



# DIGITAL ACCESS TO SCHOLARSHIP AT HARVARD

## Rapid, Field-Deployable Method for Genotyping and Discovery of Single-Nucleotide Polymorphisms Associated with Drug Resistance in *Plasmodium falciparum*

The Harvard community has made this article openly available. [Please share](#) how this access benefits you. Your story matters.

<b>Citation</b>	Daniels, Rachel, Daouda Ndiaye, Mikeal Wall, Jason McKinney, Papa Diogoye Séne, Pardis Christine Sabeti, Sarah K. Volman, Souleymane Mboup, and Dyann Fergus. 2012. Rapid field-deployable method for genotyping and discovery of single-nucleotide polymorphisms associated with drug resistance in <i>Plasmodium falciparum</i> . <i>Antimicrobial Agents and Chemotherapy Journal</i> 56(6): 2976-2986.
<b>Published Version</b>	<a href="https://doi.org/10.1128/AAC.05737-11">doi:10.1128/AAC.05737-11</a>
<b>Accessed</b>	February 19, 2015 10:52:15 AM EST
<b>Citable Link</b>	<a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:9894539">http://nrs.harvard.edu/urn-3:HUL.InstRepos:9894539</a>
<b>Terms of Use</b>	This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Open Access Policy Articles, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#OAP">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#OAP</a>

*(Article begins on next page)*

# Rapid, Field-Deployable Method for Genotyping and Discovery of Single-Nucleotide Polymorphisms Associated with Drug Resistance in *Plasmodium falciparum*

**Rachel Daniels,<sup>a,f</sup> Daouda Ndiaye,<sup>b</sup> Mikeal Wall,<sup>c</sup> Jason McKinney,<sup>c</sup> Papa Diogoye Séne,<sup>a</sup> Pardis C. Sabeti,<sup>a,d,f</sup> Sarah K. Volkman,<sup>a,e,f</sup> Souleymane Mboup,<sup>b</sup> and Dyann F. Wirth<sup>a,f</sup>**

Broad Institute, Cambridge, Massachusetts, USA<sup>a</sup>; Université Cheikh Anta Diop, Dakar, Sénégal<sup>b</sup>; Idaho Technology, Inc., Salt Lake City, Utah, USA<sup>c</sup>; Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts, USA<sup>d</sup>; School for Nursing and Health Sciences, Simmons College, Boston, Massachusetts, USA<sup>e</sup>; and Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts, USA<sup>f</sup>

Despite efforts to reduce malaria morbidity and mortality, drug-resistant parasites continue to evade control strategies. Recently, emphasis has shifted away from control and toward regional elimination and global eradication of malaria. Such a campaign requires tools to monitor genetic changes in the parasite that could compromise the effectiveness of antimalarial drugs and undermine eradication programs. These tools must be fast, sensitive, unambiguous, and cost-effective to offer real-time reports of parasite drug susceptibility status across the globe. We have developed and validated a set of genotyping assays using high-resolution melting (HRM) analysis to detect molecular biomarkers associated with drug resistance across six genes in *Plasmodium falciparum*. We improved on existing technical approaches by developing refinements and extensions of HRM, including the use of blocked probes (LunaProbes) and the mutant allele amplification bias (MAAB) technique. To validate the sensitivity and accuracy of our assays, we compared our findings to sequencing results in both culture-adapted lines and clinical isolates from Senegal. We demonstrate that our assays (i) identify both known and novel polymorphisms, (ii) detect multiple genotypes indicative of mixed infections, and (iii) distinguish between variants when multiple copies of a locus are present. These rapid and inexpensive assays can track drug resistance and detect emerging mutations in targeted genetic loci in *P. falciparum*. They provide tools for monitoring molecular changes associated with changes in drug response across populations and for determining whether parasites present after drug treatment are the result of recrudescence or reinfection in clinical settings.

**T**he Malaria Eradication Research Agenda (malERA) initiative recently reported (19) that development of new tools for surveillance and detection of drug-resistant parasites is imperative for any successful eradication effort. Rapidly expanding genetic data sets for malaria can be leveraged to identify molecular biomarkers related to important parasite characteristics, which could be key for surveillance.

Arguably, the most well studied and clinically important sets of molecular biomarkers are genetic loci associated with resistance to antimalarial compounds. Resistance to aminoquinolines, such as chloroquine, and antifolates, such as pyrimethamine and sulfadoxine, is widespread, and molecular markers associated with these resistant phenotypes have been identified. Specifically, researchers have identified genetic changes in the *pfprt* locus for chloroquine resistance (12), the *dhfr* locus for pyrimethamine and proguanil resistance (23, 24), and the *dhps* locus for sulfadoxine resistance (5). Other candidate molecular biomarkers for drug resistance are *pfmdr1* (13, 27), associated with resistance to amodiaquine and other aminoquinolines; *cytb*, associated with atovaquone resistance (15, 17); and PfATPase6, implicated in artemisinin resistance (6, 16, 25). New discovery efforts, including genome-wide association studies, are beginning to identify additional candidate loci related to drug resistance (20, 26, 31); these loci will need to be validated in independent parasite populations.

It is critical that assays identify additional candidate loci related to drug resistance for known and newly discovered molecular biomarkers to be developed in order to easily and reliably examine samples from infected patients identified through positive blood smears. Because the parasite population changes readily and radically in response to selective pressure from drugs and other intervention methods, eradication policies must be equally responsive and adaptable. The data from these assays must allow real-time observation of parasite population changes to inform timely protocol changes and further global eradication efforts to reduce or even eliminate child deaths from malaria.

While a number of assays have been developed for drug resistance loci, they rely on technologies that are not suited to real-time detection and discovery of single-nucleotide polymorphisms (SNPs) associated with reduced drug sensitivity. Many of these technologies, like pyrosequencing and real-time PCR (29, 32), require extensive

training and the use of expensive reagents on instruments in regional clinic settings, which means that samples collected in the field can wait months or years for analysis. Other technologies, such as restriction fragment length polymorphism (RFLP) and *msp1*, *msp2*, and *glurp* marker typing, are economical to implement, but interpretation of results can be complicated and dependent on the user's skill level or lead to results that cannot be directly compared between studies (21). These limitations reduce the utility of existing methodologies for global surveillance campaigns and emphasize the need for assays that are portable, cost-effective, and uncomplicated.

In this report, we describe a novel modified high-resolution melting (HRM) method for real-time genotyping of malaria, using parasites derived from culture-adapted isolates. We validate these highly sensitive and robust assays by examining known drug resistance loci found in patient blood samples from Senegal. We also report the discovery of new genetic variants for several genetic loci known to be involved in modulating drug resistance.

## MATERIALS AND METHODS

**DNA extraction and quantification.** We utilized culture-adapted parasites and clinical isolates from a number of sources. Culture-adapted *Plasmodium falciparum* parasites obtained from the Malaria Research and Reference Reagent Repository (<http://MR4.org>) included 3D7 (MRA-151, deposited by D. Walliker), 7G8 (MRA-152, deposited by D. Walliker), Dd2 (MRA-156, deposited by T. E. Wellems), D10 (MRA-201, deposited by Y. Yu), FCR3 (MRA-731, deposited by W. Trager), V1/S (MRA-176, deposited by D. E. Kyle), W2 (MRA-157, deposited by D. E. Kyle), W2mef (MRA-615, deposited by A. F. Cowman), K1 (MRA-159, deposited by D. E. Kyle), and TM90C6B (MRA-205, deposited by D. E. Kyle). Culture-adapted *P. falciparum* parasites obtained from the Walter Reed Army Institute of Research (WRAIR) included W2, W2mef, and WMCI. We obtained an additional 27 *P. falciparum* clinical samples under human subject informed consent conditions from patients being evaluated at the SLAP clinic in Thies, Senegal, during 2007. The research and ethics study was approved by the Harvard School of Public Health Institutional Review Board (IRB) and the Ministry of Health in Senegal. Written informed consent was obtained in the subjects' own language.

