Highly multiplex PCR assays by coupling the 5′-flap endonuclease activity of *Taq* DNA polymerase and molecular beacon reporters

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Real-time PCR is the most utilized nucleic acid testing tool in clinical settings. However, the number of targets detectable per reaction are restricted by current modes. Here, we describe a single-step, multiplex approach capable of detecting dozens of targets per reaction in a real-time PCR thermal cycler. The approach, termed MeltArray, utilizes the 5′-flap endonuclease activity of *Taq* DNA polymerase to cleave a mediator probe into a mediator primer that can bind to a molecular beacon reporter, which allows for the extension of multiple mediator primers to produce a series of fluorescent hybrids of different melting temperatures unique to each target. Using multiple molecular beacon reporters labeled with different fluorophores, the overall number of targets is equal to the number of the reporters multiplied by that of mediator primers per reporter. The use of MeltArray was explored in various scenarios, including in a 20-plex assay that detects human Y chromosome microdeletions, a 62-plex assay that determines *Escherichia coli* serovars, a 24-plex assay that simultaneously identifies and quantitates respiratory pathogens, and a minisequencing assay that identifies *KRAS* mutations, and all of these different assays were validated with clinical samples. MeltArray approach should find widespread use in clinical settings owing to its combined merits of multiplicity, versatility, simplicity, and accessibility.

multiplex PCR | melting curve analysis | molecular diagnostics | real-time PCR

Homogeneous detection of amplified nucleic acid targets in a closed-tube format, as occurs in real-time PCR, is the mainstay of current nucleic acid testing (1). Detection of the amplification products without opening the reaction tube essentially eliminates carry-over contamination, saves time and labor, simplifies automation, and enables target quantification. However, a fundamental limitation of real-time PCR assays is their low capacity for simultaneously detecting a variety of different targets in a single reaction. The limited ability to distinguish a variety of different fluorescent colors in a fluorometric thermal cycler restricts the number of detectable targets to no more than six in a reaction (2–4). The addition of a melting analysis step following PCR enables multiple targets to be detected by their distinct melting temperature (T_m) values (5), which can be regarded as a second dimension for target identification, in addition to the fluorescent color of each probe used in real-time PCR. Nevertheless, this direct detection format often requires the same number of target-specific fluorogenic probes as the number of target sequences. Thus, with an increase in the number of targets that it is desirable to detect, there is an increase in the number of fluorogenic probes required, resulting in an increase in the fluorescence background and an increase in the cost. An added difficulty is that any variations that occur in the probe-binding region will induce a T_m shift that confuses the results. These limitations prevent these assays from detecting dozens of targets in a single reaction (6).

To date, the largest number of targets detected homogeneously in one reaction has been achieved by the use of a ligation-based two-dimensional (2D) labeling strategy, which relies on ligating a "barcode" sequence to each target as an exogenous barcode comprising the combination of fluorescence color and T_m (7). Up to 32 targets (alleles) can be detected in one reaction by using a library of barcode sequences and a set of universal linear probe reporters. The resulting assays are easy to standardize in terms of set-up and readout. Nevertheless, this strategy requires a separate ligation step for adding the barcodes, leading to restrictions in many respects, including 1) added complexity and lower throughput, 2) reduced analytical sensitivity due to nonspecific amplification caused by the presence of multiple oligonucleotides, and 3) nonquantitative end-point detection restricted to screening rather than quantitative detection (7–9).

Here, we describe a single-step, multiplex homogeneous detection approach, termed "MeltArray," to address the limitations described above. MeltArray employs the 5′-flap endonuclease (FEN) activity of *Taq* DNA polymerase to cleave the

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Significance

We describe a highly multiplex PCR approach that can identify 10-fold more targets in current real-time PCR assays without additional enzymes or separate reactions. This single-step, single-tube, homogeneous detection approach, termed MeltArray, is achieved by coupling the 5′-flap endonuclease activity of the *Taq* DNA polymerase and multiple annealing sites of the molecular beacon reporters. The 5′-flap endonuclease cleaves a probe specifically into a "mediator" primer, and one molecular beacon reporter allows for the extension of multiple "mediator" primers to produce a series of fluorescent hybrids with different melting temperatures unique to each target. The overall number of targets detectable per reaction is equal to the number of the reporters multiplied by the number of mediator primers per reporter.

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target-specific "mediator probes" into "mediator primers," which serve as endogenous barcodes during PCR (10). Through the use of molecular beacon reporters, each of which serves as a template for the extension of multiple mediator primers, generating a series of fluorescent double-stranded DNAs of different length, each target sequence in a sample is identified by a unique combination of fluorescence color and T_m. The overall number of targets detectable in one reaction is therefore the number of molecular beacon reporters multiplied by the number of different mediator primers that can bind to different sites on the loop of each molecular beacon reporter. In addition, the Hom-Tag Assisted Non-Dimer System (HANDS) (11) is utilized to suppress primer-dimer formation and to enhance specific amplification. MeltArray was tested in a variety of scenarios entailing the use of multiplex PCR assays, including a 20-plex assay to detect human Y chromosome microdeletions, a 62-plex assay to determine *Escherichia coli* serovars, a 24-plex assay for the concurrent qualitative and quantitative detection of respiratory pathogens, and a minisequencing assay that identifies *KRAS* mutations, and all of these scenarios were validated with clinical samples.

Results

Molecular Beacon Reporters for Multiple Mediator Primers. We envisioned that if multiple mediator probes, each designed to bind to a different target sequence, can be used in the same reaction and all the mediator primers released from the mediator probes by the 5′-FEN activity of *Taq* DNA polymerase can bind with a common fluorogenic reporter, the resulted 5′-FEN PCR assays would be simpler and more cost-effective. We examined two different types of dual-labeled, self-quenched probes—a linear probe and a molecular beacon—as candidate fluorogenic reporters for such 5′-FEN PCR assays (Fig. 1). The hybridized mediator sequence cleaved from the mediator probe serves as a primer for its own enzymatic extension on the

reporter, generating a strong fluorescent signal during the extension stage of PCR. Then, a melting analysis is carried out to determine the temperature at which the extended mediator primer dissociates from the reporter, causing a significant reduction in the reporter's fluorescence intensity. Both probes generated typical real-time PCR amplification curves as well as melting curves. The linear probe generated two melting peaks (Fig. 1A), and the molecular beacon generated one melting peak (Fig. 1B). We attributed this difference to the higher specificity of molecular beacons compared to linear probes (i.e., molecular beacons cannot form a fluorescent hybrid with a mediator probe, whereas the linear probe can form a fluorescent hybrid to produce a peak of lower T_m). These observations showed that molecular beacons are superior to linear probes in a 5′-FEN PCR melting curve analysis owing to their significantly lower fluorescence background.

We next investigated whether one molecular beacon reporter could capture more than one mediator primer. We designed a molecular beacon and two mediator probes, a *KRAS* probe and a *BRAF* probe, the mediator sequences of which could hybridize to different locations in loop of the same molecular beacon reporter. According to their hybridization position, the melting peak of the *KRAS* probe should have a higher T_m value than that of the *BRAF* probe. The results of the duplex 5′-FEN PCR (*SI Appendix*, Fig. S1A) showed that the melting peak of *KRAS* appeared at 67.5 °C and that of *BRAF* appeared at 79.0 °C. This result proved that it is feasible for a duplex 5′-FEN PCR to use two mediator probes that share one molecular beacon reporter.

