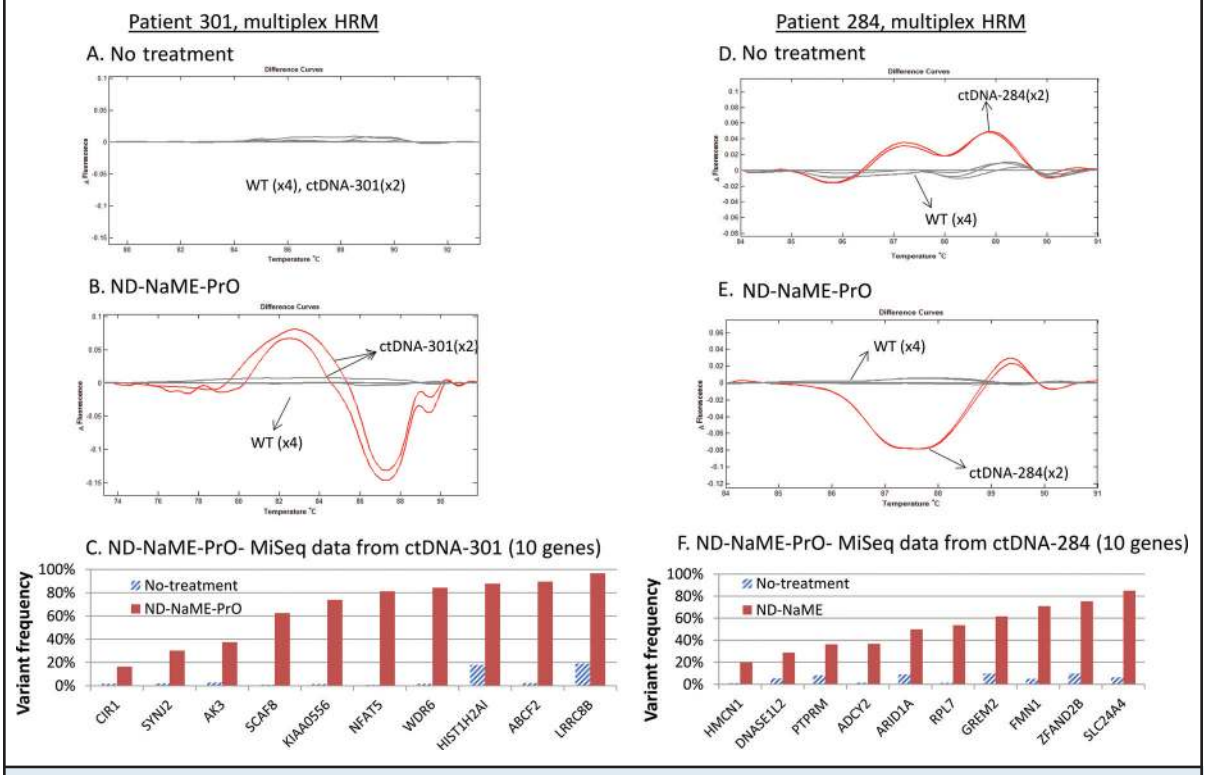

Fig. 4. ND-NaME-PrO HRM MiSeq performed in circulating tumor DNA (ctDNA) containing mutations in 10 different targets obtained from patients with metastatic breast cancer.

No-treatment samples without DSN were processed in parallel comparison (A and D). Multiplexed mutation scanning prescreening performed by multiplexed HRM (B and E). Variant frequency at the 10 mutated targets on cfDNA as derived with targeted resequencing (MiSeq) for no-treatment samples (hatched bars) and ND-NaME-treated samples (solid bars) (C and F).

profiles were clearly distinguished from WT DNA, presumably because of mutation enrichment. Mutation abundance down to 0.1% was detectable using multiplexed HRM mutation scanning in this case. Taken together, these data show the potential of using multiplexed HRM in combination with mutation enrichment on 10–20 targets, in which mutations in any 1 target can be identified before sequencing.