We isolated genomic DNA from culture-adapted parasites using the QIAmp DNA Blood Mini Kit (Qiagen catalog number 51106), Qiagen G-100 (catalog number 13343), and Promega Maxwell 16 Blood DNA Purification Kit (Promega catalog number AS1010). We preserved DNA from patient blood samples by using Whatman FTA filter paper (Whatman catalog number WB120205), and isolated DNA using the QIAmp DNA Blood Mini Kit (Qiagen catalog number 51106), the GenSolve Blood Spot Kit (GenVault catalog number GVR-50), or the Promega Maxwell DNA IQ Casework Sample Kit (Promega catalog number AS1210). We quantified DNA using a Nanodrop 1000 (Thermo Scientific) or a real-time quantitative PCR assay with a labeled probe for hypothetical *P. falciparum* gene PF07\_0076, as previously described (8). We used 0.01 ng of *P. falciparum* patient sample template material per reaction.

**Primer and probe design.** We imported genomic sequence of the laboratory strain 3D7 from PlasmoDB version 6.3 (<http://www.plasmodb.org>) into Idaho Technology, Inc. Primer Design Software (version 1.0.R.84). We selected primer and probe sets ranking in the top 10 out of 1,000 potential set designs for SNPs located in six genes across the *P. falciparum* genome: *pfprt* (C72, M74, N75, K76, H97, A220, N326, and I356), *pfdhfr* (N51, C59, I164, and S108), *pfdhps* (S436, G437, K540, A581, and A613), *cytB* (Y268), *pfATPase 6* (L263, E431, A623, and S769), and *pfmdr1* (N86, Y184, R371, S1034, N1042, and D1246). We designed the probes to contain the SNP of interest. Where several SNPs existed in close proximity on the genome, we designed the probe to cover as many of the SNPs as possible. We determined likely melting peak separation between mutant and wild-type alleles on these probes using Hybridization Probe Melting Temperature Software v1.5 and uMelt (Idaho Technology, Inc.) (10). These applications allowed us to determine peak separation, but the actual melting peak temperatures depended on the actual amplification reaction conditions. We discarded designs that did not show at least 1.5°C difference between melting peaks and redesigned many to increase that difference. We ordered probes with a 3' block (a C3 spacer phosphoramidite attached to a standard 3' CpG) to prevent extension during PCR amplification. Table 1 lists primer and probe sequences.

**Amplification conditions and equipment.** We optimized the assay amplification conditions on a 96-well microtiter plate system using a gradient thermocycler (Bio-Rad iCycler), followed by HRM analysis on a LightScanner-96 (Idaho Technology, Inc., Salt Lake City, UT). We performed all PCR amplification reactions using 2.5ul LightScanner master mix with LCGreen Plus double-stranded DNA (dsDNA) dye (Idaho Technology, Inc.) and primers and probes at various concentrations for assay optimization. We used cultured *P. falciparum* strains with previously reported genotype profiles for wild-type and mutant SNPs to test the performance of the assays in detecting both alleles. Where we did not have samples with all possible mutant SNPs for an assay, we generated constructs containing those mutated positions and used them as controls for the assays (miniGenes; Integrated DNA Technologies). We verified all products by sequencing by Genewiz (Genewiz, Inc., Cambridge, MA) on an ABI 3730xl DNA analyzer and with BigDye Terminator v3.1 chemistry using the company's sample submission requests.

Once optimized for 96-well plate systems, we transferred the technology directly to a LightScanner-384 to increase assay economy.

We performed PCR using a gradient thermal cycler to determine optimal annealing temperatures for asymmetric PCR

conditions, with one primer in 5:1 or 7:1 excess and the probe at 80 to 100% of the concentration of the excess primer (data not shown). Asymmetric PCR is essential to probe-based amplification, producing excess single-stranded material that binds to the blocked probe, which increases the signal needed for probe melting analysis. For assays crtH97, cytBY268, and mdrS1034, the optimal asymmetric ratio of forward to reverse primers was 5:1; for the remaining assays, the asymmetry was 1:5. The final primer concentration was 0.5  $\mu$ M excess primer and 0.1  $\mu$ M limiting primer. The final reaction concentration of the 3'-blocked probes was 0.4  $\mu$ M.

The best amplification and melting conditions depended on the instrument used for HRM. On the LightScanner-96 or -384, 55 cycles of a three-step program performed best. For assays dhfrS108, mdrD1246, ATPase6S769, crtI356, and crtA220, the reaction mixtures were denatured for 2 min at 95°C and denatured, annealed, and extended at 94°C, 63°C, and 74°C for 30 s each, with a final step at 95°C for 30 s. All remaining assays had an annealing temperature of 66°C instead.

With glass capillaries used in the LightScanner-32 (LS32; Idaho Technology, Inc.), which transfer heat much more efficiently than the plate-based LightScanner-96 and -384, the amplification cycle was 95°C denaturation for 2 min, followed by 55 cycles of 94°C for 5 s and 66°C for 30 s, and then a premelt cycle of 5 s each at 95°C and 37°C. Following PCR, the amplified products were analyzed using HRM on the LightScanner instruments. Products were heated from 40 to 90°C and the change in fluorescence recorded incrementally. Standard software included with the instruments were used for unlabeled probe analysis to visualize melting peaks based on these changes in fluorescence.

Once we selected an optimal annealing temperature for pure allelic samples, we implemented a novel amplification protocol to preferentially increase the amplification of mutant alleles in mixed samples. The mutant allele amplification bias (MAAB) technique is an experimental approach used in samples suspected of containing very small fractions of mutant alleles in a single sample. MAAB uses decreased annealing temperature during PCR amplification to increase the melting signal from the mutant allele in mixtures. Amplification bias of the mutant allele is accomplished by using a probe design that is perfectly matched to the normal, or wild-type, allele.

We developed MAAB protocols on a LightScanner-32 to take advantage of the high temperature transition rate capability, which is critical for optimal performance of the MAAB technique. This instrument utilizes capillary tubes in a 5- to 10- $\mu$ l reaction volume and offers real-time PCR and automated high-resolution melting analysis capability. The glass capillary reaction vessels accommodate the requisite rapid temperature transitions required for MAAB. Optimal annealing temperatures under MAAB were reduced to 52 to 56°C.