We then designed another 5′-FEN PCR assay to see whether multiple mediator probes can share one molecular beacon reporter, even if their binding sites on the loop of the molecular beacon reporter partially overlap each other. This was carried out in a quadruplex 5′-FEN PCR for the detection of four chromosome-specific sequence-tagged site markers (sY242,
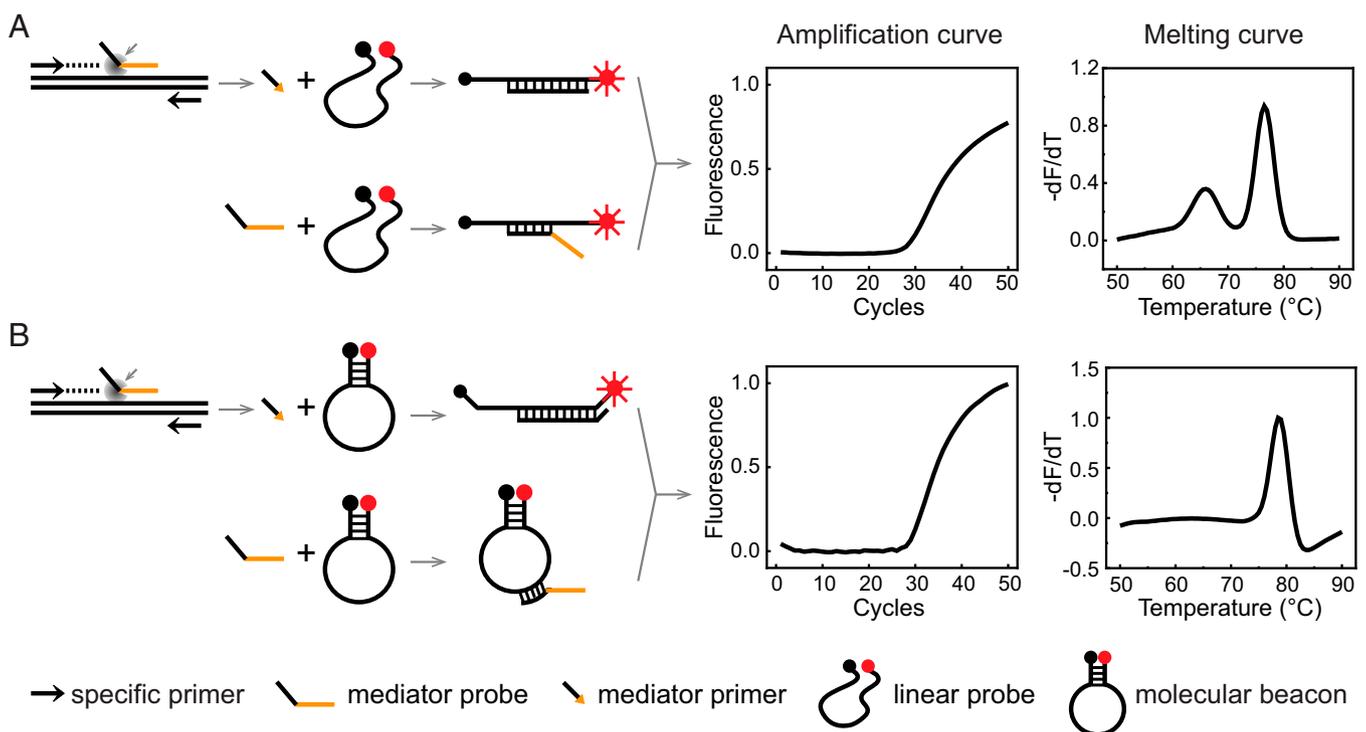
Fig. 1. Comparison of two types of dual-labeled, self-quenched probes when used as the reporter in a 5′-FEN PCR assay. (A) The linear fluorogenic probe. (B) The molecular beacon probe. Both types of probe gave real-time PCR and melting curve results generated from the cleaved mediator probes. The linear probe gave an additional melting peak (at a lower temperature), which resulted from uncleaved mediator probes, whereas the molecular beacon probe did not produce this background peak.

ZFX/Y, sY82, and sY86) in different azoospermia factor regions of the human Y chromosome. The relative positions of the four mediator primers on the reporter are shown in *SI Appendix*, Fig. S1*B*, and the T_m values of the four targets, in order by temperature, were sY242 < ZFX/Y < sY82 < sY86. Notably, all of the mediator primer binding sites on the loop of the molecular beacon reporter were overlapped by 7 to 10 nucleotides. The results showed that all the four targets were successfully detected with T_m values of sY242 (49.6 °C) < ZFX/Y (55.6 °C) < sY82 (63.2 °C) < sY86 (68.4 °C). This result demonstrated that four mediator probes could share one molecular beacon reporter to form a quadruplex 5′-FEN PCR assay. We therefore concluded that multiplex 5′-FEN PCR assays can be designed that utilize multiple mediator probes and one common molecular beacon reporter, provided that the released mediator primers generate differentiable T_m values regardless of whether or not the binding sites for these mediator primers are partially overlapped.

Construction of a MeltArray for Multiplex PCR Assays. We began by building a library that is composed of molecular beacon reporters and the corresponding mediator sequences. The sequences of the reporters and mediators were noncomplementary to any known species. In a 5′-FEN-PCR, the highest T_m would be generated by the mediator primer and its extension binding to the entire loop portion of the molecular beacon reporter and the entire arm sequence of the molecular beacon reporter, and the lowest T_m would be generated by a mediator primer whose extension binds only to the arm sequence of the molecular beacon reporter. In order to determine the maximum number of mediator primers that could be used with one molecular beacon reporter, the T_m range generated should be as wide as possible. We assumed that lowest T_m should be higher than the temperature for efficient annealing (e.g., ~40 °C) and the highest T_m should be smaller than the 100 °C boiling point of water. As an example, a molecular beacon was designed with a loop sequence of 49 nucleotides and an arm sequence of 6 nucleotides. A series of oligonucleotides were synthesized representing the extended mediators, each of which differs by one nucleotide in length. The T_m value of each hybrid was obtained through melting analysis. As shown in *SI Appendix*, Fig. S2, all the oligonucleotides generated typical melting curves, yielding a series of T_m values ranging from 56.1 to 91.1 °C. Given that a regular real-time PCR thermal cycler can resolve a T_m difference (ΔT_m) of ~3 °C, we could obtain a set of 12 differentiable T_m values, corresponding to the same number of the mediator primers, mediator probes, and targets in a 5′-FEN PCR assay. Alternatively, two and more molecular beacon reporters of different lengths but labeled with an identical fluorophore could also be used to capture their respective mediator primers and collectively form a set of differentiable T_m values. By changing the sequences and the labeling fluorophore types, additional molecular beacon reporters labeled with Atto 425, FAM, HEX, Cy5, and Quasar-705, respectively, and their corresponding mediator sequences could be utilized. Collectively, these oligonucleotides formed an off-the-shelf universal library composed of molecular beacon reporters and mediator sequences (SI Appendix, Dataset 1), which can be used as a universal reporting system for any 5′-FEN PCR assay.

To examine the above assumption and test the library as well, we designed an experiment to display 12 T_m transitions in the temperature range from 45 to 95 °C in one detection channel. This was done by using two molecular beacon reporters labeled with ROX and 12 corresponding oligonucleotides representing the extended mediators chosen from the constructed library. Slightly different concentrations of the oligonucleotides were used for better recognition of the curves from each preparation. For comparison, both the melting curves (−dF/dT plots)

and the corresponding thermal denaturation curves (F/T plots) were obtained (*SI Appendix*, Fig. S3*A*). The results showed that all the 12 T_m peaks could be identified in the −dF/dT plots (*Upper* panel). By contrast, the fluorescence change could be hardly observed in the F/T plots (*Lower* panel) at the corresponding T_m values. Thus, the −dF/dT plots are better suited to distinguish a large number of T_m transitions within the temperature range studied. We repeated the above experiment with three different batches of preparations each in six replicates in three consecutive days. The reproducibility of the T_m measurement was evidenced by the small threefold SDs (3 SDs < 0.5 °C, $n = 54$; *SI Appendix*, Table S1) of all the 12 averaged T_m values in the −dF/dT plots (*SI Appendix*, Fig. S3*B*). These results together demonstrate that 12 melting peaks can be simultaneously generated using two molecular beacon reporters with high reproducibility.

The flowchart of a MeltArray scheme for a multiplex assay is illustrated for a 12-plex 5′-FEN PCR using one optical channel (Fig. 2*A*). The MeltArray assay includes 12 different mediator probes, and each of these 12 mediator probes includes a unique 5′-mediator primer sequence that is complementary to a different predefined region in the loop of the molecular beacon reporter. Consequently, when each of these 12 different-sequence mediator primers binds to the loop of the molecular beacon reporter and after those mediator primers are enzymatically extended, each different mediator primer will generate a partially double-stranded reporter whose double-stranded section will be of a different length. Following the final melting analysis, the combination of the measured T_m and the color of the fluorophore allows for 12 different target sequences to be identified (Fig. 2*A*). If the fluorometric thermal cycler is able to distinguish six different fluorescent colors (as are usually distinguishable in current real-time PCR instruments), then six different molecular beacon reporters can be used, each of which possesses an entirely different loop sequence. This opens up the possibility of simultaneously being able to identify as many as 72 different target sequences in a single closed-tube PCR assay.