MULTIPLEXED ND-NaME-PrO HRM AND MiSeq SEQUENCING ON CIRCULATING DNA FROM PATIENTS WITH CANCER

To perform ND-NaME-PrO HRM and MiSeq sequencing on circulating DNA from plasma obtained from 2 patients with metastatic breast cancer (patients 301 and 284), we selected 2 samples in which exome sequencing had been performed on matched tumor biopsies *(36, 37)*. We selected 10 mutations that were confidently detected by whole-exome sequencing of the tumor biopsies. These were detected at random, without consideration of whether they were potential driver mutations or passenger mutations, to assess our ability to enrich any tumor mutation in a customizable manner. Primers and probes corresponding to these 10 mutated DNA target regions were designed for each sample (see Tables 1 and 4 in the online Data Supplement). The workflow depicted in Fig. 1B was then applied. In samples that contained multiple mutations, multiplexed HRM in the absence of ND-NaME-PrO could differentiate melting profiles from WT samples run in parallel in just 1 of 2 samples (Fig. 4, A and D). The differentiation of melting profiles in HRM is important, as it can distinguish WT from clinical samples. When mutation enrichment with ND-NaME-PrO was applied, melting profile differentiation from WT was evident in both samples (Fig. 4, B and E). With MiSeq sequencing, the presence of mutations was shown in all 10 targets for both samples. Although in the no-treatment samples the mutations were close to the noise limit (1%–2%), in the ND-NaME-PrO-treated samples, mutations were enriched in all 10 targets and were clearly evident (Fig. 4, C and F). The mutation enrichment generated by ND-NaME-PrO is variable (Fig. 4). Mutations having lower original mutation abundance are enriched the most (see Fig. 5 in the online Data Supplement; see also, Fig. 2), providing for sensitive de-

tection of low-level mutations. Overall, after multiplex ND-NaME-PrO, cfDNA mutations from metastatic breast cancer samples were enriched by up to 98-fold, with an average of 24-fold.

Discussion

We previously reported a homogeneous closed-tube approach to reduce or eliminate WT DNA from multiple targets of interest based on the preferential digestion of double-stranded, mismatch-free DNA by a double-strand specific nuclease, DSN. The originally described approach *(28)* involved DNA denaturation followed by cooling, open-tube addition of DSN enzyme, and contamination-prone sample manipulations. In addition, we have shown that if the DNA is single-stranded, such as in bisulfite-treated DNA, DNA denaturation is not required *(38)*. The present work reveals that this process also works with dsDNA in a closed-tube format that can be performed on a standard PCR machine. We observed that, under certain temperatures and probe and enzyme concentration conditions, partly denatured DNA enables WT DNA matching oligonucleotide probes to transiently bind their target and enable local DSN digestion. Consistent with previous reports *(28, 29)*, probes fully matching their DNA targets are digested by DSN at a substantially higher rate than mismatch-containing targets, thereby leading to preferential WT elimination. The thermal stability properties of the crab hepatopancreas-derived nuclease DSN *(29)* enable enzymatic activity at 65 °C, which matches the Tm of the oligonucleotide probes used in this work. Additional dsDNA-specific nucleases have been reported *(39)*, and these can also be potentially adapted to produce mutation enrichment in a similar manner by matching oligonucleotide probe Tm, denaturation conditions (buffer), and enzyme incubation temperature.

The ability to enrich multiple targets simultaneously for mutations led to a novel adaptation of HRM for multiplexed mutation scanning. Although HRM genotyping has been used for multiplexing up to 3 or 4 targets *(40)*, to our knowledge, multiplexing of multiple amplicons for HRM scanning has not been reported. Mutation scanning with HRM provides a straightforward, low-cost method for identifying DNA variations on single PCR amplicons *(32, 33)*, and it may be performed before Sanger sequencing to avoid screening of noninformative WT samples. Application of HRM scanning with next-generation sequencing platforms required the following 2 improvements: adaptation of HRM scanning to a multiplexed format after nested multiplexed PCR (Fig. 1B) and boosting HRM sensitivity by mutation enrichment to ensure low-level mutations present in clinical samples like cfDNA are not missed. Indeed, as for single PCR amplicons, the HRM scanning sensitivity is of the order of 5% *(33, 41)*, multiplexed HRM with 10 amplicons might not be sensitive enough to detect a mutation in only 1 of the amplicons, unless the mutation is present at a high level. Here, combining mutation enrichment by ND-NaME-PrO with nested multiplexed PCR and HRM resulted in sensitive HRM scanning for single mutations over 10 targets simultaneously (Fig. 3). This development opens up the possibility of filtering out uninformative samples and concentrating targeted resequencing efforts and resources to just the mutation-containing samples.

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