TABLE 1 High-resolution melting assay primer and probe sequences Assay SNP

Gene	Assay SNP ID	Forward Primer (5'>3')	Reverse Primer (5'>3')	Probe (5'>3')
pfATPase6	L263	CATGCTGTTATAGAATCAAATAGTGAAGATACTC	AATTAATAATCCATACAGTTACACATATTACAAAATGA	AAAATCGATTTATTTGGTCAACA-block
	E431	TTTTTTAATAAATAAAAGATGAAGGAAATGTTGAAGC	CATTTTCTACTATCAGTAAAAAATAACACTATATGG	ATTGATCCTTCTCCATCATCC-block
	A623	TGAATGAAAATGATAAGAATTTAAAGAATGCTAACC	ATGCTCAAATGATTTTCTCCTATAGCTT	TACAGCTCAGGCAACAACAAT-block
	S769	AAATGAAATTCATAAATAGATTCAAATATGGGA	AAATCTGTCTAATTTATAAATCATCTGTATTCT	CTTTGCTTAAAAAATTAAGTAGTAAAGAT-block
pfct	C72/M74/N75/K76	GTAAAACGACGGCCAGTTTCTGTCTGGTAAATGTGCTCA	CAGGAAACAGCTATGACCGGATGTACAAAACATAGTTACCAAT	GTGATGTGTAATGAATAAAATTTTGG-block
	H97	TTTGCTAAAAGAACITTAACAAAATGGTAACTA	ATTTATCTTACTTTTGAATTCCTTTTATTCCA	CATACAAAATAAGTTGTGAGTTCCGATGTTAC-block
	A220	GCTCAGTCTGTTGAAACACAANGAAGAAATCTATC	GCTCAGGCTGAAACAAGTTTAAAGTGTAAATATATAAATATTAC	GTCTTAATAGTGCTTAATGTA-block
	N326	CGAGCATTTTTAGAAAACCTTCGCATTGT	TTATCTCTTTTATTCTACATAGCTG	CCTCTTTGACATTTGTAAT-block
	I356	GCTCAGGTCGAAATTTCTACCATGACATATACTATTG	CACCTGACTGATTATATATTTATCTTTTAAATCTTACGGC	GTCCAGCAACAGCAATTGCT-block
pfctB	Y268/M270	GTAAAACGACGGCCAGTTGATAATGCTATCGTAGTAAATACATATGTTACTC	CTTTGTTCTGCTAATAAGAATAAATGTAATGA	AACATTGCATAAAATGGTAGAAAGTAC-block
pFDHFR	N51/C59	ACATTTAGAGGTCTAGGAAATAAAGGAGT	ATATTTACATCTCTTATATTTCAATTTTTCATATTTGATTCTCAC	AAATGTAATCCCTAGATAGAAATTTTGTGCAG-bloc
	I164	ACAAAGTTGAAGATCTAATAGTTTACTTGGG	CTGGAAAAAATACATCACATTCATATGACTATTTATTCTA	AAATGTTTTATTATAGGAGGTTCCG-block
	S108	CTGTGATAATGTAATGATATGCCTAATTCTA	GACAATAAACATTTTCTATTGCTTAAAGGT	GGAAGAACAGCTGGAAAGCAT-block
pFDHPS	S436/A437	GAATGTTTAAAATGATAAATGAAGGTGCTA	CAGGAAACAGCTATGACGAAATAATGTAATCAGGTACTACTAAATCTCT	ATCCTCTGGTCTTTTGTATACC-block
	K540	GTGTTGATAATGATTTAGTTGATATATTAATGATATTAGTGC	GTTTATCCATTGTATGGATTCCCTT	TAATCCAGAAATATAAAATTTAAAAAACAAC-bloc
	A581	CTTGATTAATGGAATACCTCGTTATAGGA	AGTGGATACTCATCATATACATGATATTTTGTAAAG	TTGGATTAGGATTTGCGAAGAAACATGATCA-block
	A613	CTCTTCAAAAATACATGATATGATGAGTATCCACTT	CATGTAATTTTGTGTATTTATTACAACATTTGA	AAGATTATTGCCCATGATGA-block
pMDR	N86	TTATTTTATATCATTTGATGTGCTGATTATCAGG	CAGGAAACAGCTATGACATCATTGATAATATAAATGACTAAACCTATAGACT	GAACATGAATTAGGTGATGATTAATCC-block
	Y184	AGTTCAGGAATGGTACGAAATTTATAACA	ACGCAAGTAATACATAAAGTCAAAGC	CCTTTTTAGGTTTATATTTTGGTAT-block
	R371	ATGGTGCCCTCAGTTATATCCATT	TTTGTGCTCTAAAGCTTTCATATATTCTGT	GGGTACTTATAGTATGTTTATGTTAAC-block
	S1034	AAATAAAGGACAAAAAGAAGAATTATTGTAATGC	AGAAGGATCCAAACCAATAGGC	AGCGCTTGAAGTAAATCCCA-block
	N1042	AAGAATTTATTGAAATGCAGCTTTATGGG	GGATTTTATAAAGTCATCACTAATATAGTACTCTC	CAATTTTTTAAATAGTTTGGCTAT-block
	D1246	GCAGAAGATTACTGTATTTAATAATAATGGAGA	TTTCATATATGGACATATAAATAACATGGGT	GTGATTATAACTAAGAGATCTTAGAAACT-block

