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Technical Advance

DNA Diagnostics by Surface-Bound Melt-Curve Reactions

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Melting-curve procedures track DNA denaturation in real time and so provide an effective way of assessing sequence variants. Dynamic allele-specific hybridization (DASH) is one such method, based on fluorescence, which uses heat to denature a single allele-specific probe away from one strand of a polymerase chain reaction product attached to a solid support. DASH is a proven system for research genotyping, but here we evaluate it for DNA diagnostics under two scenarios. First, for mutation scanning (resequencing), a human genomic sequence of 97 bp was interrogated with 15 probes tiled with 12-base overlaps, providing up to fourfold redundancy per base. This test sequence spanned three high-frequency single nucleotide polymorphisms, all of which were correctly revealed in 16 individuals. Second, to score multiple different mutations in parallel, 18 alterations in the *gyrA* gene of *Salmonella* were assessed in 62 strains by using wild-type- and mutation-specific probes. Both experiments were performed in a blinded manner, and all results were confirmed by sequencing. All DNA variants were unambiguously resolved in every sample, with no false-positive or false-negative signals across all of the investigations. In conclusion, DASH performs accurately and robustly when applied to DNA diagnostic challenges, including mutation scoring and mutation scanning. *(J Mol Diagn 2007, 9:30–41; DOI: 10.2353/jmoldx.2007.060057)*

In general terms, DNA diagnostic challenges involve testing the DNA of an individual for sequence variations that could be relevant to the genetic etiology of a particular phenotype in that individual. Applications could range from scoring single base alterations (eg, known pathogenic mutations) in one or a few individuals, through to population-level screening for known or novel changes that might cause disease. At this latter extreme, the challenge overlaps with research into the genetic basis of disease. In practice, most real-world DNA diagnostic activities involve testing particularly likely disease genes in small numbers of individuals to answer one or two clinically relevant questions, are any of a limited set of known pathogenic mutations present in that gene (mutation scoring), and/or are any suspicious changes present anywhere in that gene (mutation scanning)? In addition, chromosome level and structural variation analyses may be conducted, but such investigations are beyond the focus of this current report.

The technologies used for mutation scoring and mutation scanning are mostly distinct, but it would be preferable if diagnostics procedures could be applied equally well to both challenges, using standardized and convenient reaction formats. Such a truly generic DNA diagnostics system is probably still some way off, but it is nevertheless desirable that current method development efforts emphasize solutions that are as flexible as possible. Toward that goal, many factors might be considered, such as:

1. The assay target—Is the objective to score one critical bp, to assess all suspect sites in one or several genes, to resequence several genes/genome regions, or to test extremely large numbers of bases to report on a complete-genome (at least to some degree of depth)?

2. The result precision—Will it be sufficient to determine merely whether a sample DNA is the same as a

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reference sequence, whereas in other scenarios, the precise location and nature of extant changes relative to the reference must be elucidated?

3. The reaction chemistry—How important are factors such as the need for standard run conditions versus assay-specific optimization, the cost per result, and the speed of data generation?

4. The reaction format—How desirable is it that the procedure is homogenous (sealed tube) rather than nonhomogenous or single-step rather than multistep in its design?

5. The required equipment—Will the method be run on generic or method-specific devices, how expensive and easy to use will those instruments be, and what throughput potential will they have?

Given so many diverse method features to consider, it is hard to imagine that one technology will ever fully satisfy the most extreme requirements imposed by all these criteria. It is also likely that complex and consequentially temperamental solutions will probably not be the way forward because such technologies are more sensibly reserved for highly specialized applications. Instead, to progress toward a generic solution it will be most effective to use robust and elegant underlying reaction principles. In this respect it is, therefore, not surprising that straightforward DNA hybridization—the simplest and most direct way to assess a DNA sequence—is now being increasingly exploited in the field of DNA diagnostics.

The power of hybridization for DNA diagnostics stems from the fact that subtle sequence changes impose substantial changes in duplex stability such that, when assays are suitably formatted, each and every DNA sequence change can be reliably detected by direct or indirect measurement of that duplex stability. No additional enzymes or processing steps are required, and assays can be kept simple, cheap, and convenient. Hybridization methods may be designed to detect (but not specify) any difference relative to a reference sequence—as in denaturing high-performance liquid chromatography,[1,2] denaturing and temperature gradient gel electrophoresis,[3–5] single-strand conformational polymorphism analysis,[6,7] and high-resolution amplicon melting analysis.[8,9] These procedures subject test samples to dynamically changing environments that transition from low to high stringency and thereby exploit duplex stability differences and enable underlying mutations to be revealed. This same dynamic melting concept has also been used to locate and identify individual base changes by tracking the melting behavior of short normal or mutant oligonucleotides (probes) hybridized in solution to one strand of a target fragment produced by polymerase chain reaction (PCR) amplification. Melting profiles from single-labeled probes[10–12] or dual-labeled probes[13,14] can be recorded by their change in fluorescence during denaturation. Far higher throughput hybridization analyses are made possible by using array formats on solid surfaces—such as dot blot and related methods[15,16]—wherein static, high-stringency reactions are used to quantitatively assess probe-target binding. Such arrays have since been developed into microarray formats[17,18] so that many thousand different dispersed target sites can be tested in parallel. The same technology has also enabled long contiguous runs of bases to be examined on so-called resequencing chips.[19,20]

Unfortunately, the static conditions used in high-throughput array hybridization systems fail to distinguish all sequence changes in all contexts. Logically then, it would make sense to couple the generic utility and power of dynamic hybridization to the throughput capabilities provided by array-formatted assays. This has previously been explored in the dynamic allele-specific hybridization (DASH) research genotyping method, which uses heat to control probe-target denaturation[21–23] and electronic microchips (eg, Nanogen, San Diego, CA), which uses an electrical field to control probe-target denaturation.[24,25] The DASH system is further detailed in Figure 1, and our extensive experience with this method for genotyping single nucleotide polymorphisms (SNPs) (more than 4000 target sites examined, producing ~2 million genotypes) has shown that single probes applied by this method reliably detect >95% of all sequence variants under standard run conditions, with a routine accuracy of ~99.9%. DASH has been implemented as a microtiter plate-based version (DASH-1[21]) and as a membrane-based macroarray format that interrogates up to 10,000 samples per array (DASH-2[23]). DASH has also shown itself highly effective as a means to score insertion/deletion variants[26] and, via its quantitative capabilities, has recently enabled us to reveal the existence of extensive copy number variation in the human genome.[27]

Given the proven capabilities of DASH in the SNP genotyping arena, and the considerable needs of DNA diagnostics, we reasoned that DASH might be effective as an advanced hybridization platform for DNA diagnostics. We therefore explored this possibility, both in the context of scanning for mutations in a human genomic sequence and for the simultaneous scoring of many commonly mutated sites in quinolone-resistant *Salmonella* strains. This latter application represents a significant real-world example of troublesome bacterial antibiotic resistance. Quinolone resistance often arises because of spontaneous point mutations that cause amino acid substitutions within the topoisomerase subunits, often in combination with decreased expression of outer membrane porins or overexpression of multidrug efflux pumps.[28–30] We report the findings here, leading to the conclusion that both DASH-1 and a newly adapted version of DASH-2 are highly accurate and useful for DNA diagnostic applications.