The use of a MeltArray enables each different amplicon to be identified by virtue of the generation of a unique combination of fluorescent color and T_m, allowing multiple targets to be detected simultaneously in a sample. To ensure efficient target amplification, primer-dimer formation among the many primers included in the reaction needs to be suppressed. This is accomplished through the use of HANDS, which enables the exponential amplification of the targets to outcompete the linear amplification of the primer-dimers (11). To this end, a 20-nucleotide–long "tag sequence" that is not complementary to any of the target sequences is added to the 5′-end of each target-specific primer. After the first few rounds of amplification, the resulting amplicons possess an entire primer sequence on their 5′-ends (including the tag sequence) and the entire complement of the other member of the primer pair on their 3′-ends (including the complement of the tag sequence). Also present in the assay mixture is a high concentration of a single "universal primer," whose sequence is identical to the tag sequence. Consequently, these abundant universal primers hybridize to their complementary sequence on the 3′-ends of the amplicons, efficiently priming the exponential synthesis of all of the amplicons generated from all of the target sequences present in the sample. Meanwhile, any primer-dimers that are formed cannot be exponentially amplified because they possess the universal tag sequence on their 5′-ends and also possess the complement of the universal tag sequence on their 3′-ends, and as a consequence of the relatively small size of these primer-dimers, they form hairpin structures that restrict the access of the universal primers and therefore cannot be amplified efficiently (Fig. 2*B*).
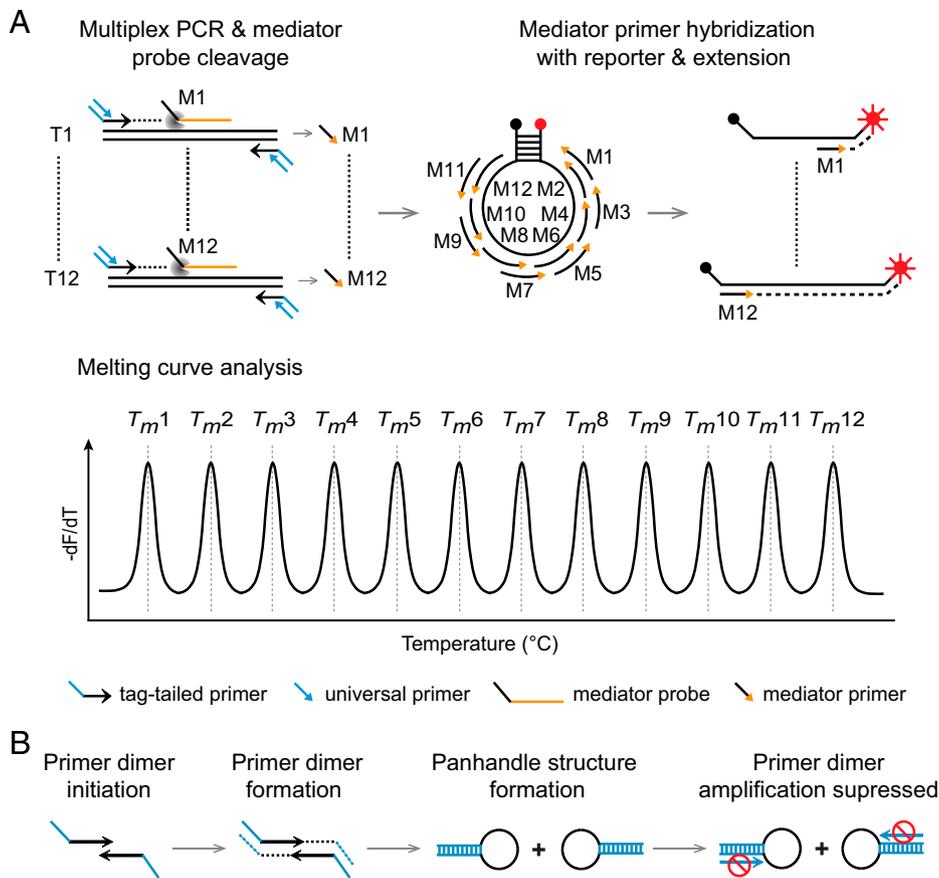
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Fig. 2. Detection of target sequences in MeltArray assays. (*A*) A 12-plex MeltArray assay utilizing one fluorophore. In this example, the reaction contained 12 pairs of target-specific primers, 12 target-specific mediator probes (each possessing a different 5′-mediator sequence, labeled M1 to M12), and one molecular beacon reporter. Each of the 12 different mediator primers was designed to hybridize to a different portion of the loop sequence of the molecular beacon reporter. After elongation of the mediator primers, each of the 12 different-length double-stranded fluorescent reporters resulted in a unique T_m value following melting curve analysis. (*B*) Elimination of primer-dimer exponential amplification. Primer-dimers form a thermodynamically preferred single-stranded hairpin structure that cannot be amplified.

A 20-plex PCR MeltArray Assay for Simultaneous Detection of 20 Targets. In order to explore the potential of MeltArray for multiplex PCR detection, we tested the feasibility of using multiple molecular beacon reporters labeled with differently colored fluorophores for a 20-plex PCR assay. This assay was used to detect 18 chromosome-specific sequence-tagged site markers in different azoospermia factor regions (AZFa, AZFb, AZFc, and AZFd) of the human Y chromosome, in addition to detecting the *SRY* gene as a positive control for the presence of Y chromosome DNA and the *ZFX/Y* gene as a positive control for the presence of both X and Y chromosome DNA (Fig. 3*A*). These targets were identified using three pairs of molecular beacon reporters labeled with FAM, ROX, and Cy5, respectively, and each pair of the reporters were labeled with the same fluorophore (Fig. 3*B*). This 20-plex assay produces 20 peaks in a healthy male that has all of the target sequences. However, in an infertile male, one or more of these peaks may be missing. After optimization, the MeltArray assay was used to analyze 83 genomic DNA samples from healthy males. All of the 20 target regions present in a healthy male were correctly detected (Fig. 3*C*), with a negligible deviation of the T_m values, regardless of whether one sample was tested repeatedly (*SI Appendix,* Fig. S4*A*) or whether multiple samples were examined simultaneously (*SI Appendix,* Fig. S4*B*).

To evaluate the effect of genomic DNA concentration on the performance of the MeltArray assay, we studied the working range of the template concentration using both healthy male and female genomic DNA samples. The results showed that human genomic DNA, ranging from 100 ng/reaction down to 100 pg/reaction, could reproducibly be analyzed. The heights of the melting peaks gradually decreased with reduced DNA quantity. However, the T_m values of all 20 peaks remained unchanged (*SI Appendix,* Fig. S5).

This MeltArray assay was then used to screen 757 samples that included 92 samples from the Liuzhou Maternity and Child Healthcare Hospital and 665 samples randomly chosen from normal human DNA samples stored in our laboratory. Of the hospital samples, 57 were Y chromosome microdeletion-positive (i.e., at least one of the target sequences was absent and did not produce a peak in the melting curve analysis), 34 were negative, and 1 was female (Fig. 3*C*). Of the 665 random samples, 538 were determined to be from normal males, 126 were from females, and 1 was Y chromosome microdeletion-positive. These results were confirmed by comparing the MeltArray assay results with the results obtained by a routinely used gel electrophoresis-based PCR assay. The microdeletion types that were detected are summarized in *SI Appendix,* Table S2.