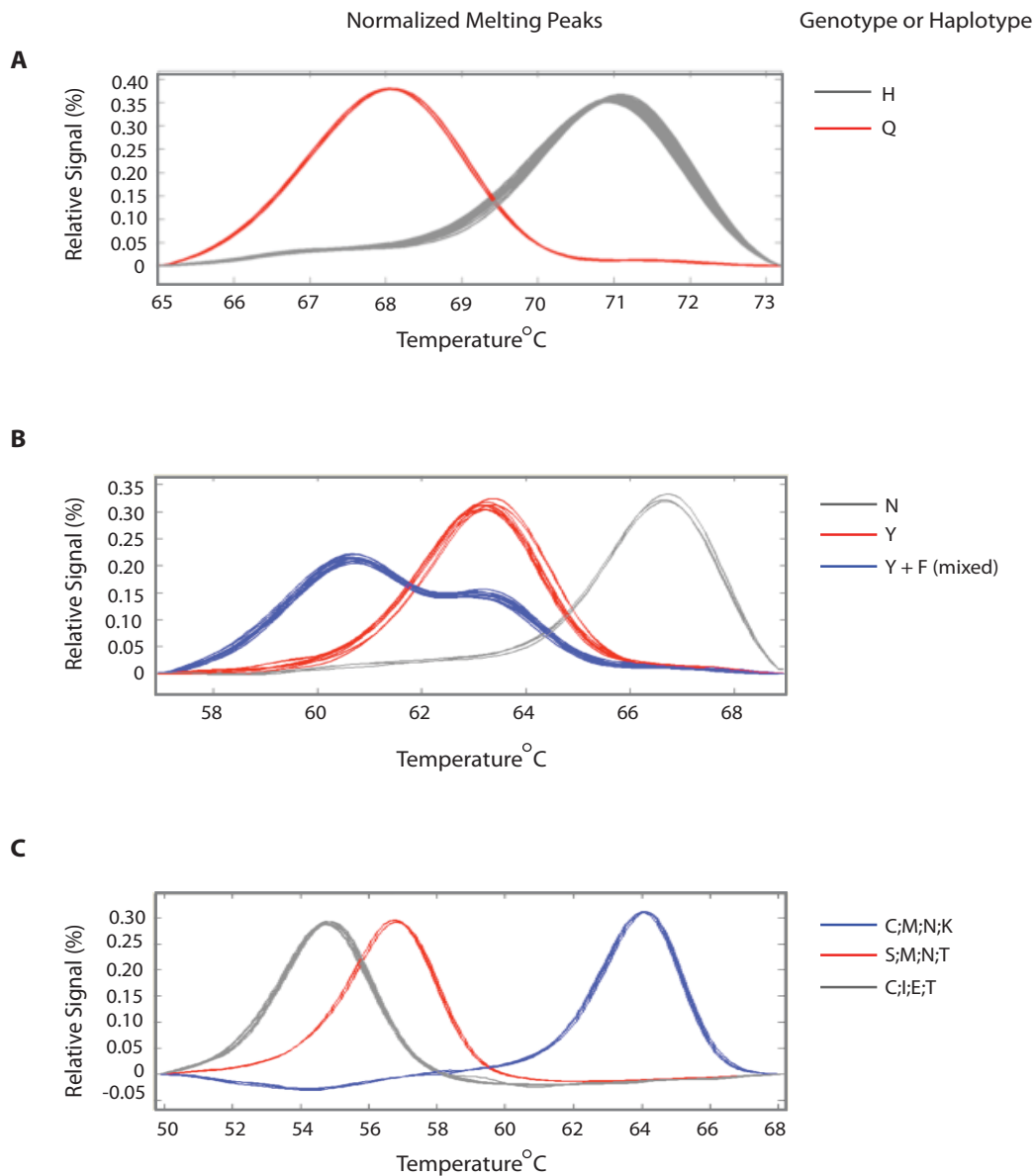
**Figure 1**

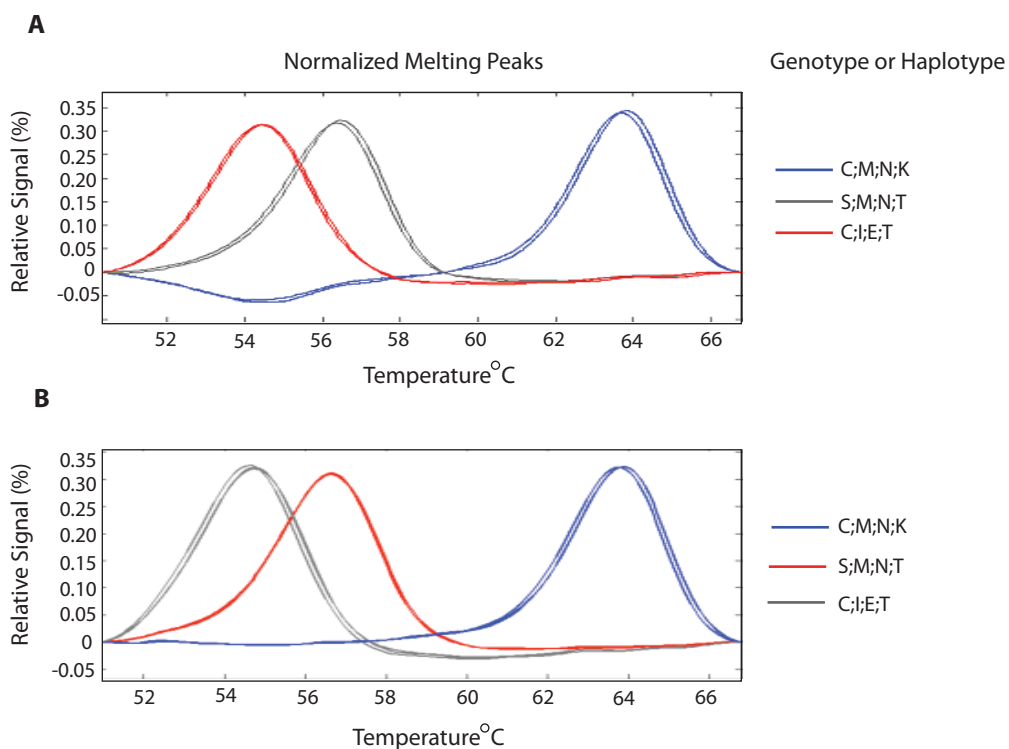
FIG 1 Representative melting peaks of HRM assays. (A) Example of a single-SNP probe assay; *Pfprt* H97 shown. The probe is designed to be a perfect match to the wild type (3D7). Any mutations in this probe region lower the melting peak. Shown here is TM90C6B with the mutation H97Q that results from a nucleotide base change from A to T. This class IV SNP is the most challenging for HRM to distinguish. (B) Example of a single-SNP probe assay with multiple alleles. *Pfmdr* 86 shows wild-type N86 in 3D7 (gray), mutant N86Y in strains K1 and FCR3 (red), and a recently reported mutation in field isolates, N86F in Dd2 (blue). Note that different sources of Dd2 have varying numbers of copies of *pfmdr1*. In this instance, Dd2 has copies with different SNP mutations that have been sequenced to verify the presence of both alleles. Both N86Y and N86F derive from an A<sub>i</sub>T nucleotide change. Additionally, the SNPs tested are adjacent to one another in the genome and can be clearly differentiated by the assay. (C) Representative SNP haplotype probe assay. Shown is the assay developed for *pfprt* residues 72, 74, 75, and 76. The probe is designed as a perfect match to the 3D7 allele at each locus (codons 72, 74, 75, and 76); thus, sample 3D7 (blue) displays the highest melting peak ( $T_m$ ) possible with this probe. This peak corresponds to a genotype at these four loci of C,M,N,K. Sample 7G8 (red) is mismatched under the probe at codons 72 (TT mismatch) and 76 (CT mismatch) for a genotype of S,M,N,T. Samples Dd2, V1/S, and FCR3 (gray) are all the same genotype and are mismatched to the probe at codons 75 (GT and AA mismatch; 1st and 3rd bases of codon 75) and 76 (CT mismatch), making the genotype C,I,E,T. If a sample were mismatched with the probe at only a single base site, the melting peak would be somewhere between the red and blue peaks. If a sample were mismatched at all 4 sites, the melting peak would be even lower than the gray peak.

## RESULTS

We designed a set of 23 HRM assays using the 3'-blocked LunaProbe technology to genotype SNPs across six genes previously associated with reduced drug sensitivity. These assays failed traditional TaqMan primer/probe design requirements, but HRM design was successful. The LunaProbe technology has been shown to increase assay sensitivity: the addition of 3'-blocked probes covering short (less than 50-bp) regions within the primer amplicon results in enhanced peak melting-temperature ( $T_m$ ) separation between alleles, especially class IV SNPs (A->T or T->A) that are a challenge to differentiate with traditional HRM methodologies (Fig. 1A and B). LunaProbes also allow separation of SNPs located very close or adjacent to each other in the genome (Fig. 1B and C). This improved design resulted in better peak separation than previously reported with HRM (1). We confirmed the genotypes determined by HRM analysis through direct sequencing of the amplicons, finding 100% correspondence of the observed genotypes to the reported sequences.

Our genotyping assays proved to be robust and specific. They were able to detect and differentiate alleles with low concentrations of template material even in the presence of excess contaminating genomic material. Using cultured isolates without excess human material, as would be found in filter-extracted patient samples, the limit of detection was  $10^{-5}$  ng, similar to that found by other groups (Fig. 2A) (1, 14) and also similar to other PCR-based methodologies and comfortably within the yield from extraction of patient samples collected on filter paper in the field. The assays remained specific for their target regions in the presence of an excess of human genomic material that we introduced in order to mimic the ratios of human and parasite DNAs obtained from patient samples; however, the absolute limit of detection with contaminating human DNA was reduced to approximately 10 pg (Fig. 2B).