Materials and Methods

DNA Samples

Human DNA samples from 16 unrelated Swedish females were prepared by standard phenol-chloroform extraction procedures. *Salmonella* DNA was prepared from 62 *Salmonella enterica* isolates received at the Health Protection Agency Centre for Infections *Salmonella* Reference Unit between 1991 and 2002. DNA from these strains was prepared from 24-hour cultures using a Qiagen DNeasy

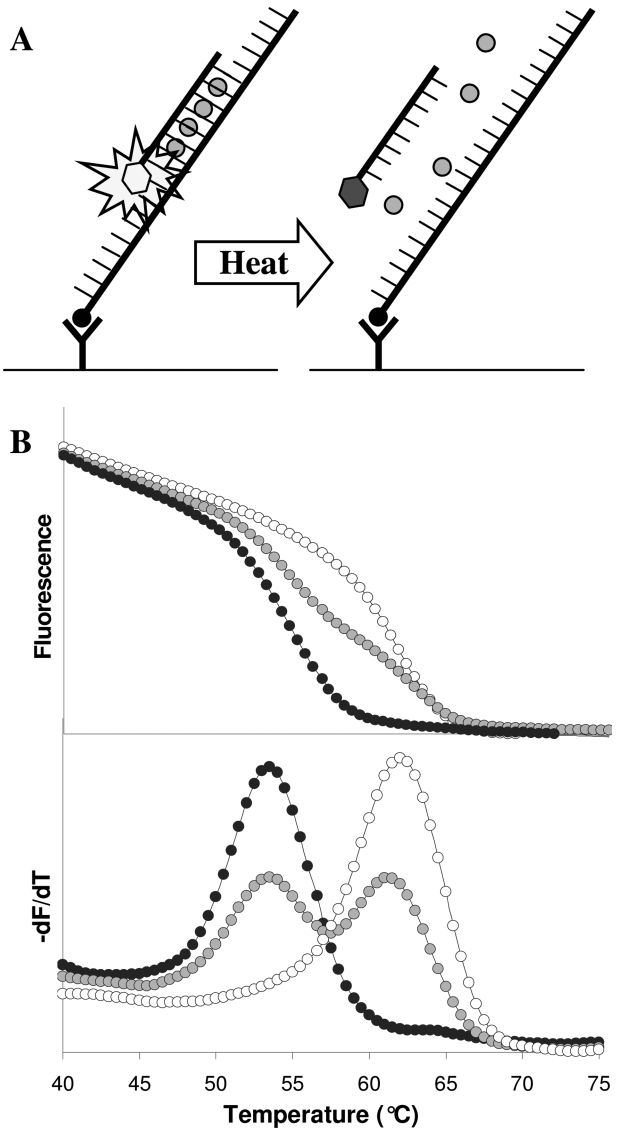

Figure 1. The DASH procedure. **A:** One strand of a PCR product is bound to a solid surface by means of a capture molecule built into one of the PCR primers. To interrogate a polymorphic position in this fragment, a single sequence-specific oligonucleotide probe is hybridized to it at low stringency. Fluorescence signals proportional to the amount of hybridized probe are generated, eg, by means of iFRET[33] using a ROX fluorescence acceptor (hexagon shape) at one end of the probe plus a double-strand-specific dye (**gray circles**) that functions as a fluorescence donor. The sample is steadily heated under standard run conditions, which will cause the probe-target duplex to denature and the ROX fluorescence to decrease. The temperature at which the rate of fluorescence decrease is maximal indicates the melting temperature (Tm) of the probe-target duplex, and this is directly related to the degree of bp matching between the probe and the target. **B:** The melting behavior of the probe-target duplex is analyzed by constructing fluorescence versus temperature melt-curves. By plotting the negative derivative of fluorescence versus temperature, melting events are revealed as peaks at high (**white circle plots**) or low (**black circle plots**) temperatures, representing matched and mismatched probe-target duplexes, respectively. Heterozygous targets (**gray circle plots**) show two peaks of melting behavior.

tissue kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions. In addition, DNA from seven of the strains were also prepared using a simpler method: a single colony was resuspended in 100 μl of distilled water with 15% (w/v) Chelex 100 molecular biology grade resin (Bio-Rad, Hertfordshire, UK) and boiled for 10 minutes. The cell suspension was then centrifuged for 5 minutes at 13,000 rpm, and the supernatant removed and stored at −20°C until required.

Primers and Probes

Oligonucleotides (PCR primers and probes) were obtained from Thermo Electron GmbH (Ulm, Germany) and Biomers.net GmbH (Ulm, Germany). One of the primers in a primer pair contained a 5′-biotin group, and all probes were labeled with a 3′-ROX moiety. Primers were designed using the OLIGO software (Molecular Biology Insights, Inc., Cascade, CO). Basic rules for primer design were primer length restricted to 20 to 24 bp, primer Tm difference <5°C for each primer pair, and a maximum of 3-bp complementarity to the 3′ end of any primer. A full list of primer and probes sequences is provided in Tables 1 and 2.

PCR

PCR reactions of 5 to 20 μl contained 0.375 μmol/L 5′-biotinylated primer, 0.75 μmol/L of nonlabeled primer, 0.03 U/μL AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 1× AmpliTaq Gold buffer, 1.5 mmol/L $MgCl_2$, 5% dimethyl sulfoxide, and 0.2 mmol/L of each dNTP. Human genomic DNA PCRs contained 0.1 to 0.5 ng of DNA per μl. *Salmonella* PCRs contained typically 0.5 to 2.5 ng of DNA per μl. Thermal cycling was performed on either a 96-well (for DASH-1) or a 384-well (for DASH-2) MultiBlock system (Thermo Scientific, Basingstoke, UK). Thermal cycling consisted of an initial activation step of 94°C for 10 minutes, followed by 40 cycles of 94°C for 15 seconds and 55°C for 30 seconds. For longer PCR products (>200 bp), thermal cycling consisted of an initial activation step of 94°C for 10 minutes, followed by 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 1 minute.