A 62-plex PCR MeltArray Assay for Target Identification. Encouraged by the above results, we believe that an even higher multiplex PCR assay could be carried out using six detection channels present in a fluorometric thermal cycler. Therefore, we designed a 62-plex MeltArray assay that distinguishes 61 O-antigen biosynthesis genes, allowing for the identification of
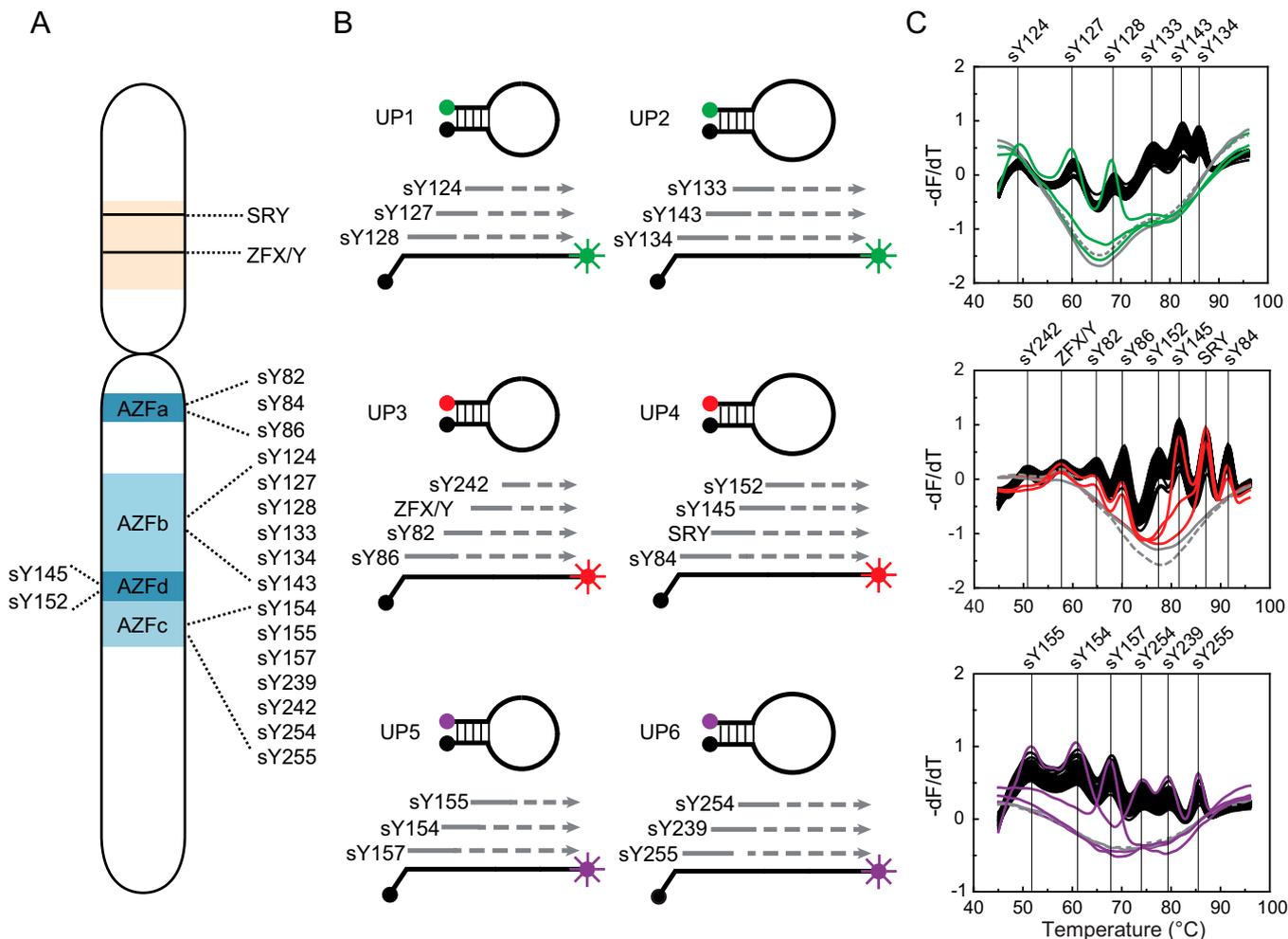
Fig. 3. MeltArray assay for the detection of microdeletions in the human Y chromosome. (*A*) The relative position of each sequence-tagged site marker and the *SRY* and *ZFX/Y* genes in the Y chromosome. (*B*) Design of the MeltArray assay using six universal fluorogenic molecular beacon reporters labeled with FAM (green), ROX (red), or Cy5 (purple). (*C*) Detection results obtained by the MeltArray assay for 88 human genomic DNA samples. Black lines indicate the melting curve results obtained with 83 male DNA samples that did not possess microdeletions; colored lines indicate the melting curve results obtained from the corresponding channels with four male DNA samples that possessed microdeletions; dashed gray lines indicate the melting curve results obtained with a female DNA sample; and solid gray lines indicate the melting curve results obtained with the no-template control.

54 individual O serogroups possessing unique O-antigen biosynthesis genes, and 7 *E. coli* strain groups (Gp1, Gp2, Gp4, Gp6, Gp9, Gp13, and Gp15) that contain 18 individual O serogroups with identical or very similar O-antigen biosynthesis genes. The 62-plex MeltArray assay was performed on a six-color fluorometric thermal cycler that is able to carry out thermal melting analyses after exponential amplification is completed. For each differently colored fluorophore, we used 8 to 12 different mediator probes, each releasing a different-sequence mediator primer that results in a predefined T_m if its corresponding target sequence is amplified. This MeltArray assay was able to generate 62 different fluorophore-T_m combinations for the identification of 61 O genotypes and 1 *E. coli*-specific gene, *yccT* (Fig. 4*A*). A reproducibility study showed that the resulting T_m values showed little fluctuation, thereby enabling each potential target to be unambiguously distinguished (Fig. 4*B*).

This MeltArray assay was used to screen a collection of 167 isolates of *E. coli*, which included 150 isolates exhibiting 33 different O serotypes (determined by conventional serotyping) and 17 isolates that failed to give phenotypic results by conventional serotyping. One isolate among the 150 isolates possessing known serotypes gave a discordant MeltArray result compared

to conventional serotyping. However, the MeltArray result of this isolate was subsequently confirmed to be correct by whole-genome sequencing (WGS) (Fig. 4*C*; *SI Appendix*, Fig. S6). Of the 17 isolates that failed to give conventional serotyping results, MeltArray successfully identified 11 isolates that exhibited 7 different O genotypes. These results were also confirmed by subsequent WGS. The remaining six isolates gave no signal. However, four of these six isolates were found to possess genotypes that were outside the genotypes covered by the MeltArray assay. The other two isolates were not identified, even by WGS (*SI Appendix*, Fig. S6). These results demonstrate that the MeltArray target detection technique is more robust than conventional serotyping for serovar determination. Moreover, MeltArray assays generate results comparable to the results of WGS.

Inclusion of Real-time PCR Detection. As an end-point melting analysis, one natural question to be asked is whether a MeltArray assay can also carry out real-time PCR detection. To answer this question, we chose respiratory pathogens as model targets since some of the pathogens are better at providing quantitative results, while others need only provide qualitative identification. We designed a 24-plex respiratory assay that detects 14
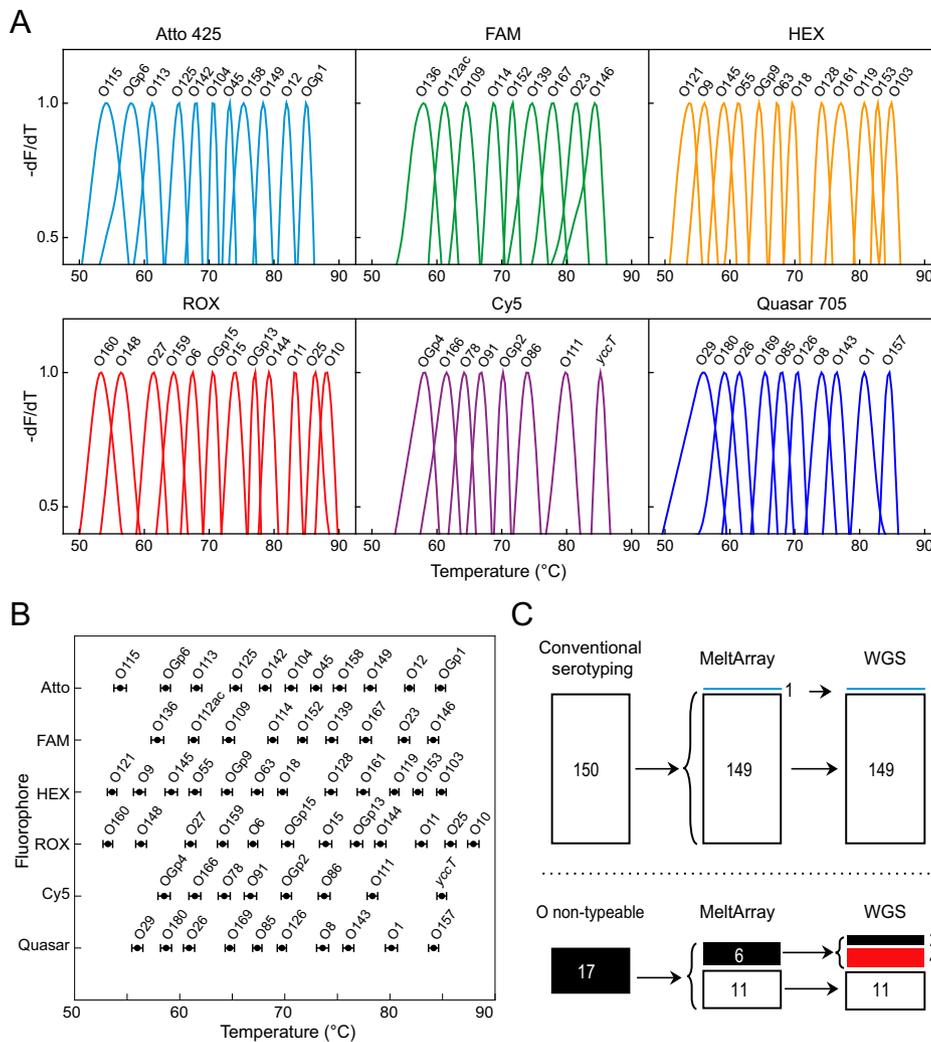
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Fig. 4. MeltArray assay for the identification of 61 O genotypes of *E. coli*. (*A*) T_m value and fluorescence detection channel for each O genotype (and for the *yccT* gene of *E. coli*). (*B*) Reproducibility of the T_m value of each target that can be detected in the 62-plex MeltArray assay. Black circles represent the mean T_m value of each O genotype, and horizontal bars show ±3-fold differences in the SDs of each mean T_m value. (*C*) Comparison of the results obtained for 167 *E. coli* isolates with MeltArray assays to the results obtained by conventional serotyping and by WGS. The height of the hollow bars indicates the number of isolates that were detected by each method. The blue bar indicates the one isolate that gave discordant results between conventional serotyping and the MeltArray assay. Solid black bars indicate the number of isolates that were not identified by each serotyping method. The red bar indicates the four isolates whose O genotype was detected by WGS but was not detected by either the MeltArray assay or by conventional serotyping.

