Genotyping drug resistance markers in *Plasmodium falciparum*

Application of refined methods of High Resolution Melting Analysis to a human malaria pathogen

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With increasing global interest in malaria eradication, new tools are required that offer cost-effective, fast, and unambiguous methods to monitor biological markers such as SNPs associated with drug resistance in order to track drug resistance and inform eradication strategies in real time.

High Resolution Melting technology meets many of these requirements and has been applied to infectious diseases including *Plasmodium falciparum*; however, refinements to this method offer increased sensitivity to both genotype known markers and to discover novel polymorphisms.

We developed and validated a set of genotyping assays to classify biomarkers associated with drug resistance across six genes in *Plasmodium falciparum*, utilizing refinements and extensions of High Resolution Melting analysis. With LunaProbes and Mutant Allele Amplification Bias (MAAB), the set of assays are robust and sensitive. They are able to unambiguously differentiate genotypes and SNP haplotypes in mixed and single patient infections. In most instances, the drug resistance phenotype corresponded to the genotype derived by the assays. In addition, these assays were able to detect emerging SNPs in the regions associated with drug resistance.

Using existing sequence data, we designed a set of 23 assays to genotype SNPs reported to be associated with drug resistance or reduced sensitivity.

Design utilized 3'-blocked LunaProbe assays to improve performance for SNPs adjacent to each other in sequence or that had changes expected to result in small melt differences.

We optimized assay amplification and melt conditions using culture-adapted and sequenced *P. falciparum* strains and genotypes from melting peaks were verified by PCR re-sequencing.

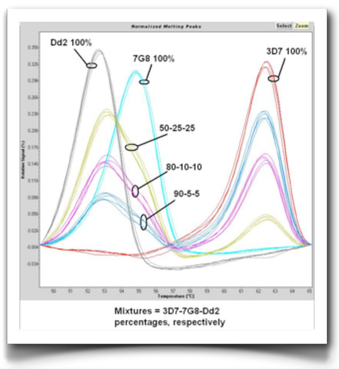
Following optimization, assays were further optimized for Mutant Allele Amplification Bias.

After optimization, we applied the assays to patient samples collected from our field site in Thíes, Senegal. DNA was extracted from Whatman FTA filter paper punches and cultured strains.

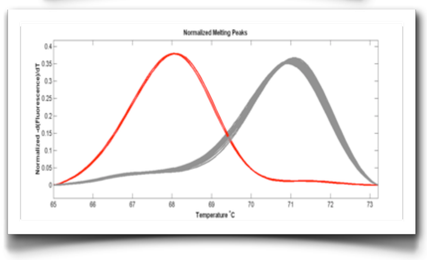
10pg of template material was used per 5-10ul reaction in a LightScanner-384 or LS-32 from Idaho Technology, Inc.

1 Assays are Robust and Sensitive

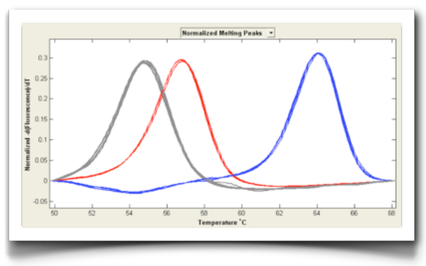
Assays performed well with mixed genomes. At right is a mixture of three genomes in various ratios.



The assays showed clear differentiation of melting peaks between mutant and wild-type SNPs. Shown here is a single-SNP assay for *pfcrt* H97. Wild-type (grey) has a higher melting peak than the mutated allele (red).

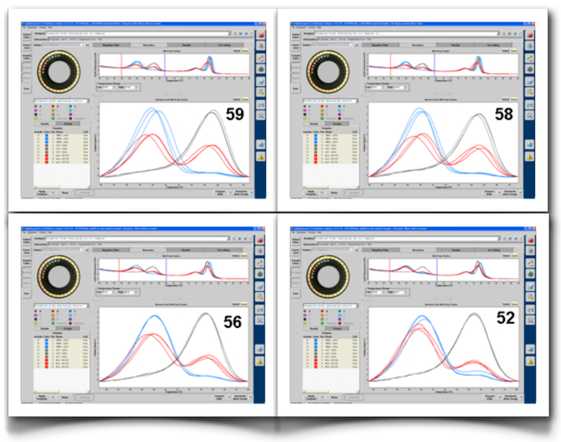


They also produced clear melting profiles for SNP haplotypes. Shown is an assay for SNPs located on *pfcrt* at positions C72, M74, N75, and K76. Each combination of SNPs produces a distinctive melting peak.