Sequencing

To sequence the human genomic DNA fragment, a 300-bp fragment encompassing the 97-bp region of interest using the primers LSCAN-23F and LSCANb22R (Table 1). Fifty-μl PCR products were purified using a MinElute PCR purification kit (Qiagen) following the manufacturer's protocol and eluting the PCR products in water. One μl of each eluted product (~25 ng) was used as template for cycle sequencing that used a BigDye 3.1 terminator kit (Applied Biosystems), using 0.16 μmol/L of either of the initial amplification primers in 20-μl reactions. Cycling conditions consisted of 96°C for 1 minute followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes using a 96-well MultiBlock system (Thermo Electron Corporation). Sequencing products were purified using DyeEx 2.0 spin columns (Qiagen) following the manufacturer's protocol, and the samples were finally sequenced on a 48-capillary 3730 DNA analyzer (Applied Biosystems).

Sequence data for the *Salmonella gyrA* gene was generated from a 342-bp fragment amplified using primers P1 (5′-TGTCCGAGATGGCCTGAAGC-3′) and P2 (5′-TAC-

Table 1. Primer and Probe Sequences for Mutation Scanning

Name	Chemistry	Sequence
LSCAN-01F	None	5′-TAACTTACTAGGAGCTTTTAATGG-3′
LSCAN-02F	None	5′-TCATTGTAGACTGTCTTAATTGC-3′
LSCAN-03F	None	5′-TCTATCTGTCTTACCTCATCACC-3′
LSCANb04R	5′-Biotin	5′-AATAGAGTGAAATGTATGATTGG-3′
LSCANb05R	5′-Biotin	5′-TATTTGCTAATCTCTGAGAAGGC-3′
LSCANb06R	5′-Biotin	5′-CTGTCAGTTTTACACAATTCATC-3′
LSCANb22R	5′-Biotin	5′-ATATTAGCCTCATATTTGGGAAG-3′
LSCAN-23F	None	5′-AGATGGGTAAGAAATGTAGTTAG-3′
LSCAN-24F	None	5′-TAAAACAAGACTACGTGTCACTG-3′
LSCAN + 07P	3′-ROX	5′-ATTTATCCGTCTTGCCA-3′
LSCAN + 08P	3′-ROX	5′-TCCGTCTTGCCATATGA-3′
LSCAN + 09P	3′-ROX	5′-CTTGCCATATGACTAAG-3′
LSCAN + 10P	3′-ROX	5′-CATATGACTAAGCAGAT-3′
LSCAN + 11P	3′-ROX	5′-GACTAAGCAGATCAACA-3′
LSCAN + 12P	3′-ROX	5′-AGCAGATCAACAAATAC-3′
LSCAN + 13P	3′-ROX	5′-ATCAACAAATACCTATC-3′
LSCAN + 14P	3′-ROX	5′-CAAATACCTATCACTCA-3′
LSCAN + 15P	3′-ROX	5′-ACCTATCACTCACACAC-3′
LSCAN + 16P	3′-ROX	5′-TCACTCACACACTAGAA-3′
LSCAN + 17P	3′-ROX	5′-CACACACTAGAAATGAA-3′
LSCAN + 18P	3′-ROX	5′-ACTAGAAATGAAACAAG-3′
LSCAN + 19P	3′-ROX	5′-AAATGAAACAAGCTTTA-3′
LSCAN + 20P	3′-ROX	5′-AAACAAGCTTTAATGCA-3′
LSCAN + 21P	3′-ROX	5′-AGCTTTAATGCACACAG-3′

CGTCATAGTTATCCACG-3′).[31] Fifty-μl PCR products were purified using a Unifilter 96-well microplate (Whatman, Middlesex, UK) following the manufacturer's protocol. Two μl of each eluted product was used as template for cycle sequencing using 10 pmol of primer P1 and a CEQ Dye Terminator cycle sequencing quick start kit (Beckman Coulter, Buckinghamshire, UK) in 20-μl reactions. Cycling conditions consisted of 30 cycles of 96°C for 20 seconds, 50°C for 20 seconds, and 60°C for 4 minutes using a 96-well MultiBlock system (Thermo Electron Corporation). Sequencing products were purified by following the Dye Terminator cycle sequencing quick start kit protocol and the

Table 2. Primer and Probe Sequences for *gyr*A

Name	Chemistry	Sequence	Probe allele
LGyrAb03R	5′-Biotin	5′-GCTGCGCtATACGAACGATG-3′	
LGyrA-04F		5′-CAATGACTGGAACAAAGCCTATAA-3′	
LGyrAb07R	5′-Biotin	5′-CCGTACaGTCATAGTTATCCACG-3′	
LGyrA-08F		5′-GTTCTATTGAaGGCGACTCCG-3′	
GyrA1 + 01p	3′-ROX	5′-AAAAATCTGCCCGTGTC-3′	Ala67wt
GyrA1 + 02p	3′-ROX	5′-AAAAATCTCCCCGTGTC-3′	Pro67
GyrA2 + 01p	3′-ROX	5′-CGTTGGTGACGTAATCG-3′	Asp72wt
GyrA2 + 02p	3′-ROX	5′-CGTTGGTGGCGTAATCG-3′	Gly72
GyrA3 + 01p	3′-ROX	5′-CCACGGCGATTCCGCAG-3′	Gly81Asp82Ser83wt
GyrA3 + 02p	3′-ROX	5′-CCACTGCGATTCCGCAG-3′	Cys81
GyrA3 + 03p	3′-ROX	5′-CCACAGCGATTCCGCAG-3′	Ser81
GyrA3 + 04p	3′-ROX	5′-CCACGGCAATTCCGCAG-3′	Asn82
GyrA3 + 05p	3′-ROX	5′-CCACGGCGGTTCCGCAG-3′	Gly82
GyrA3 + 06p	3′-ROX	5′-CCACGGCGATTTCGCAG-3′	Phe83
GyrA3 + 07p	3′-ROX	5′-CCACGGCGATTACGCAG-3′	Tyr83
GyrA3 + 08p	3′-ROX	5′-CCACGGCGATGCCGCAG-3′	Ala83
GyrA4 + 01p	3′-ROX	5′-CAGTGTATGACACCATC-3′	Asp87wt
GyrA4 + 02p	3′-ROX	5′-CAGTGTATGGCACCATC-3′	Gly87
GyrA4 + 03p	3′-ROX	5′-CAGTGTATAACACCATC-3′	Asn87
GyrA4 + 04p	3′-ROX	5′-CAGTGTATTACACCATC-3′	Tyr87
GyrA5 + 01p	3′-ROX	5′-CGCGGCGGCAATGCGTT-3′	Ala119wt
GyrA5 + 02p	3′-ROX	5′-CGCGGCGGTAATGCGTT-3′	Val119
GyrA5 + 03p	3′-ROX	5′-CGCGGCGTCAATGCGTT-3′	Ser119
GyrA5 + 04p	3′-ROX	5′-CGCGGCGGAAATGCGTT-3′	Gly119
GyrA6 + 01p	3′-ROX	5′-GAAAATCGCCCACGAAC-3′	Ala131wt
GyrA6 + 02p	3′-ROX	5′-GAAAATCGGCCACGAAC-3′	Gly131
GyrA7 + 01p	3′-ROX	5′-CGATCTCGAAAAAGAGA-3′	Glu139wt
GyrA7 + 02p	3′-ROX	5′-CGATCTCGCAAAAGAGA-3′	Ala139
GyrA8 + 01p	3′-ROX	5′-ACGGTGGATTTCGTGGA-3′	Asp144wt
GyrA8 + 02p	3′-ROX	5′-ACGGTGGACTTCGTGGA-3′	Asp144mut