**Figure 2**



**FIG 2** Limit of detection and performance with human genomic material. (A) Limit of detection of assays. A representative assay (pfdhps 436/437) shows the limit of detection. The melting peaks are still distinguishable, with 10<sup>-5</sup> ng of Plasmodium template as the lowest curves. Shown are the normalized derivative melting peaks of both the probe and full amplicon (the higher-temperature melting peaks outside the vertical bars of the probe amplicon). (B) Limit of detection of assays with software normalization. The same assay shown in panel A after software normalization of the probe melting peaks (Idaho Technology, Inc., standard software suite analysis of unlabeled probes) still shows strong peaks for the mutant and wild-type alleles. (C) Performance with an excess of human material. Shown are mock samples of culture-adapted parasite genomic material with an excess of human material added. The Pfprt K72-76 assay is shown. The limit of detection is 10 pg of parasite template combined with 1 ng of human DNA.



The genotyping assays are also sensitive and were made more so by our technology modifications. We were able to detect alleles comprising 2% to 5% of the total sample; by introducing the novel amplification method MAAB on a LightScanner-32 instrument using glass capillary tubes, we were able to increase the sensitivity to detect mutant alleles present at less than 1% of the mixture (Fig. 3B). There is some vertical spread between the mixture peaks; however, the inflection for each mixture does not change compared to the negative or peak curve seen with the unmixed samples. This inflection difference is consistent across repeated trials and is reliable as long as unmixed controls are present.

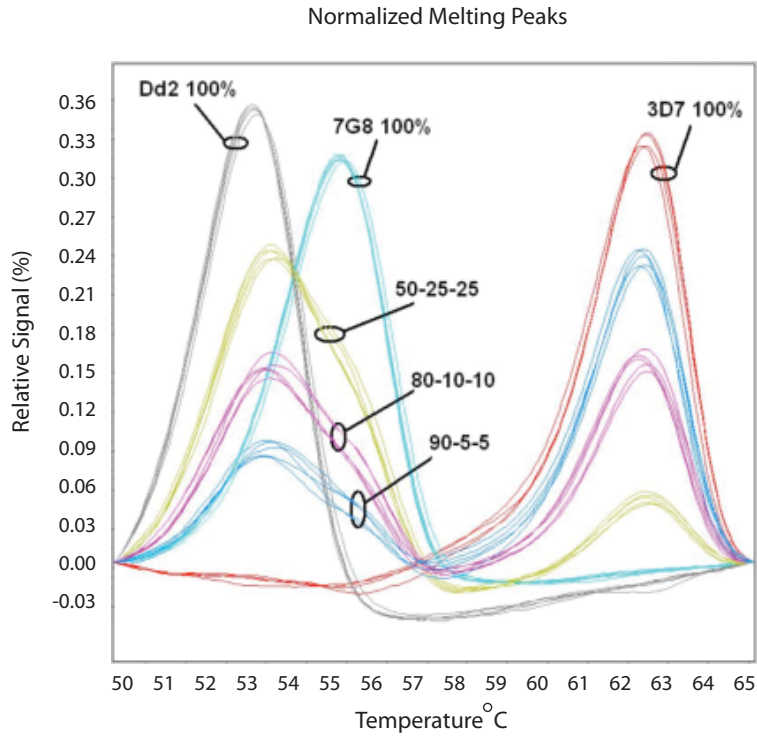
Remarkably, we could discriminate individual alleles even in more complex mixtures containing three or more alleles using our modified HRM approach (Fig. 3A). These results demonstrate that the assays are sensitive and specific and are able to identify alleles present at less than 1% of a mixture when multiple alleles exist in a sample. Comparable amplification technologies, such as TaqMan, fail to consistently detect mutant alleles at less than 10% of the sample mixture (8). Without the refinements to HRM using blocked probes, the typical sensitivity for detection is still an improvement at 2 to 5%.

Additional methods, such as nested-PCR amplification and RFLP analysis, rely on more subjective and complicated interpretation of gel bands. Here, we show that HRM analysis results in clearly separated and unambiguous melting peaks to determine sample genotypes.

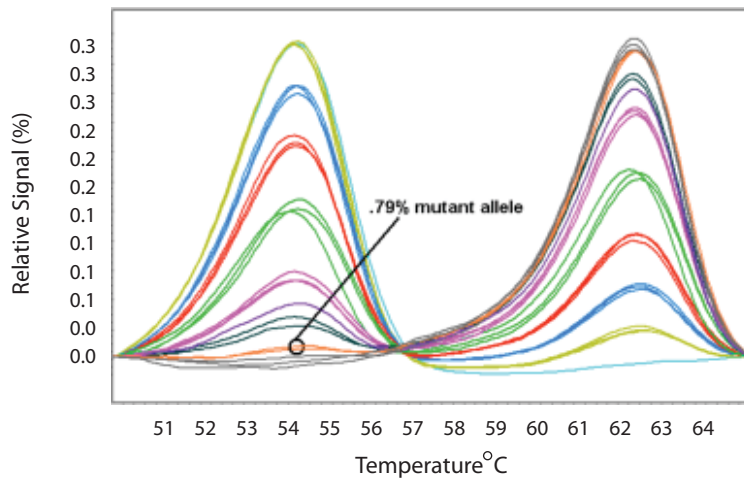
To assess the accuracy and reliability of these assays, we applied them to genotype 44 independent, culture-adapted parasites from a number of different geographical regions and verified our results by sequencing (Genewiz, Inc., South Plainfield, NJ) (Table 2). From our assays, we found that 40% of the samples had the mutant genotype for *pfcr* K76 and 18% and 40% displayed variants at *dhps* residues 436 and 437, respectively. For *dhfr*, 65% were mutants at residues 51 and 59, and 70% were mutants at residue 108. While many other SNPs associated with drug resistance were primarily wild-type genotypes, there were variant polymorphisms in this international collection for each of the following loci: *pfcr* 220 (40%) and 356 (12.5%), *pfmdr1* 86 (32.5%) and 184 (70%), and *PfATPase6* (40%). All mutations detected by genotyping assays were confirmed by sequencing (Genewiz, Inc., South Plainfield, NJ), and our assays showed 100% correlation with the sequencing results, as well as those reported on PlasmoDB version 6.3 (<http://www.plasmodb.org>). The genotyping assays are also sensitive and were made more so by technology improvements. These data

**Figure 3**

**A**



**B**



**FIG 3** Assay performance with mixtures of genomes and MAAB. (A) Mixture of three genomes showing clear differentiation between fractions: 3D7-7G8- Dd2. The pfcrK72-76 assay is shown. (B) MAAB. Shown in the pfcr K72-76 assay, MAAB in the LightScanner-32 increases the likelihood of detecting low- frequency allele fractions in a mixed population. Different proportions of mutant DNA were mixed with wild-type DNA as follows: 100% wild-type DNA (3D7), a 50/50 mixture of mutant DNA (7G8) with wild-type DNA (3D7), a 25/75 mixture of mutant DNA (7G8) and wild-type DNA (3D7), and so on down to less than 1% mutant DNA mixed with wild-type DNA in 1 ng total template concentration.