different RNA viral species, two different DNA viral species, three different atypical bacterial species, four different typical bacterial species, and one human control gene (*RNase P*). Among these targets, it is important to quantitate the amount of each of the four typical bacterial species to differentiate infection from colonization in the sample.

In this multiplex assay, the presence or absence of 20 different target sequences in the sample was determined at end point by qualitative MeltArray analysis. Significantly, the amount of each of the four different bacterial species potentially present in the sample was determined via the real-time measurement of the fluorescence intensity increase by 5′-hydrolysis of molecular beacon probes during the extension stage. These real-time fluorescence intensity measurements were collected during the 95 °C denaturation stage of each thermal cycle (Fig. 5*A*), at which temperature all of the uncleaved molecular beacon probes and all of the molecular beacon reporters exist as short single-stranded random coils, whose fluorophore and quencher interact with each other by fluorescence resonance energy

transfer, creating a relatively low-intensity background signal. Moreover, each of these four probes is a "shared-stem molecular beacon," in which their 3′-arm sequence, as well as their loop sequence, is designed to be complementary to its target sequence, assuring that the bound molecular beacon will be cleaved rather than displaced when it is encountered by the DNA polymerase. Consequently, simultaneous real-time PCR detection and MeltArray analysis was achieved (Fig. 5*B*).

The limit of detection (LOD) of the assay was ~10 copies/µL for each of the pathogens, and the quantitative range of detection for each of the four bacterial pathogens was between 10 and 100,000 copies/µL. The assay was evaluated using bronchoalveolar lavage fluid (BALF) samples collected from 67 hospitalized pediatric patients who were diagnosed with either pneumonia or another respiratory tract infection. The results showed that 53 (79.1%) samples contained at least one pathogen. Among these, 26 (38.8%) contained nonbacterial pathogens only, 10 (14.9%) contained bacterial pathogens only, and 17 (25.4%) contained both bacterial and nonbacterial
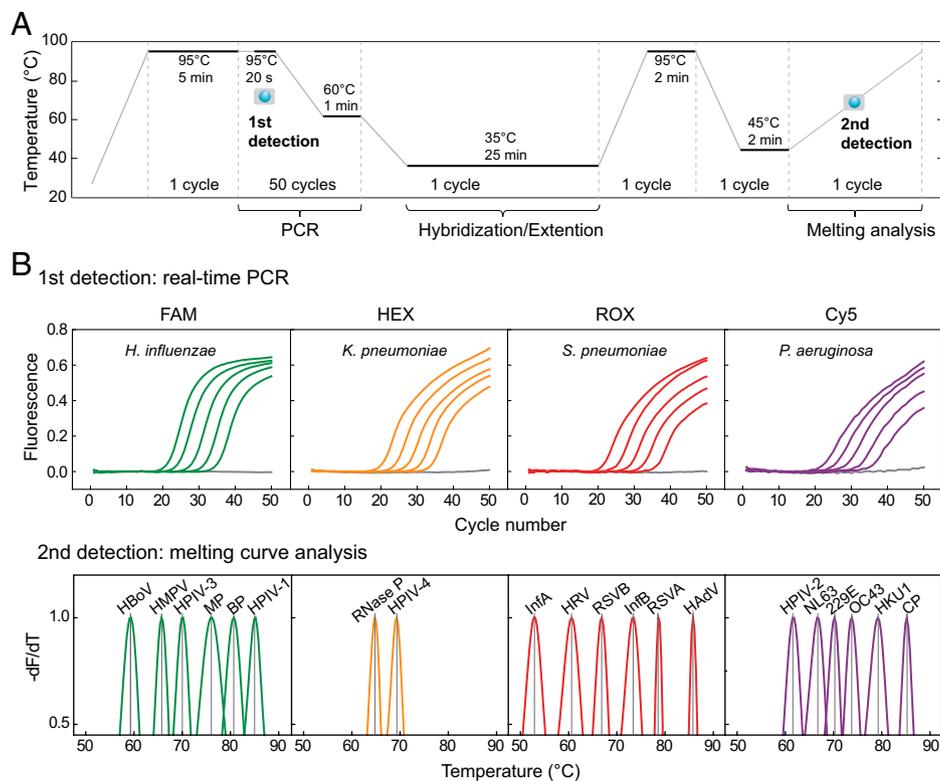
Fig. 5. Simultaneous real-time PCR and melting curve analysis for the identification and quantification of respiratory pathogens. (*A*) Running program for coupling MeltArray with real-time PCR detection. Real-time PCR detection fluorescence intensity was measured at the denaturation stage of each thermal cycle during target amplification. MeltArray detection, on the other hand, was carried out during subsequent melting curve analysis. (*B*) Concurrent real-time PCR and melting curve analysis in a single reaction. Four bacterial species (*Haemophilus influenzae*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa*) were quantitated by determination of the quantification cycle (C_q value) that occurred during real-time PCR, and the other 19 pathogens were each identified by the unique combination of the fluorescent color and the T_m value of their melting peak.

pathogens. The frequency determined for each pathogen is shown in *SI Appendix*, Table S3. We compared these results to those obtained from the singleplex real-time PCR assays, and a 100% concordance was obtained.

Minisequencing of Single-Nucleotide Mutations. Finally, we explored whether MeltArray assays could be used to distinguish sequence variants at the single-nucleotide level, thereby enabling identification of the genotypes of certain mutations relevant to choosing an appropriate therapeutic drug for cancer patients. This was accomplished by detecting single-nucleotide mutations that can occur at three different nucleotide positions within the six-nucleotide–long sequence of codons 12 and 13 of the human *KRAS* gene. There are three different nucleotide substitutions that can occur at each of three different nucleotide locations within the six-nucleotide *KRAS* target sequence (at positions c.34, c.35, and c.38). Thus, there exists one wild-type target sequence and nine different mutant target sequences, each containing a single mutant nucleotide.

We designed ten different mediator probes, each specific for a different target sequence (T_m difference > 7 °C between matched and mismatched targets) and each designed to release a unique mediator primer for interaction with one of four differently colored hairpin reporters (Fig. 6*A*). The results showed that the assay yielded a distinguishable signal for each mutant type, and the assay tolerated a large amount of wild-type genomic DNA (up to 500 ng). The lowest mutant allele frequency (MAF) detected was 5 to 10% (Fig. 6*B*), which is better than the sensitivity of conventional Sanger sequencing, whose lowest MAF is 20%. The LOD of the assay was calculated to be 30 target sequence copies/reaction.

We utilized the MeltArray assay for the analysis of 167 different tissue samples, comprising 107 freshly frozen samples and 60 formalin-fixed paraffin-embedded (FFPE) samples, obtained from colon cancer patients. The detection results were compared with the results obtained by Sanger sequencing, and the discordant samples were subjected to allele-specific PCR. The results are summarized in *SI Appendix*, Table S4. In the 107 frozen tissue samples, the MeltArray assays picked up three more mutant samples than did Sanger sequencing, and in the 60 FFPE samples, the MeltArray assay detected two more mutant samples than did Sanger sequencing. The identity of all of the mutant samples was confirmed by allele-specific PCR. These results demonstrate that MeltArray assays are more sensitive for detecting low MAF mutations than Sanger sequencing assays. The mutation types, together with their frequencies in the 167 samples, are summarized in Fig. 6*C*. The observed distribution of the mutations in these samples is similar to the distribution of these mutations listed in the Cosmic database (https://cancer.sanger.ac.uk/cosmic).