samples analyzed using a CEQ 8000 DNA analysis system (Beckman Coulter) using the LFR-1 run conditions. Resulting sequencing data are available as supplemental material at *http://jmd.amjpathol.org*.

DASH-1

DASH-1 was conducted as described previously.[21] In brief, PCR products were diluted 1:1 with HEN buffer (0.1 mol/L Hepes, 10 mmol/L ethylenediaminetetraacetic acid, and 50 mmol/L NaCl, pH 7.5), and 20 μl per well was bound to a 96-well streptavidin-coated microtiter plate. The solution was then removed, and the wells were rinsed once with 25 μl of 0.1 mol/L NaOH to elute the unbound (nonbiotinylated) strand of the PCR product. A 25-μl solution containing HEN plus 15 pmol allele-specific probes was added. The microtiter plate was sealed, heated to 85°C, and air-cooled to 25°C for ~5 minutes, enabling the probe to hybridize to the bound PCR product (regardless of which alleles were present). The solution was replaced with HEN containing SYBR Green I dye (Molecular Probes/Invitrogen, Paisley, UK) at a 1:10,000 dilution. The plates were analyzed in a DASH instrument (Thermo Hybaid, although any Q-PCR machine would suffice), and fluorescence was recorded while heating from 35 to 85°C at a rate of 0.3°C/second.

DASH-2

DASH-2 was conducted in a manner similar to that previously described,[23] with a few changes that enabled the diagnostics application. PCR products were first transferred from a 384-well microtiter plate to a streptavidin-coated polypropylene membrane via centrifugation as previously described.[32] To achieve this, the membrane (DynaMetrix Ltd., Hertfordshire, UK, *http://www.dynametrix-ltd.com*) was premoistened in HE buffer (0.05 mol/L Hepes and 5 mmol/L ethylenediaminetetraacetic acid, pH 7.5) and placed over the open wells of the microtiter plate. The arrangement was compressed in a clamping device and centrifuged at 1500 rpm for 30 seconds in a suitable device (S20 rotor; B4i Jouan; Thermo Scientific). After binding at room temperature for 30 minutes, the clamped structure was inverted and briefly centrifuged to return the bulk fluid into the microtiter plate wells. The membranes were then rinsed once in a 0.1 mol/L NaOH bath for 2 minutes to remove nonbiotinylated PCR product strands and once in HE for neutralization.

To apply different probes to distinct locations on the same membrane, 10 pmol/μl of appropriate probe solution in HE buffer was placed in the matching well of a 384-well plate, and this was transferred to cover the membrane area where PCR product had been bound using the same clamping device and centrifuge as described above. Excess probe solution was immediately transferred back to the wells of the plate. The membrane was then recovered from the clamp and placed in a sandwich of two 8 × 12-cm glass plates (slightly larger than the membrane) to form a hybridization chamber. This was heated to 85°C on a flat PCR block (PCR Express; Thermo Electron Corporation) and air-cooled to room temperature to assist probe annealing. A final rinse was performed in HE to remove excess probe.

To execute the dynamic melt procedure, membranes were soaked for 1 hour in HE buffer containing a 1:20,000 dilution of supplied stock SYBR Green I dye (Molecular Probes), and they were then individually sandwiched between two glass plates and placed into a DASH-2 genotyping device (DynaMetrix Ltd.). Fluorescence images and feature intensity values were collected while heating the membrane assembly from 35 to 85°C (with a heating rate of 3°C/minute) by imaging every 0.5°C.

Melt-Curve Analysis

Output fluorescence data files were imported into purpose-built software (DynaScore; DynaMetrix Ltd.). Using this tool, melt-curves were examined for each microtiter plate well (DASH-1) or array feature (DASH-2), and probe-target denaturation events were visualized by plotting negative derivatives curves of the fluorescence signal versus temperature. A single high-temperature peak indicated the sample was completely matched to the probe sequence. A single low temperature peak indicated a 1- or 2-bp mismatch compared with the probe sequence (two-base mismatches cause much larger decreases in melting peak temperature, and more than two-base mismatches fall below the temperature window examined). If peaks were seen at both temperatures, this indicated that two alleles were present in the PCR product, such as would occur with a human heterozygous sample.

Results

Scanning for Unknown Mutations

To reliably scan all bases in a gene for mutations, a method must be able to detect essentially all possible base changes in any sequence context, preferably under standard run conditions. To explore the ability of DASH to achieve this, we conducted a blinded experiment in which a series of probes were designed to detect any sequence variation that might exist in a 97-bp (typical coding exon sized) human genomic DNA fragment. This target fragment was chosen based on the fact it was known to span three common SNPs, for which the dbSNP rsIDs are rs917188[C/T], rs917189[C/G], and rs917190[C/A]. Figure 2 illustrates the target genomic region, showing the relative SNP, PCR primer, and DASH probe locations.