validate the assays as able to reliably detect previously characterized mutations in the six drug resistance loci. We next tested the correspondence of our genotyping assays with drug phenotypes among the culture-adapted parasites (2). We evaluated the K76 locus of *pfprt* associated with chloroquine resistance and the N51, C59, and S108 loci of *dhfr* associated with pyrimethamine resistance. We observed 100% correspondence between the *pfprt* K76T mutation and chloroquine resistance among culture-adapted parasites. We also observed 100% correspondence between the N51I allele (100%) and the C59R allele (100%) in *dhfr* for pyrimethamine resistance. For the S108N allele, four of the parasites deviated from direct correspondence with pyrimethamine resistance. Three of the parasites that had the S108N allele tested sensitive to pyrimethamine. These parasites were wild type for the N51 and C59 alleles and had pyrimethamine resistance levels near the cutoff (2,000 nM) for resistance (3). One parasite that had the S108 allele tested resistant to pyrimethamine. This parasite, however, was mutant for the N51I and C59R alleles and also had a level of pyrimethamine resistance that was just over the resistance cutoff level of 2,000 nM. Thus, we observed almost complete correspondence between the genotyped alleles and the expected drug responses for chloroquine and pyrimethamine among the culture-adapted parasites in this analysis, confirming that these molecular markers are useful for interpreting drug responses in *P. falciparum* parasites.

We then demonstrated the usefulness of our HRM assays on materials derived from blood collected on filter paper from patients seeking treatment for malaria at the Thies, Senegal, clinic. Table 2 shows the prevalences of mutations in the following loci: *pfprt* 76 (15%), *dhps* 436 (26%) and 437 (48%), and *dhfr* 51 and 59 (85%) and 108 (93%). Other SNPs with variant alleles include *pfprt* 220 (26%) and 356 (22%), *pfmdr1* 86 (7%), *pfmdr1* 184 (70%), and *PfATPase6* 623 and 431 (7%). Based upon comparison to data from previous years, there has been a decrease in the prevalence of mutations associated with chloroquine resistance and an increase in mutations associated with pyrimethamine resistance. The decrease in chloroquine resistance but increase in pyrimethamine resistance is consistent with the use of these drugs for malaria treatment in Senegal. While chloroquine use has largely been discontinued, use of pyrimethamine has continued (initially in a combination treatment with sulfadoxine and amodiaquine) and remains the mainstay treatment for intermittent preventative treatment for pregnant women (IPTp) in Senegal to date (30).

While traditional probe-based technology approaches fail to amplify if novel variants appear within the amplicon region, our assays allowed us to detect both known and novel haplotypes and mutations in genetic loci associated with drug resistance. Techniques such as TaqMan require prior knowledge of all sequence variants for successful genotyping in order to design labeled probes for every polymorphism. We have demonstrated that our method, on the other hand, is far more robust and can identify novel variants. First, we detected previously unreported haplotypes in Senegal for *pfdhps* 436/437 in three of the culture-adapted parasite lines, including the 436A/437A haplotype for two parasites (P05.02 and Th105.07), as well as the 436Y/437G haplotype for another parasite (Th28.04) (Fig. 4A). Second, we detected a new mutation in the *cytB* locus from a single patient sample (Fig. 4B). This novel mutation, confirmed by sequencing (Genewiz, Inc., South Plainfield, NJ), is the M270I variant, located near the amino acid associated with atovaquone resistance in the *cytB* gene at Y268 (17). Because this novel allele was detected in direct patient material, we were unable to test for any biological effects of the mutation on drug sensitivity. Because the probe-based analysis also allows study of the larger amplicon, we were able to discover SNPs 22 bp upstream of the probe region but still within the amplicon targeted by the *ATPase6* 431 assay in D10 (MRA-201) (data not shown). The additional utility of focusing on genomic regions known to be under selection is the increased likelihood that additional mutations associated with reduced drug sensitivity will be found in the regions targeted by the assay primers that may confer drug resistance. While samples with variant melting peaks must be sequenced to characterize the emerging mutation, HRM technologies offer the unique ability to quickly and economically scan samples for emerging variants.

Table 2: Sample and Genotype Information

Sample	Origin	Sample Type	Gene and SNP location																														
			prt	prf	prf	prf	prf	prf	prf	prf	prf	prf																					
HB3	Honduras	culture adaptation	C72	M74	N72	K76	H97	A220	N268	U356	N06	Y184	R71	R71	S1034	N1042	D1246	B436	A437	K540	A81	A613	Y268	M270	L263	A623	E431	S769	N51	C59	I164	S108	
Dd2	Indonesia	culture adaptation	C	I	E	T	H	A	S	T	I	N	F	R	S	N	D	S	A	K	A	A	A	Y	M	L	A	E	S	N	C	I	N
MP2475	Malawi	culture adaptation	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
MP2533	Malawi	culture adaptation	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
PS1189	Mali	culture adaptation	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	N	C	I	S	
3D7	Netherlands	culture adaptation	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	N	C	I	S	
AP041	Nigeria	culture adaptation	C	I	E	T	H	S	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
N29.07	Nigeria	culture adaptation	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	N	C	I	S	
N59	Nigeria	culture adaptation	C	I	E	T	H	S	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
N69	Nigeria	culture adaptation	C	I	E	T	H	S	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
N92	Nigeria	culture adaptation	C	I	E	T	H	S	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
P31.01	Pikine, Senegal	culture adaptation	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
P05.02	Pikine, Senegal	culture adaptation	C	I	E	T	H	S	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
P11.02	Pikine, Senegal	culture adaptation	C	I	E	T	H	S	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
P27.02	Pikine, Senegal	culture adaptation	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	N	C	I	S	
P51.02	Pikine, Senegal	culture adaptation	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	N	C	I	S	
P60.02	Pikine, Senegal	culture adaptation	C	I	E	T	H	S	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
P08.04	Pikine, Senegal	culture adaptation	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
P09.04	Pikine, Senegal	culture adaptation	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
P19.04	Pikine, Senegal	culture adaptation	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
P26.04	Pikine, Senegal	culture adaptation	C	I	E	T	H	S	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4901.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4906.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4960.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4903.07	Thies, Senegal	filter paper	C	I	E	T	H	S	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4931.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4923.07	Thies, Senegal	filter paper	C	I	E	T	H	S	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4907.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4909.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4911.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4912.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4913.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4915.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4918.07	Thies, Senegal	filter paper	C	I	E	T	H	S	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4925.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4926.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4929.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4933.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4934.07	Thies, Senegal	filter paper	C	I	E	T	H	S	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4935.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4936.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4937.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4938.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4939.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4940.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4941.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4942.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4943.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4944.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4945.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4946.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4947.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4948.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4949.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4950.07	Thies, Senegal	filter paper	C	M	N	K	H	A	N	I	N	F	R	S	N	D	S	G	K	A	A	A	Y	M	L	A	E	S	I	R	I	N	
T4951.07	Thies, Senegal	filter paper	C	M	N	K	H																										