Discussion

In this study, a highly multiplex homogeneous PCR approach was proposed following the observation that molecular beacon reporters can bind to multiple released mediator primers in generated by 5′-FEN PCR. Using multiple molecular beacon reporters labeled with different fluorophores, the overall number of targets that can be detected per reaction equals the number of different molecular beacon reporters multiplied by of the number of different mediator primers that can be generated per molecular beacon reporter. Also, by introducing the HAND
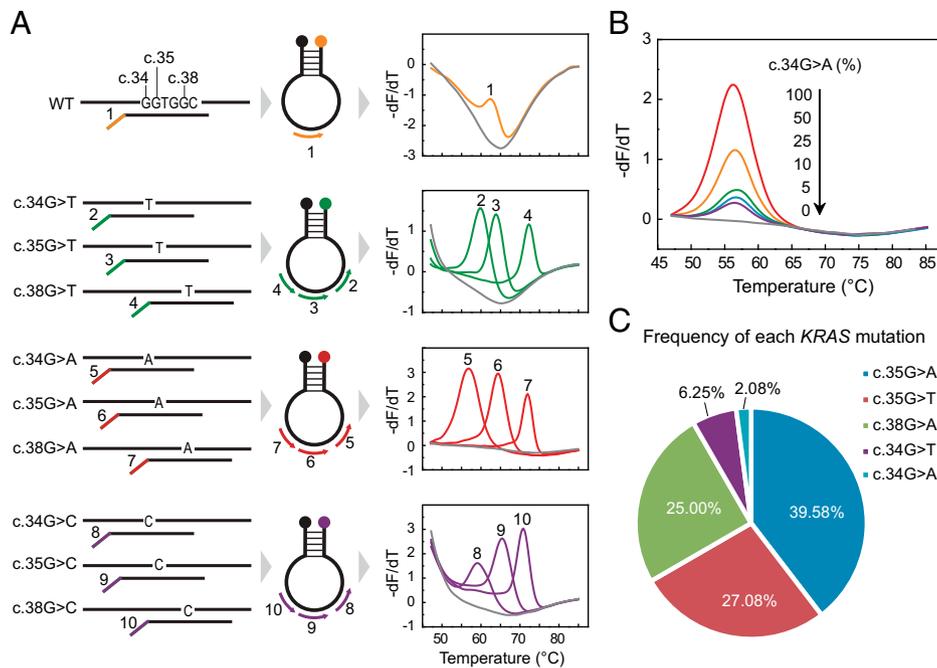
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Fig. 6. Minisequencing of *KRAS* mutations in a MeltArray assay. (*A*) The design of the MeltArray assay for the minisequencing of the nucleotides present at positions c.34, c.35, and c.38 in codons 12 and 13 of the human *KRAS* gene. Colored curves indicate the melting curves of the corresponding targets and gray curves indicate the melting curves of the no-template control. (*B*) Demonstration of the detection sensitivity of the MeltArray assay. Melting curves were obtained with mutation-mimicking plasmid DNA samples containing different proportions (100, 50, 25, 10, 5, and 0%) of the *KRAS* c.34G > A mutation. (*C*) Mutation frequencies detected in 167 human tissue samples with the *KRAS* minisequencing MeltArray assay.

system to suppress primer-dimer formation, MeltArray successfully detects a very large number of different targets in a homogeneous single-step reaction. Furthermore, MeltArray can include real-time detection, thereby including the quantitative power of real-time PCR. Moreover, MeltArray can achieve single-nucleotide resolution, enabling the minisequencing of multiple nucleotides simultaneously in a single reaction. Collectively, MeltArray provides single-step, multiplex, homogeneous PCR detection with improved capacity and versatility, which cannot be achieved by current approaches.

Homogenous PCR detection was originally invented to enable closed-tube detection of amplified products, thereby preventing carry-over contamination of amplicons from one assay tube to another, creating false-positive results (12). The success of these assays was enhanced by the introduction of real-time PCR detection, allowing for the quantitative detection of nucleic acids (13), and was further boosted by the introduction of melting curve analysis, which permits sequence variations to be distinguished (14). These advantages, together with ease of use, rapidity, and cost-effectiveness, have made homogeneous detection the most popular nucleic acid testing tool in clinical settings. However, after nearly 30 years of advances, it is often regarded as a somewhat "low-end" technique, owing to the fact that its use is limited to the detection of one or a few targets in a single reaction. In practice, difficulties increase markedly when the number of different targets is more than the number of different optical channels available in the fluorometric thermal cycler, thereby preventing the development of multiplex PCR assays that can detect the dozens of different targets that are often required to characterize, define, and diagnose complex diseases.

We previously showed that a ligation-based 2D-labeling strategy could detect up to 32 targets (alleles) in one reaction by introducing a tag sequence ("barcode") to each target as a unique label, comprising a combination of fluorescence color

and T_m (7). This ligation-based 2D-labeling strategy, as its name suggests, uses a ligation reaction to add the barcode to the ligation probe before amplification. This barcode is exogenous by nature. By contrast, MeltArray uses a mediator probe that produces the barcode only if the mediator probe binds to its target and is cleaved as amplification occurs. The barcodes in a MeltArray assay are therefore endogenous. Endogenous barcoding endows MeltArray with merits in many respects: being free from nonspecific amplification, removal of an extra ligation step, and omission of the DNA ligase, among others.

One key mechanism in MeltArray is the use of the 5′-FEN activity of *Taq* DNA polymerase to cleave the mediator probes into mediator primers. The 5′-FEN activity of *Taq* DNA polymerase had already been used to establish a "mediator PCR" scheme, which allows the use of universal, standardized reporters due to decoupling of amplification and fluorescence detection (10). The universal reporters therein adopt a hairpin-shaped secondary structure, the 3′-unpaired stem (~45 nucleotides) that contains the mediator hybridization site (~20 nucleotides), which is complementary to the mediator sequence. Such a reporter has one capture site and can therefore receive only one mediator primer. To detect more than one target, the same number of universal reporters, as well as the same number of mediator probes, are required. For example, in a pentaplex assay, "mediator PCR" uses five reporters in addition to five mediator probes (15), leading to increased cost and complexity in comparison with a regular probe-based real-time PCR in which no more than one fluorogenic probe is used for each target. It is therefore difficult, if not impossible, for "mediator PCR" to be utilized in a multiplex PCR assay that is able to detect dozens of targets in one reaction by either real-time PCR detection or by melting curve analysis.

In our MeltArray approach, the single use of universal reporters encountered in "mediator PCR" is overcome by replacing it with multiuse molecular beacon reporters, (i.e., one molecular beacon reporter allows the extension of multiple

mediator primers to yield a series of fluorescent hybrids with different melting temperatures that are unique for each assay target sequence). After melting curve analysis, all of the targets that share one molecular beacon reporter can be identified. An obvious benefit of the multiple use of a molecular beacon reporter is its cost-effectiveness, due to saving the cost of expensive fluorogenic reporters. More importantly, the use of fewer reporters produces a lower fluorescence background, which is critical for higher analytical sensitivity and multiplexity. The choice of molecular beacons as reporters has many advantages: 1) Molecular beacons are more specific than corresponding linear probes (16, 17), so they are less likely to hybridize to the mediator sequences of the mediator probes to form an undesirable fluorescent hybrid when target amplification has not occurred, thereby assuring that there is a low background in the lower T_m region of the melting curve; 2) molecular beacons give a narrower melting peak than linear probes (18), such that more melting peaks can be distinguished within the limited T_m range; and 3) molecular beacons themselves have a lower background fluorescence than linear probes (18, 19). These advantages are significant when multiple reporters are used for highly multiplex detection.

A third mechanism of MeltArray is the use of the HANDS approach, in which all primers have a common 5'-tag that favors the formation of a hairpin structure by the primer-dimers, but not by the considerably longer target-specific amplicons (11). Hairpin structures hinder the access of the universal primer, preventing primer-dimer amplification, and facilitate the amplification of the target-specific amplicons with high analytical sensitivity. For example, in the 20-plex MeltArray assay for Y chromosome microdeletions, as low as 100 pg of human genomic DNA per reaction could reliably be detected. By contrast, the ligation-based 2D-labeling strategy, which did not have a mechanism to eliminate nonspecific signals derived from the large number of ligation probes, required at least 20 ng of human genomic DNA per reaction for the reproducible detection of 16 different single nucleotide polymorphism genotypes (7). In this regard, the MeltArray assay was 200-fold more sensitive than the 2D-labeling approach. Similar high analytical sensitivities were also observed in the other MeltArray assays presented in this study.