The experiment was first conducted using the DASH-1 platform. PCR products of 139 to 1904 bp in length (14 combinations of five forward and four reverse primers), all encompassing the 97-bp test sequence, were bound separately to microtiter plate wells and interrogated by 15 different probes in parallel. The probes were 17 bp long (known optimum length for DASH), and their sequences partially overlapped to create a redundant tiling path across the assayed region. In this way, each base was being scanned by at least three probes (providing redun-

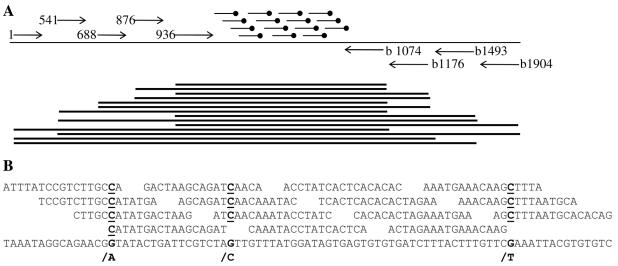

Figure 2. Schematic diagram of the human genomic sequence scanned for mutations. **A:** Overview of the target region taken from chromosome 7. PCR primers are drawn as **black arrows** (→) indicating 5′ to 3′ orientation, and the number behind each primer indicates the relative position of its 5′ base in the target sequence. Primers labeled with a "b" carried 5′-biotin residues to enable surface immobilization in the DASH procedure. A total of 14 different PCR products, with lengths from 139 to 1904 bp, were amplified using different combinations of the forward and reverse primers, indicated by **black lines**. Those products were analyzed by the 15 DASH probes, here labeled with **black circles** representing the 3′-ROX groups. **B:** The sequences of the DASH probes relative to the 97-bp region they are designed to interrogate. SNP locations are marked as bold underlined characters in the probes, and the two possible alleles of the target sequence are shown in the complementary target sequence.

dancy of information), and any altered bases would be located at various positions within probe molecules (in case this affected performance). In cases in which probes covered known SNP positions, they were designed to match the highest frequency allele. The full set of probes was used to interrogate four human genomic DNAs, and several sequence variants were thereby revealed. Shorter PCR products produced particularly strong and clear results in terms of peak height of the derivative signal, and only when target fragments were more than 500 to 600 bp in length did the signals start to become too weak to allow definitive melting points to be determined (Figure 3).

Given the above findings, we next concentrated on one of the targets, a 387-bp PCR product (PCR primers LSCAN-24F and LSCANb04R) amplified from the full test set of 16 unrelated individuals, and examined this with

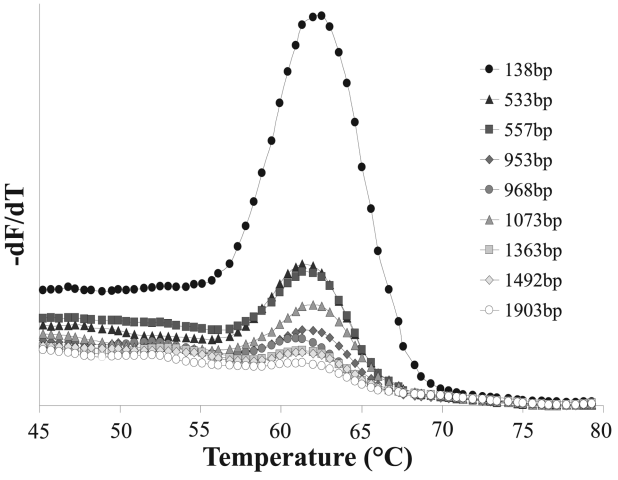

Figure 3. The effect of PCR product length on result quality. A DNA sample was amplified with different primer pairs to create products of the indicated lengths from 138 to 1903 bp, all spanning the core 97-bp target sequence of interest. The melt curves that were generated by interrogating these amplicons with one example probe (LSCAN + 18P) are shown, illustrating the effect of increasing product length on data quality.

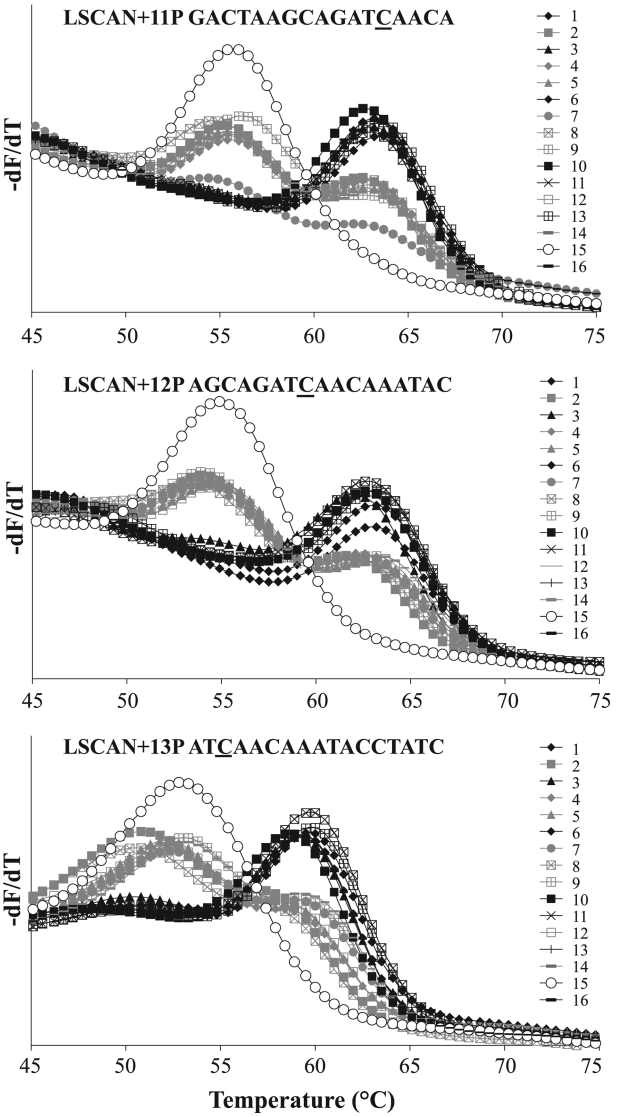

Figure 4. Example data from mutation scanning. One C/G SNP, rs917189, is detected by three overlapping probes that all match the C allele. The three probes detect sequence alterations consistently, as shown by comparing melting temperatures for 16 different human DNA samples. The samples labeled in black are perfectly matched to the probe, giving the highest temperature melting peak, the sample labeled in white is homozygous for the other allele (has 1 bp mismatched to the probe) and therefore produces a peak at a lower melting temperature, and the samples labeled in gray are heterozygous and generate peaks for both alleles.

the full set of tiled probes. Homozygous and heterozygous patterns were consistently detected by the probes for every known SNP site, and all probes covering the same SNP sites detected the mutations equally well (Figure 4), with only one exception. The solitary case in which one probe failed to detect a real sequence variation was arranged such that the polymorphic site was located at the very 5′ base of the probe. Only this probe (LSCAN + 10P) for SNP rs917188 was aligned in this extreme manner, and this caused it to generate very similar melting profiles for both alleles every time it was used. Homozygous and heterozygous versions of rs917188 were, however, clearly revealed by the three other probes that overlapped this site. Outside the SNP positions, no other