**Figure 4**

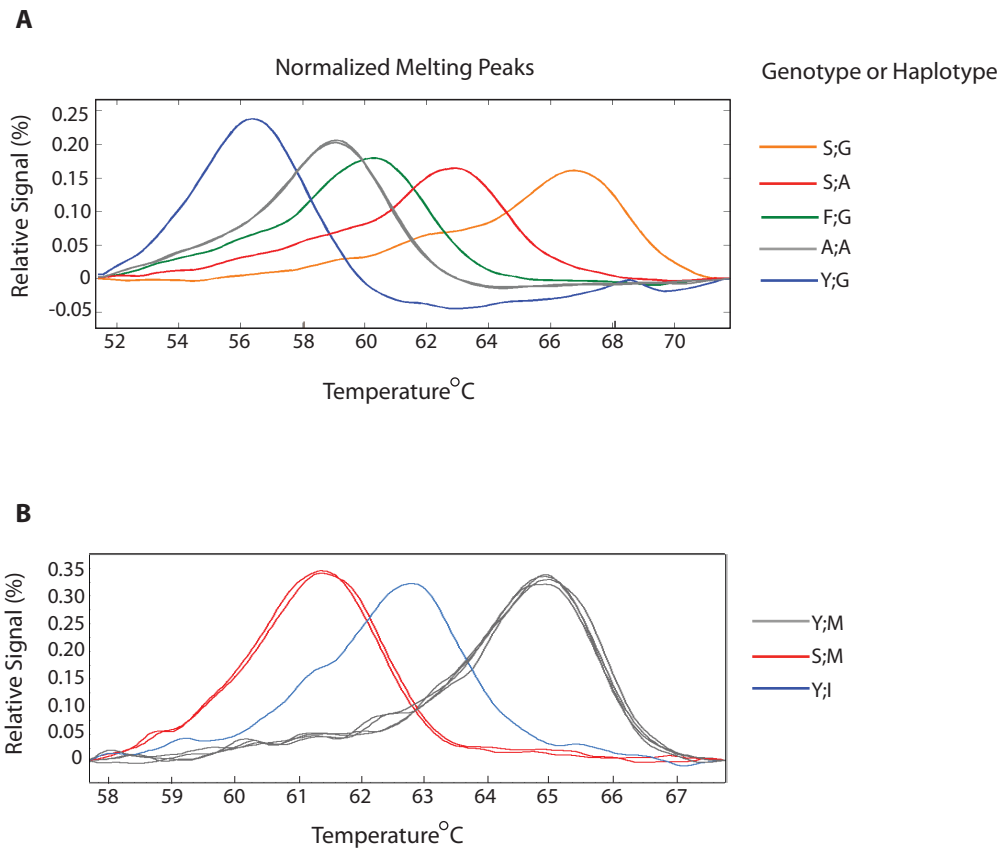


FIG 4 Detection of emerging and new mutations. (A) New SNP haplotype detected in Senegal patient samples. The pfdhps 436A/437A and 436Y/437G haplotypes have not previously been reported in Senegal, though they have been reported in other regions. (B) Novel mutation in cytB. The plot of melting peaks shows the expected wild-type Y268 and mutant S268 peaks as controls in gray and red, respectively. The new mutation, M270I, confirmed by sequencing, is shown in blue.

In addition, the assays differentiated between copies of a genetic locus. The *pfmdr1* locus is known to be present in multiple copies in some drug-resistant parasites, particularly those resistant to mefloquine (13, 28). As part of this analysis, we observed different genotypes for the *pfmdr1* locus in the Dd2 parasite and confirmed the presence of the newly reported *pfmdr1* N86F mutation in Dd2 (4, 9). This demonstrates the ability of these assays to detect mutations among loci subject to copy number variation, which has been implicated in some drug-resistant parasites (11, 18, 22). Copy number variation has also been identified as a source of reduced sensitivity to antimalarial drugs. Instruments with real-time capacity (Idaho Technology LightScanner-32 and Qiagen Rotorgene-Q) offer additional utility of these assays for direct detection of these variations. Finally, we demonstrated that our assays distinguish between single and mixed alleles, in both culture-adapted and clinical samples. Multiple melting peaks appearing in single-genome infections determined by molecular barcode or other methods (8) suggest that there are variant copies of the genetic locus in the parasite genome (Fig. 1).

## DISCUSSION

High-resolution melting technology offers a new tool for first-line surveillance of known and emerging markers associated with reduced sensitivity to drug treatments. While it does not replace standard protocols, such as sequencing, to characterize the specific genetic changes, the method is economical and easy to implement in a large number of global regions to scan the parasite population for changes that could signal the emergence or importation of known mutations. As new mutations are detected through the technology, they can be characterized by sequencing, and new HRM assays can be rapidly developed in an iterative process of population tracking.

Several other groups have presented HRM assays applied to *P. falciparum* (1, 7, 14); however, the techniques presented here show clear advances over the previous works. We have implemented a probe-based technique that improves the sensitivity of these assays over the whole-amplicon methodology presented in the work of Andriantsoanirina et al. and similar to the technology presented by both Gan and Loh and Cruz et al. Compared to the previous publications, we have developed and validated an extended set of assays for SNPs related to reduced drug sensitivity and have made notable advances in the ability to detect mutant alleles present in small amounts within a sample, an improvement on both studies, which noted a 10% limit of detection of mutant alleles while we have detected mutant alleles at less than 1%. While MAAB is most effective in instruments that use glass capillaries for their improved heat transfer properties, the method could be especially useful for surveillance of emerging resistance where the primary causative SNP is known. The ability to detect these mutations at very low levels can inform control efforts. MAAB could be applied following larger-scale plate-based screens where the results may indicate the presence of a minor population of mutants. MAAB could confirm these observations.

Further, we applied these assays to determine the SNP genotypes of 27 patient samples with both single and mixed infections and were able to detect the emergence of haplotypes new to Senegal. Like Gan and Loh, we identified new mutations within the probe region but also found evidence of new mutations outside the probe but within the larger amplicon.

With this technology, results are robust, consistent, and repeatable; however, several caveats exist. Care must be taken in preparing the reaction mixtures. Salt concentrations have a strong effect on the melting temperature of the complexes, so it is advised that all samples be in the same buffer for the most consistent results. Not all samples have to be at the same concentration, but the reactions are most reliable with low concentrations of template. They become less so with an excess of template (more than 10 ng/ul), and the melting peaks may shift even if all samples are in the same buffer if any of them are at a very high template concentration relative to the other samples. While most groups report femtogram levels of detection within culture-adapted strains without human genomic material, the sensitivity of the assays decreases when this human contaminant is present in the sample. Still, we observed universally robust amplifications and unambiguous genotypes from 0.005 to 0.01 ng of *P. falciparum* template from patient-derived samples that had not been depleted of white blood cells. HRM is a promising technology for field-based studies; however, the success of the results also depends on the quality of the analysis software that is included with the HRM analysis instruments. Currently, no standalone application exists for melting analysis. We have had the best success with the suites offered by Idaho Technology, Inc., as a combination of sensitive analysis and ease of peak calling without extensive and complicated user training. User training will be further reduced as researchers publish additional validated assays that require no further optimization. Our initial panel of 23 assays is a strong start toward this goal. Because our HRM assays are sensitive, work on mixed infections, and are easily deployed to study field samples, they have the capacity to track both current and emerging infections. Our genotyping assays can be used to

monitor the frequencies of known drug resistance alleles or parasite types within a population. Furthermore, they can elucidate changes in these frequencies as interventions or other factors that impact transmission dynamics in a given population. For example, as molecular markers of emerging drug resistance are discovered, these markers can be monitored across a population to determine the likelihood that current drug therapies would remain effective or to inform policies to contain the spread of emerging drug resistance and detect the importation of drug-resistant parasites. Because these assays do not require fluorescence-based probes, the reagent cost per sample is much lower than for alternative technologies, and storage and preparation conditions are much less stringent. In our hands, HRM costs approximately 2/3 of what TaqMan-based SNP genotyping costs and have less than 1/10 the cost for sequencing all of these regions. In addition, the assays are reliable for samples collected on filter paper, a low-cost method for sample preservation, transportation, and storage. The fast readout of the melting analysis is clear and unambiguous, allowing easy interpretation of results and less specialized researcher training.