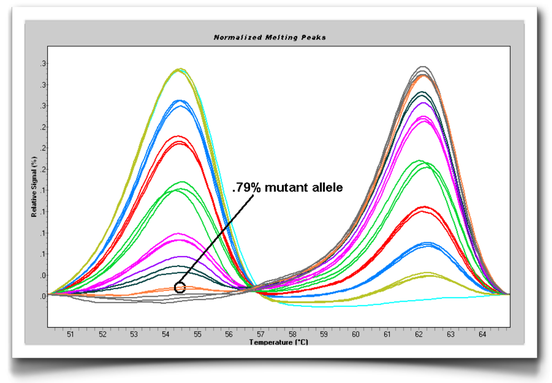
2 Mutant Allele Amplification Bias Increases Sensitivity

Mutant Allele Amplification Bias takes advantage of enzyme mechanics during amplification to favor the production of products with the mutant allele. By decreasing the annealing temperature from the previous optimized temperature, the sensitivity to the presence of the mutant in a sample can be increased.



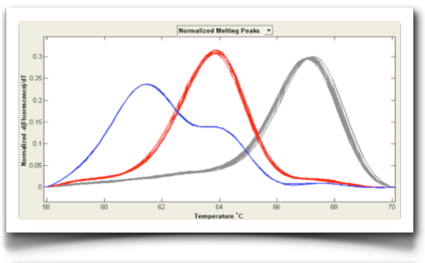
MAAB is able to improve the sensitivity to detect a mutant allele in a mixture from 2-5% to less than 1% of the total alleles.

Shown is the sensitivity of the *pfcrt* N86 assay with MAAB.

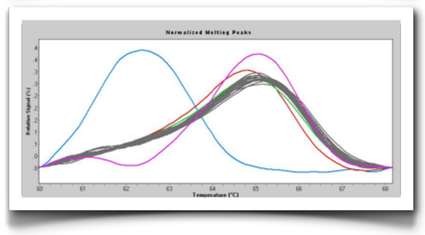


3 Detection of New Mutations...

HRM with LunaProbes and MAAB allowed detection of new mutations. Here is *pfmdr* N86, clearly showing the presence of multiple and unique copies of the allele (blue).



The Senegal patient data set revealed a new mutation in *pfcytB* at M270 instead of the known Y268 change.



...and Haplotypes

Several samples showed a previously unreported AG haplotype from Senegal in *pfdhps* residues 436 and 437.

In addition, a new mutation in DHPS 436 resulting in a change to tyrosine was seen.

Isolate	*pfdhps* S436/A/F/Y	*pfdhps* G437A
P05.02	A	G
P08.04	A	G
Th105.07	A	G
Th28.04	Y	G

4 Biological Validation: Genotype Matches Phenotype

HRM-derived drug resistance SNP genotypes were compared to phenotype data generated by a previously described *ex vivo* drug assay that directly determined the resistance status of patient-derived parasites for a variety of common anti-malarials using IC_{50} measurements[1].

Shown is the correlation of genotype and phenotype of chloroquine for a set of samples from Senegal. Similar positive correlations were seen in other drugs tested in a variety of samples from other global regions, including Senegal.

Where phenotype and genotype differ (*), the most common explanation is that the reported IC_{50} is very close to the resistant/sensitive threshold.

Isolate	Drug	Sensitive/ Resistant	Genotype / Phenotype Match?
P05.02	chloroquine	R	✔
P09.04	chloroquine	S	✔
P11.02	chloroquine	S	✔
P19.04	chloroquine	S	✔
P26.04	chloroquine	R	✔
P27.02	chloroquine	S	✔
P31.01	chloroquine	S	✔
P51.02	chloroquine	R	✔
P60.02	chloroquine	S	✔
Th10.04 D10	chloroquine	S	✔
Th10.04 H7	chloroquine	S	✔
Th105.07	chloroquine	S	✔
Th15.04	chloroquine	S	✘*
Th230.08	chloroquine	S	✔
Th231.08	chloroquine	S	✘*
Th232.08	chloroquine	S	✔
Th26.04	chloroquine	R	✔
Th28.04	chloroquine	R	✔
Th74.08	chloroquine	S	✔
V34.04	chloroquine	R	✔
V35.04	chloroquine	S	✔
V42.05	chloroquine	R	✔
V92.05	chloroquine	S	✔
Th130.09	chloroquine	S	✔
P08.04	chloroquine	S	✔

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Literature Cited:

1. Ndiaye et al. A non-radioactive DAPI-based high-throughput in vitro assay to assess *Plasmodium falciparum* responsiveness to antimalarials--increased sensitivity of P. falciparum to chloroquine in Senegal. Am J Trop Med Hyg (2010) vol. 82 (2) pp. 228-30