Table 3. Results of Mutation Scanning by DASH Compared with DNA Sequencing

	rs917188		rs917189		rs917190	
Sample	DASH	Sequence	DASH	Sequence	DASH	Sequence
1	Match	C/C	Match	C/C	Match	C/C
2	Match	C/C	Het	C/G	Het	C/A
3	Match	C/C	Match	C/C	Match	C/C
4	Het	C/T	Het	C/G	Het	C/A
5	Match	C/C	Het	C/G	Het	C/A
6	Match	C/C	Match	C/C	Match	C/C
7	Het	C/T	Het	C/G	Het	C/A
8	Het	C/T	Het	C/G	Het	C/A
9	Het	C/T	Het	C/G	Het	C/A
10	Het	C/T	Match	C/C	Match	C/C
11	Het	C/T	Match	C/C	Match	C/C
12	Match	C/C	Het	C/G	Het	C/A
13	Match	C/C	Match	C/C	Match	C/C
14	Match	C/C	Het	C/G	Het	C/A
15	Match	C/C	Mism.	G/G	Mism.	A/A
16	Het	C/T	Match	C/C	Match	C/C

Mutation scanning with DASH detects sequence variants consistent with DNA sequencing. The DASH result is combined for all probes overlapping each SNP site. The DASH probes always had a C at the polymorphic positions, and the Match result indicates a homozygous sample completely complementary to the probe, Mism indicates a homozygous sample with one base pair mismatch compared with the probe, and Het indicates a heterozygous sample. The sequencing data are shown for the same strand as the probe.

altered sequences were detected by any probe on any DNA sample. All detected variants at the three SNP sites were subsequently confirmed by sequencing of the 16 test samples (Table 3), and no other sequence variants were revealed by this direct sequence analysis. Thus, in this blinded experiment, DASH correctly identified all of the mutations in their homozygous and/or heterozygous states, with false-negative and false-positive rates of zero (given 3104 bases examined, including 23 heterozygous and two homozygous differences relative to the reference sequence).

Core parts of this experiment were next rerun on the DASH-2 platform, as a basis for ensuring our subsequent high-throughput experiments would give equivalent data quality to the DASH-1 test. For this, the 387-bp PCR products from three informative samples were bound to membrane arrays with a density of 384 features per membrane, and the full tiling set of probes was used to interrogate the immobilized amplicons. In DASH-2, a single probe is normally hybridized to the whole membrane, but in the current experiment we needed to apply different probes to distinct membrane features (the different bound target amplicons). To solve this problem, we adapted the method used for transferring the PCR products to the membrane, as detailed in Materials and Methods. This worked effectively, and the results of the DASH-2 mutation scanning experiment were fully equivalent to the findings produced by DASH-1.

Simultaneous Scoring of Multiple Known Mutations

A common DNA diagnostics challenge entails scoring for the presence of a fairly limited number of mutations that are known to often cause the phenotype of interest. Current methods usually struggle if the number of potential mutations is more than ~5, except when the target sites are fortuitously closely located and a sequencing procedure is applied. Therefore, to provide a particularly challenging real-world test case for mutation scoring by DASH, we assessed how well it could detect quinolone resistance-determining SNPs in the *gyrA* gene of *Salmonella enterica*—a scenario entailing an ~300-bp target region that must be assessed in parallel for 18 different base mutations that may occur singly or in any combination.

As detailed in Table 2 and Figure 5, eight probes corresponding to the wild-type sequence of this gene and 18 mutation-specific probes (all probes being 17 bases long) were designed to interrogate 18 previously reported mutations[28] in 10 codons of the *gyrA* gene. For each short coding region to be interrogated, a set of one normal and several mutation probes were designed that spanned a fixed stretch of nucleotide positions and had the potentially mutated bases located toward the center. DNA samples from 62 different *Salmonella* stock strains, for which the mutation patterns were known (from previous DNA sequencing), were then examined by DASH-2 using these probes. The DNA samples were delivered to our analysis laboratory and processed blindly to the mutations present in each sample. The target gene was amplified as two separate PCR products, and these were examined in parallel by DASH-2 using standard run conditions. Several (24 × 16) arrays were used in parallel with one sample per row, and the probes were applied column by column.

The DASH results produced by this experiment showed that specific probe melting temperatures for different samples fell into distinct classes (matched and mismatched patterns), spaced by 2.5 to 8°C—consistent with the usual resolution of DASH.[21,22] The findings are summarized in Table 4 and Figure 6. From the melt curves we were able to readily interpret the mutational status at each site of interest in each sample (ie, by simply considering whether or not the tested sites matched each of the normal and mutant probes). This analysis was aided in this particular experiment by the

Figure 5. Alignment of primers and probes for the *gyrA* gene. Primers and probes are aligned to the wild-type (wt) sequence of the *Salmonella gyrA* gene (GenBank accession no. X78977). There are probes complementary both to the wt sequence and to 18 different mutations within the gene. The name after each probe indicates which mutation to which it is complementary.

fortuitous fact that each tested site was normal in many different samples (giving a benchmark Tm in each case for the normal sequence against the normal probe). The fact that the samples were normal was obvious from the total set of melt-curve patterns we observed. We therefore did not need to include additional known reference samples, although this might obviously be done in any routine use of the method. General use of the method would, of course, require that inclusion of a probe for every mutation to be detected. Data analysis was aided by the fact that the assay design entailed several levels of redundancy. Specifically, mutant sample sites could first be identified by the fact they were mismatched to the normal probe, whereas all other samples (the majority of samples) matched the normal probe at those sites. Using mutation-specific probes then provided secondary confirmation that the set of nonnormal sample sites were indeed mutated (ie, they matched one of these probes, unlike the normal samples), at the same time elucidating the exact identity of each mutation. For all codons for which more than one mutation was being assessed (including some mutations that involved two base changes relative to the wild-type gene), various distinct melt-curve patterns were observed across the set of normal and mutant probes that additionally confirmed what mutations must be present at each site. For example, mutation A at a site in a sample would have two base mismatches relative to a probe for a mutation B at that site (in contrast to zero or one base mismatches relative to the mutation A probe or the normal probe, respectively), and those extra mismatched bases imposed informative greater decreases in probe-melting temperatures.

On unblinding the experiment, we found that all 62 strains had been accurately genotyped at all their mutant and all their normal sites (Table 4), showing consistent results for the seven samples prepared in duplicate. These included 101 instances of seven different mutations affecting amino acids 83, 87, and 144, and these were distributed across 59 DNA samples. Among these, 31 had two or three different mutations within the gene. The other three strains carried no mutations whatsoever, and in these DNAs no mutations were reported by DASH. Thus, in this single-run blinded experiment, DASH correctly identified all of the existing mutations, with false-negative and false-positive rates of zero [given interrogation of 1116 (18×62) different potentially mutated loci].