With increased efforts to establish global networks of research centers based on-site across the globe, such as the International Centers of Excellence for Malaria Research (ICEMR) and the collaborative efforts of the Gates Foundation, the initial capital expenditures for equipment capable of high-resolution melting analysis can be distributed across sites. In addition, many of these instruments are multipurpose and are able to offer real-time analysis in addition to HRM.

Our rapid and field-deployable genotyping tools have great promise for surveillance and clinical diagnosis as we transition from malaria control to malaria elimination. For malaria elimination to be successful, tools that can provide quick and reliable information about the changing dynamics of parasites in the natural setting are key. Such information includes monitoring drug-resistant loci in parasites or determining whether a parasite persists after drug treatment. Our genotyping assays work on small amounts of patient-derived material and can disentangle information from mixed parasite infections. These assays can be used to track parasites and determine the sources of emerging infections.

## REFERENCES

1. Andriantsoanirina V, et al. 2009. Rapid detection of point mutations in *Plasmodium falciparum* genes associated with antimalarial drugs resistance by using High-Resolution Melting analysis. *J. Microbiol. Methods* 78:165–170.
2. Baniecki ML, Wirth DF, Clardy J. 2007. High-throughput *Plasmodium falciparum* growth assay for malaria drug discovery. *Antimicrob. Agents Chemother.* 51:716–723.
3. Basco LK, Ramiliasoa O, Le Bras J. 1994. In vitro activity of pyrimethamine, cycloguanil, and other antimalarial drugs against African isolates and clones of *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* 50:193–199.
4. Beshir K, et al. 2010. Amodiaquine resistance in *Plasmodium falciparum* malaria in Afghanistan is associated with the pfcrt SVMNT allele at codons 72 to 76. *Antimicrob. Agents Chemother.* 54:3714–3716.
5. Brooks DR, et al. 1994. Sequence variation of the hydroxymethyl-dihydropterin pyrophosphokinase: dihydropteroate synthase gene in lines of the human malaria parasite, *Plasmodium falciparum*, with differing resistance to sulfadoxine. *Eur. J. Biochem.* 224:397–405.
6. Cojean S, Hubert V, Le Bras J, Durand R. 2006. Resistance to dihydroartemisinin. *Emerg. Infect. Dis.* 12:1798–1799.
7. Cruz RE, Shokoples SE, Manage DP, Yanow SK. 2010. High-throughput genotyping of single nucleotide polymorphisms in the *Plasmodium falciparum* dhfr gene by asymmetric PCR and melt-curve analysis. *J. Clin. Microbiol.* 48:3081–3087.
8. Daniels R, et al. 2008. A general SNP-based molecular barcode for *Plasmodium falciparum* identification and tracking. *Malar. J.* 7:223.
9. Dlamini SV, Beshir K, Sutherland CJ. 2010. Markers of anti-malarial drug resistance in *Plasmodium falciparum* isolates from Swaziland: identification of pfmdr1-86F in natural parasite isolates. *Malar. J.* 9:68.
10. Dwight Z, Palais R, Wittwer CT. 2011. uMELT: prediction of high-resolution melting curves and dynamic melting profiles of PCR products in a rich web application. *Bioinformatics* 27:1019–1020.
11. Eastman RT, Dharia NV, Winzeler EA, Fidock DA. 2011. Piperaquine resistance is associated with a copy number variation on chromosome 5 in drug-pressured *Plasmodium falciparum* parasites. *Antimicrob. Agents Chemother.* 55:3908–3916.
12. Fidock DA, et al. 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Molecular Cell* 6:861–871.
13. Foote SJ, Thompson JK, Cowman AF, Kemp DJ. 1989. Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. *Cell* 57:921–930.
14. Gan L, Loh J. 2010. Rapid identification of chloroquine and atovaquone drug resistance in *Plasmodium falciparum* using high-resolution melt polymerase chain reaction. *Malar. J.* 9:134.
15. Gil JP, et al. 2003. Detection of atovaquone and malarone resistance conferring mutations in *Plasmodium falciparum* cytochrome b gene (cytb). *Mol. Cell Probes* 17:85–89.
16. Jambou R, et al. 2005. Resistance of *Plasmodium falciparum* field isolates to in-vitro artemether and point mutations of the SERCA-type PfATPase6. *Lancet* 366:1960–1963.
17. Korsinczyk M, et al. 2000. Mutations in *Plasmodium falciparum* cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. *Antimicrob. Agents Chemother.* 44:2100–2108.
18. Lim P, et al. 2009. Pfmdr1 copy number and artemisinin derivatives combination therapy failure in *falciparum* malaria in Cambodia. *Malar. J.* 8:11.

19. malERA Consultative Group on Monitoring Evaluation and Surveillance. 2011. A research agenda for malaria eradication: monitoring, evaluation, and surveillance. *PLoS Med.* 8:e1000400.
20. Mu J, et al. 2010. *Plasmodium falciparum* genome-wide scans for positive selection, recombination hot spots and resistance to antimalarial drugs. *Nat. Genet.* 42:268–271.
21. Mwingira F, et al. 2011. *Plasmodium falciparum* msp1, msp2 and glurp allele frequency and diversity in sub-Saharan Africa. *Malar. J.* 10:79.
22. Nair S, Brockman A, Paiphun L, Nosten F, Anderson TJC. 2002. Rapid genotyping of loci involved in antifolate drug resistance in *Plasmodium falciparum* by primer extension. *Int. J. Parasitol.* 32:852–858.
23. Peterson DS, Walliker D, Wellems TE. 1988. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in *falciparum* malaria. *Proc. Natl. Acad. Sci. U. S. A.* 85:9114–9118.
24. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature* 403:906–909.
25. Uhlemann A-C, et al. 2005. A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. *Nat. Struct. Mol. Biol.* 12:628–629.
26. Van Tyne D, et al. 2011. Identification and functional validation of the novel antimalarial resistance locus PF10\_0355 in *Plasmodium falciparum*. *PLoS Genet.* 7:e1001383.
27. Wilson CM, et al. 1989. Amplification of a gene related to mammalian *mdr* genes in drug-resistant *Plasmodium falciparum*. *Science* 244:1184–1186.
28. Wilson CM, et al. 1993. Amplification of *pfmdr* 1 associated with mefloquine and halofantrine resistance in *Plasmodium falciparum* from Thailand. *Mol. Biochem. Parasitol.* 57:151–160.
29. Wilson PE, Alker AP, Meshnick SR. 2005. Real-time PCR methods for monitoring antimalarial drug resistance. *Trends Parasitol.* 21:278–283.
30. World Health Organization. 2011. World malaria report 2010 (Who Global Malaria Programme). World Health Organization, Geneva, Switzerland.
31. Yuan J, et al. 2011. Chemical genomic profiling for antimalarial therapies, response signatures, and molecular targets. *Science* 333:724–729.
32. Zhou Z, et al. 2006. Pyrosequencing, a high-throughput method for detecting single nucleotide polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes of *Plasmodium falciparum*. *J. Clin. Microbiol.* 44:3900–3910.