The three mechanisms mentioned above collectively endow MeltArray with the capability to detect the largest number of targets in one closed tube in a single step with high analytical sensitivity, specificity, reproducibility, and cost-effectiveness. Moreover, MeltArray assays can include real-time PCR detection, enabling both quantitative and qualitative detection in a single reaction. In this particular application, shared-stem molecular beacons serve as 5'-hydrolysis probes for real-time PCR, and their fluorescence is detected at the denaturation stage. Thus, the net fluorescence increase per cycle occurs only from cleaved molecular beacons, whereas intact molecular beacons and molecular beacon reporters (including free molecular beacons and molecular beacons hybridized to extended mediator primers) become denatured and emit only a weak background fluorescence as a result of the interaction between the quencher and fluorophore. Therefore, real-time PCR signals arise solely from the hydrolysis probes, and their signals are separate from the signals arising from the MeltArray reaction. Similarly, the MeltArray signals arise solely from intact molecular beacon reporters, since the hydrolysis probes do not produce melting curve signals in the symmetric PCR used for the MeltArray. Consequently, the two detection modes are independent from each other, despite their coexistence in one reaction tube. Last but not least, MeltArray assays can detect single-nucleotide variations as demonstrated in the minisequencing of *KRAS* mutations. In this application, the mediator probes work in a similar manner as the genotyping probes in

real-time PCR, in which the probes generate signals only from their perfectly matched targets. Because MeltArray assays can display signals that are combinations of T_m and fluorescence color, a larger number of mutations can be genotyped or minisequenced in a single reaction. This feature should find immediate use in those scenarios in which many different mutation types need to be detected.

Although we have demonstrated the advantages mentioned above, MeltArray assays have limitations that need further improvement. First, as an end-point detection mode, its quantitative ability is restricted, despite the peak height of the melting curve being proportional to the target quantity within a certain range. Thus, MeltArray is mostly intended for screening assays rather than for quantitative detection. Coupling with real-time detection can only partially overcome this limitation because of the limited number of differently colored fluorophores that can be distinguished by a standard real-time thermal cycler. We anticipate further improvement by using fluorometric thermal cyclers equipped with an increased number of optical channels or even instruments that can analyze a full spectrum, which is an approach that has been successfully used in flow cytometers for the identification of dozens of cell markers (20). An alternative approach could be the use of digital PCR thermal cyclers that are able to carry out melting curve analyses (21), thereby enabling quantification of all of the targets by fluorophore color, T_m, and fluorescence intensity. Second, the turnaround time of MeltArray assays is lengthier than probe-based melting analysis, e.g., multicolor melt curve analysis. This is due to the existence of a roughly 30-min incubation stage required for the mediator primers to hybridize with and extend along the molecular beacon reporters before the melting analysis is carried out. This stage is absent in multicolor melt curve analysis, as its melting step can start immediately after PCR. This extra time can be offset, in part, when coupled with extreme PCR (22, 23), which allows amplification to be finished within minutes or even seconds. Also, the incubation stage might be further optimized to attain a shorter period of time.

In conclusion, the successful coupling of homogenous detection with highly multiplex PCR can find immediate applications in scenarios that currently entail nonhomogenous detection systems, e.g., bead-based Luminex assays (24), molecular weight–based matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (25), and fragment size-based capillary electrophoresis (26), all of which require specialized facilities, involve complex operations, and are cost-ineffective in comparison to the widespread use of real-time PCR thermal cyclers. Furthermore, the future use of the MeltArray approach in advanced microfluidic platforms that accommodate many reaction compartments (27, 28) should enable the development of assays that provide enhanced target coverage. We expect that in addition to improving existing multiplex assays, MeltArray will also provide homogenous detection for many currently unexplored fields.

Materials and Methods

Linear Probes Versus Molecular Beacons as Reporters in a 5'-FEN PCR Assay. The reaction was performed in a 25-μL solution containing PCR master buffer [10 mM Tris-HCl (pH 8.0) and 50 mM KCl], 3.0 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates (dNTPs), 2.0 U *Taq* HS DNA polymerase (Takara), 0.4 μM of each primer, 0.2 μM mediator probe, 0.2 μM ROX-labeled linear probe or 0.2 μM ROX-labeled molecular beacon (the sequences of which are listed in *SI Appendix*, Table S5), and 5 μL (~10 ng/μL) of human genomic DNA template. PCR and melting curve analysis was performed in an SLAN 96S real-time PCR instrument (Hongshi Medical Technology Co. Ltd.) as follows: denaturation at 95 °C for 5 min; 50 cycles of 95 °C for 20 s, 61 °C for 1 min; and then 35 °C for 10 min, 95 °C for 1 min, and 45 °C for 2 min, followed by a temperature increase from 50 °C to 90 °C (0.04 °C/step). Fluorescence data from the ROX channel were measured at the annealing step during each PCR cycle and at each step of the continuous temperature increase during the melting curve analysis procedure.

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The data were analyzed using the version 8.2.2 software of the SLAN 96S real-time PCR detection system. Human genomic DNA was extracted from peripheral blood provided by the experimenters using the Lab-Aid 820 Nucleic Acid Extraction System (Zeesan Biotech). The concentration of the genomic DNA was determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc.).

Molecular Beacon as Reporter in a Duplex 5′-FEN PCR Assay. The reaction was performed in a 25-μL solution containing PCR master buffer, 3.0 mM MgCl$_2$, 0.2 mM dNTPs, 2.0 U *Taq* HS DNA polymerase, 0.4 μM of each primer, 0.2 μM *KRAS* mediator probe, 0.1 μM *BRAF* mediator probe, 0.2 μM ROX-labeled molecular beacon (the sequences of which are listed in *SI Appendix*, Table S6), and 5 μL (∼10 ng/μL) of human genomic DNA template. PCR and melting curve analysis was performed in an SLAN 96S real-time PCR instrument as previously described.

Molecular Beacon as Reporter in a Quadruplex 5′-FEN PCR Assay. The reaction was performed in a 25-μL solution containing PCR master buffer, 3.0 mM MgCl$_2$, 0.2 mM dNTPs, 2.0 U *Taq* HS DNA polymerase, four primer pairs, four mediator probes, one ROX-labeled molecular beacon reporter (the sequences and concentrations of which are listed in *SI Appendix*, Table S7), and 5 μL (∼10 ng/μL) of human genomic DNA template. PCR and melting curve analysis was performed in an SLAN 96S real-time PCR instrument as follows: denaturation at 95 °C for 5 min; 50 cycles of 95 °C for 20 s, 60 °C for 1 min; and then 35 °C for 30 min, 95 °C for 1 min, 40 °C for 2 min, followed by a temperature increase from 40 °C to 80 °C (0.04 °C/step). Fluorescence intensity was measured in the ROX channel at each step of the continuous temperature increase.

Melting Curve Analysis of the Molecular Beacon Reporter and the Mediator Oligonucleotides. Each reaction was performed in a 25-μL solution containing PCR master buffer, 7.0 mM MgCl$_2$, 0.2 mM dNTPs, synthetic mediator oligonucleotides representing the extended mediators, and ROX-labeled molecular beacons (the sequences and concentrations of which are listed in *SI Appendix*, Tables S8 and S9). Melting curve analysis was performed in an SLAN 96S real-time PCR instrument at the following temperatures: 95 °C for 2 min, 45 °C for 2 min, followed by a temperature increase from 45 °C to 95 °C, 0.04 °C/step. Fluorescence intensity was measured in the ROX channel at each step of the continuous temperature increase.