Discussion

We have evaluated DASH—an effective SNP genotyping method used in genetics research—for its utility in DNA diagnostics. Overall, the method was found to be very effective at both mutation scanning and mutation scoring, with good utility for parallel analysis of many different sites of potential mutation. These findings are in line with a growing recognition that melt-curve analysis is an elegantly simple yet very flexible, robust, and accurate way to examine DNA sequence. This power stems from the use of dynamic hybridization conditions that enable standard run conditions to be used and essentially all base mutations to be detected in all sequence contexts. This contrasts with static hybridization procedures for which target-specific conditions must be established, with consequential limitations on what range and number of different targets can be accurately assessed in any one experiment. DASH additionally benefits from having the target sequences immobilized on a surface. This allows higher throughput array application, and because denaturation events are essentially unidirectional (the probe diffuses away from the bound target, and so it cannot rehybridize), it engenders improved quality data over alternative solution-phase melt-curve systems.

Our study, along with previously published findings, showed that DASH produces highly accurate data in terms of diminutive false-positive and false-negative rates. We actually observed no false-positive and no false-negative signals overall in the current investigation, with only one redundant probe failing to detect a real

Table 4. Results of *gyrA* Mutation Detection

Sample \ Sequence	Ala67 wt	Pro67	Asp72 wt	Gly72	Gly81,Asp82,Ser83 wt	Cys81	Ser81	Asn82	Gly82	Phe83	Tyr83	Ala83	Asp87 wt	Gly87	Asn87	Tyr87	Ala119 wt	Val119	Ser119	Gly119	Ala131 wt	Gly131	Glu139 wt	Ala139	Asp144 wt	Asp144mut
P3017450	0		0		0								0				0				0		0		0	
P3424780 (2)	0		0		0									X			0				0		0		0	
P3749380 (2)	0		0							X			0				0				0		0		0	
P379169	0		0								X		0				0				0		0		0	
P3801900 (2)	0		0		0										X		0				0		0		0	
P3907140	0		0		0										X		0				0		0		0	
P398351	0		0									X			X		0				0		0		0	
P4103430	0		0		0										X		0				0		0		0	
P4111690 (2)	0		0		0										X		0				0		0		0	
P414646	0		0		0										X		0				0		0		0	
P415310	0		0							X			0				0				0		0		0	
P4156400 (2)	0		0		0									X			0				0		0		0	
P4244791	0		0		0										X		0				0		0		0	
P4281520	0		0		0										X		0				0		0		0	
P4389690	0		0		0										X		0				0		0		0	
P4418140	0		0							X			0				0				0		0		0	
P457821	0		0									X			X		0				0		0		0	
P479862	0		0		0								0				0				0		0		0	
P490374	0		0							X					X		0				0		0		0	
P529039	0		0							X					X		0				0		0		0	
P532305	0		0							X					X		0				0		0		0	
P534461	0		0							X				X			0				0		0		0	
P535534	0		0							X					X		0				0		0		0	
P537713	0		0							X					X		0				0		0		0	
P5669150	0		0		0											X	0				0		0		0	
P5685991	0		0							X			0				0				0		0		0	
P5686210	0		0		0											X	0				0		0		0	
S144577	0		0		0										X		0				0		0		0	
S158112	0		0								X			X			0				0		0			X
S158777	0		0							X				X			0				0		0		0	
S159797	0		0							X					X		0				0		0		0	
S160376 (2)	0		0		0								0				0				0		0			X
S166488	0		0								X			X			0				0		0			X
S166492	0		0								X			X			0				0		0			X
S166495	0		0								X			X			0				0		0			X
S171493	0		0							X					X		0				0		0		0	
S173649	0		0								X			X			0				0		0			X
S176476	0		0							X				X			0				0		0		0	
S180085	0		0							X				X			0				0		0		0	
S180497	0		0								X			X			0				0		0			X
S184117	0		0								X		0				0				0		0			X
S186737	0		0								X			X			0				0		0			X
S187874 (2)	0		0								X			X			0				0		0			X
S191076	0		0								X			X			0				0		0			X
S193354	0		0							X				X			0				0		0		0	
S196400	0		0							X				X			0				0		0		0	
S198849	0		0							X				X			0				0		0		0	
S199046	0		0								X			X			0				0		0			X
S200060	0		0								X			X			0				0		0			X
VLA 153	0		0							X			0				0				0		0			X
VLA 160	0		0		0								0				0				0		0			X
VLA 250	0		0		0										X		0				0		0		0	
VLA 257	0		0							X			0				0				0		0		0	
VLA 263	0		0		0									X			0				0		0		0	
VLA 266	0		0								X		0				0				0		0		0	
VLA 275	0		0							X			0				0				0		0			X
VLA 282	0		0		0									X			0				0		0		0	
VLA 322	0		0							X			0				0				0		0			X
VLA 327	0		0							X			0				0				0		0		0	
VLA 336	0		0								X		0				0				0		0		0	
VLA 338	0		0								X		0				0				0		0		0	
VLA 400 (wt)	0		0		0								0				0				0		0		0	

A 0 indicates a sequence match to a wild-type (wt) probe, and X indicates match to a mutation-specific probe. Samples marked with (2) were analyzed in duplicate, with DNA from purified genomic DNA and boiled cell template both giving consistent results.

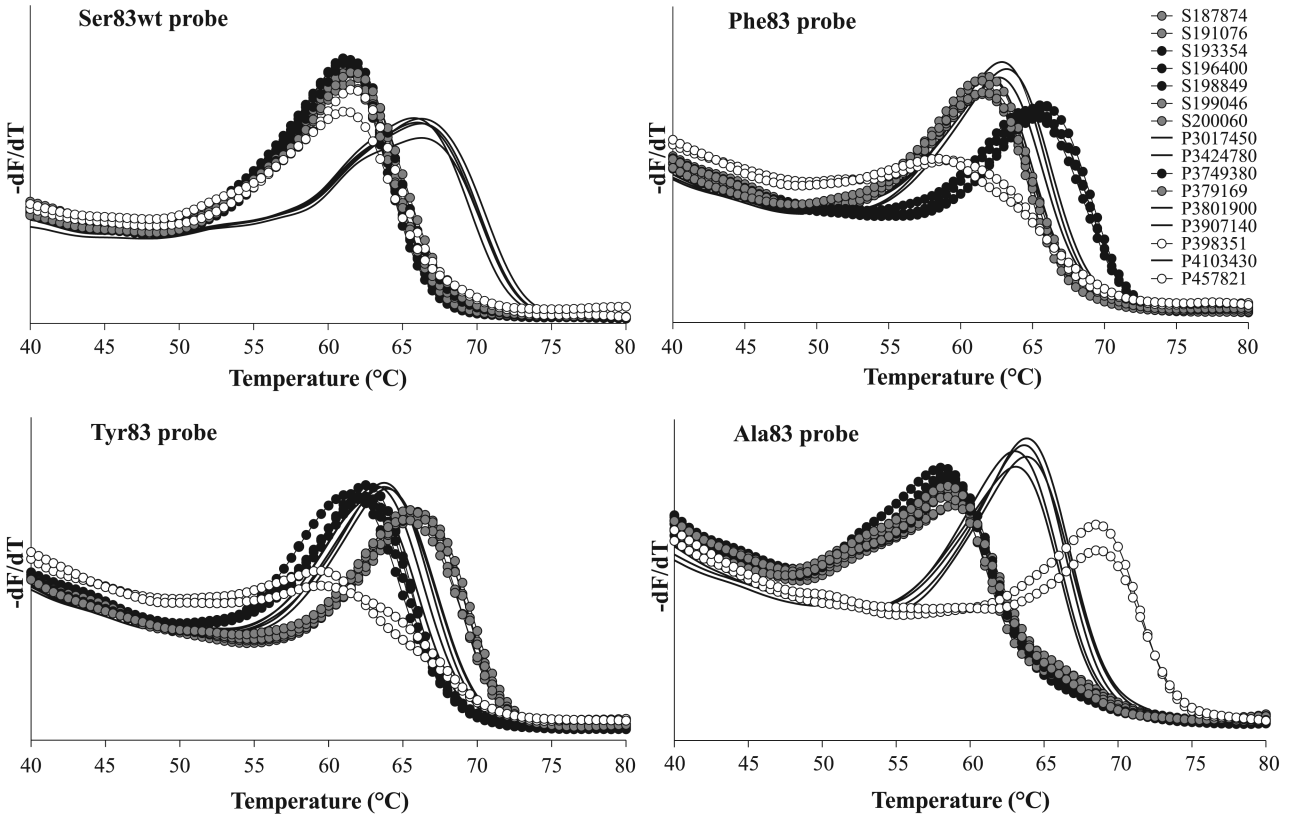

Figure 6. Example DASH results from mutation scoring in the *gyrA* gene. Example data for the mutations at amino acid 83 in the *gyrA* gene is shown for 16 *Salmonella* strains. The presence of a mutation is first detected by considering the melting curves of the wild-type (wt) probe, in this case for serine at amino acid 83. Samples shown as **solid black lines** show high temperature peaks and are therefore perfectly complimentary to the wt probe (are nonmutated). The other samples show a lower temperature peak and therefore carry some mutation compared with the wt sequence. The nature of each mutation is then determined by noting which mutation-specific probe gives a high temperature (matched) peak. In this way, we can conclude that the samples labeled with **black circles** have the Phe83 mutation, the samples labeled with **gray circles** have the Tyr83 mutation, and the samples labeled with **white circles** have the Ala83 mutation.

mutation. The three other probes overlapping the same base successfully detected this particular mutation, and the failure was because of the mutant base mismatch residing at the very 5′ position in the probe. This is in line with our general DASH experience that indicates that terminal bases are likely to be vulnerable to generate false negatives. Probe designs for DNA diagnostics use should therefore avoid placement in this manner over the base being interrogated.

Regarding general sequence applicability of the method, necessary for things such as exon scanning, the performance noted in this report is very encouraging. It also fits with findings from our extensive use of DASH for research investigations (including running assays on duplicate samples, positive and negative controls, and study replication by other methods) in which standard *in silico* assay designs successfully convert >95% of all types of single base mutation targets into working assays. Those studies use only single runs with single probes centered on the mutation sites, and yet they work in the vast majority of cases and suffer a general error rate of only ~1/1000.[22,23] This compares favorably with DNA sequencing, but in diagnostics applications, one would improve on this further by using several probes to interrogate each base of interest. We accomplished this by using an overlapping tiling set of probes for our scanning experiment and normal plus mutation probes in our scoring experiment. Using multiple redundant probes not only brings error rates down to unprecedented and exceptionally low levels, in line with the needs of DNA diagnostics, but it also improves conversion rates from 95% up toward 100%. The actual number of redundant probes used can be tailored to the needs of each diagnostics test. The ultimate sequencing scan, for example, would involve probes tiled with one-base spacing with each targeted region examined by four probes representing all base alternatives at their central location. This would enable all bases to be unambiguously determined throughout the assayed sequence. Tiling at less dense spacing and the use of only reference sequence probes (as in the scanning study we conducted) would be used when merely searching for evidence that something varies from the reference, without a need to locate this change to a precise nucleotide. The probe length could also be manipulated to give stronger signals (longer probes) or better allele resolution (shorter probes), although 17 bases is generally found to give a good balance between these parameters. Insertions and deletions can also be detected by DASH,[26] and unless such variants were greater than ~6 bp in length, they would be detected in diagnostics scenarios because they would reduce the Tms produced by reference sequence probes.

Another method variable shown to be important by our results was the length of the PCR product. Previous DASH development work[22] has shown that target strand secondary structures can significantly impact DASH data quality. Hence, to avoid this problem in research genotyping of single SNP sites, the PCR product is kept as short as possible (typically 50 to 70 bp). It is encouraging, therefore, that in this study we found DASH to work well on products up to several hundred bases in length—beyond the scale of most coding exons. If longer regions need to be investigated then they may be amplified as several overlapping PCR amplicons. This strategy was used with great effect in our analysis of mutations in the *gyrA* gene.

If using DASH for real-world DNA diagnostics, it would be wise to include some form of inbuilt controls for the normal and mutation melt-curve patterns, even though the sample data itself will often be sufficient to unambiguously assign mutations (as in the experiments conducted in the present study). These controls could comprise additional sample DNAs with known genotypes, or synthetic long oligonucleotides carrying mutant and reference versions of the test sequence. On the other hand, in environments where standard operating procedures are rigorously applied, given the excellent allele resolution and reproducibility produced by DASH, one could make use of predetermined standard melt-curves saved in the computer for comparison with test sample data.

In conclusion, array-based melt-curve procedures, such as the DASH, provide flexibility and robustness that would seem to fit ideally with current and future needs of DNA diagnostics, both in terms of mutation scoring and mutation scanning. Highly paralleled implementations can be envisaged that would score very large numbers of dispersed target sites (subject only to PCR multiplex limitations), and full resequencing of many exons in many genes could also be undertaken with greater effectiveness than achieved by static hybridization array systems. As our knowledge of the genetic etiology of disease steadily improves, we anticipate that surface-based melt-curve procedures will come to the forefront as powerful tools for increasingly high-throughput DNA diagnostics that will help underpin improved future health care and, ultimately, personalized medicine.

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