Detection of Human Y Chromosome Microdeletions. A 20-plex MeltArray assay to detect 18 Y chromosome–specific sequence-tagged site markers, the *SRY* gene, and the *ZFX/Y* gene, was performed in a 25-μL solution containing PCR master buffer, 11 mM MgCl$_2$, 0.7 mM dNTPs, 4 U *Taq* HS DNA polymerase, 3.2 μM universal primer, and 5 μL (10 ng/μL unless otherwise indicated) of human genomic DNA template. Each reaction also contained 40 tag-sequence–tailed target-specific primers, 20 mediator probes, and 6 universal molecular beacon reporters (the sequences and concentrations of which are listed in *SI Appendix*, Table S10). PCR and melting curve analysis was performed in an SLAN 96S real-time PCR instrument as follows: denaturation at 95 °C for 5 min; four cycles of 95 °C for 20 s, 60 °C for 1 min; 46 cycles of 95 °C for 20 s, 62 °C for 1 min; and then 35 °C for 30 min, 95 °C for 1 min, 45 °C for 2 min, followed by a temperature increase from 45 °C to 95 °C (0.04 °C/step). Fluorescence intensity was measured in three detection channels [FAM (510 nm), ROX (620 nm), and Cy5 (665 nm)] at each step of the continuous temperature increase.

A total of 840 genomic DNA samples extracted from peripheral blood using the Lab-Aid 820 Nucleic Acid Extraction System, including 92 clinical samples collected from the Liuzhou Maternity and Child Healthcare Hospital (Liuzhou) and 748 samples stored in our laboratory, were used to establish and evaluate the 20-plex MeltArray assay. The Research Ethics Committee of the Liuzhou Hospital and Xiamen University approved this study, and informed consent was obtained from individual patients. The concentration of the genomic DNA in each sample was determined using an ND-1000 spectrophotometer. The detection results of the 20-plex MeltArray assay were compared with those from a gel electrophoresis-based PCR detection kit (Yaneng Bio).

Identification of the O Serovar of *E. coli*. A 62-plex MeltArray assay to identify 61 O genotypes of *E. coli* was performed in a 25-μL solution containing PCR master buffer, 7 mM MgCl$_2$, 0.2 mM dNTPs, 2.5 U *Taq* 01 DNA polymerase (Zeesan Biotech), 0.8 μM universal primer, and 5 μL (∼1 ng/μL) of genomic DNA template. Each reaction also contained 124 tag-sequence–tailed target-specific primers, 62 mediator probes, and 12 universal molecular beacon reporters (the sequences and concentrations of which are listed in *SI Appendix*, Table S11). The target-specific primers were each linked at their 5′-end to a common tag primer sequence to generate the tag-sequence–tailed target-specific primers. PCR and melting curve analysis was performed in the SLAN 96S real-time PCR instrument as follows: denaturation at 95 °C for 5 min; 40 cycles of 95 °C for 20 s, 60 °C for 1 min; and then 35 °C for 30 min, 95 °C for 2 min, 45 °C for 2 min,

followed by a temperature increase from 45 °C to 95 °C (0.04 °C/step). The fluorescence intensity was measured in six detection channels [Atto 425 (450 nm), FAM, HEX (565 nm), ROX, Cy5, and Quasar 705 (705 nm)] at each step of the continuous temperature increase during the melting curve analysis procedure.

For clinical evaluation, 167 *E. coli* clinical isolates from the Shenzhen Center for Disease Control and Prevention were collected. The serovars of these isolates had been determined using 50 O antisera (Denka Seiken) according to the manufacturer's instructions. Isolates that did not react with any of the O antisera were classified as O nontypeable. Genomic DNA was purified from the isolates using a QIAsymphony DSP DNA Mini Kit (Qiagen). The concentration of the genomic DNA in each sample was determined using an ND-1000 spectrophotometer. For comparison, each genomic DNA sample was analyzed by WGS (BGI Genomics).

Concurrent Quantitative and Qualitative Detection of 24 Respiratory Pathogens. A 20-plex MeltArray that includes an additional 4-plex real-time PCR was performed in a 25-μL solution containing PCR master buffer, 7 mM MgCl$_2$, 0.2 mM dNTPs, 2 U *Taq* 01 DNA polymerase, 0.8 μM universal primer, and 5 μL of nucleic acid template (∼10^3 copies/μL plasmid DNA or reverse transcription product from ∼10^3 copies/μL armored RNA unless otherwise indicated). Each reaction also contained 48 tag-sequence–tailed target-specific primers, 7 universal molecular beacon reporters, 20 mediator probes, and 4 molecular beacon probes (the sequences and concentration of which are listed in *SI Appendix*, Table S12). PCR and melting curve analysis was performed in the SLAN 96S real-time PCR instrument as follows: denaturation at 95 °C for 5 min; 50 cycles of 95 °C for 20 s, 60 °C for 1 min; and then 35 °C for 30 min, 95 °C for 2 min, 45 °C for 2 min, followed by a temperature increase from 45 °C to 95 °C (0.04 °C/step). Fluorescence intensity was measured in four detection channels (FAM, HEX, ROX, and Cy5) at the denaturation stage of each of the 50 thermal cycles and at each step during melting curve analysis.

To determine the LOD for each pathogen, serially 10-fold diluted armored RNA or plasmid DNA of known concentration was detected 20 times for each concentration. The lowest concentration level with a detection rate of 95% for positive results was taken as the LOD for each pathogen. The armored RNA (for RNA viruses) or plasmid DNA (for DNA viruses and bacterial pathogens) were provided with the corresponding singleplex real-time PCR assay kit for each pathogen (Zeesan Biotech), which included six concentrations ranging from 1.0×10^6 copies/μL to 1.0×10^1 copies/μL. According to the instruction manual of the kits, the concentrations of the armored RNA and plasmid DNA were estimated by absorbance spectrophotometry and calibrated by reverse transcription–droplet digital PCR (RT-ddPCR) and ddPCR, respectively, using the QX200 Droplet Digital PCR System (Bio-Rad Laboratories).

The assay was evaluated using BALF samples collected from 67 hospitalized pediatric patients who were diagnosed with either pneumonia or a respiratory tract infection by the Henan Provincial Maternal and Child Healthcare Hospital. The age of the patients ranged from 2 mo to 14 y old. The Research Ethics Committee of the hospital approved this study, and informed consent was obtained from the guardians of each patient. Briefly, 200 μL of each BALF sample was used to extract nucleic acid, utilizing a viral extraction kit (Sangon Biotech). A 10-μL reverse transcription reaction containing 1× PrimeScript RT Master Mix (Takara) and 5 μL of the extracted nucleic acid was used to convert RNA to complementary DNA by incubation at 37 °C for 15 min, followed by 85 °C for 15 s to inactivate the reverse transcriptase. Then, 5 μL of each reverse transcription product was used as DNA templates for each 24-plex respiratory assay. The detection results were compared to those obtained from singleplex real-time PCR kits provided by Zeesan Biotech.

Identification of Genotypes of *KRAS* Mutations. This MeltArray assay was used to detect nine *KRAS* mutations located at c.34, c.35, and c.38 within codons 12 and 13. The assay was performed in a 25-μL solution containing PCR master buffer, 7.0 mM MgCl$_2$, 0.2 mM dNTPs, 3 U *Taq* HS DNA polymerase, 0.4 μM of each *KRAS*-specific forward and reverse primer, 10 mediator probes and 4 molecular beacon reporters (the sequences and concentrations of which are listed in *SI Appendix*, Table S13), and 5 μL DNA template (∼20 ng/μL genomic DNA or equivalent copy number of plasmid DNA unless otherwise indicated). PCR and melting curve analysis was performed in an SLAN 96S real-time PCR instrument as follows: denaturation at 95 °C for 3 min; 50 cycles of 95 °C for 15 s, 60 °C for 15 s; and then 35 °C for 30 min, 95 °C for 1 min, 40 °C for 1 min, followed by a temperature increase from 47 °C to 86 °C (0.04 °C/step). Fluorescence intensity was measured in four detection channels (FAM, ROX, HEX, and Cy5) at each step of the continuous temperature increase.

For clinical evaluation, a total of 167 colon cancer tissue samples, including 107 frozen tissue samples and 60 FFPE tissue samples, were collected from Zhongshan Hospital, which is affiliated with Xiamen University. The Research Ethics Committee of the hospital approved this study, and informed consent

was obtained from all patients. Genomic DNA was extracted using a DNeasy Blood & Tissue Kit or a QIAamp DNA FFPE Tissue Kit (both from Qiagen) according to the manufacturer's protocol. The DNA concentration of each sample was determined with an ND-1000 UV-VIS spectrophotometer. The detection results of MeltArray were compared with those of Sanger sequencing (Sangon Biotech), and the discordant results were confirmed by an allele-specific–based AmoyDx *KRAS* Mutation Detection kit that covers *KRAS* codons 12, 13, 59, 61, 117, and 146 (Amoy Diagnostics).

Statistical Analysis. Statistical analyses were carried out using IBM SPSS Statistics software version 23.0 (SPSS Inc.), where $P < 0.05$ was considered statistically significant.

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