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Video Article

A Multi-detection Assay for Malaria Transmitting Mosquitoes

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Abstract

The *Anopheles gambiae* species complex includes the major malaria transmitting mosquitoes in Africa. Because these species are of such medical importance, several traits are typically characterized using molecular assays to aid in epidemiological studies. These traits include species identification, insecticide resistance, parasite infection status, and host preference. Since populations of the *Anopheles gambiae* complex are morphologically indistinguishable, a polymerase chain reaction (PCR) is traditionally used to identify species. Once the species is known, several downstream assays are routinely performed to elucidate further characteristics. For instance, mutations known as KDR in a para gene confer resistance against DDT and pyrethroid insecticides. Additionally, enzyme-linked immunosorbent assays (ELISAs) or *Plasmodium* parasite DNA detection PCR assays are used to detect parasites present in mosquito tissues. Lastly, a combination of PCR and restriction enzyme digests can be used to elucidate host preference (*e.g.*, human vs. animal blood) by screening the mosquito bloodmeal for host-specific DNA. We have developed a multi-detection assay (MDA) that combines all of the aforementioned assays into a single multiplex reaction genotyping 33SNPs for 96 or 384 samples at a time. Because the MDA includes multiple markers for species, *Plasmodium* detection, and host blood identification, the likelihood of generating false positives or negatives is greatly reduced from previous assays that include only one marker per trait. This robust and simple assay can detect these key mosquito traits cost-effectively and in a fraction of the time of existing assays.

Video Link

The video component of this article can be found at http://www.jove.com/video/52385/

Introduction

Anopheles arabiensis, Anopheles coluzzii and *Anopheles gambiae* are the major vectors responsible for malaria transmission in Africa[1]. These three species are morphologically indistinguishable[2] and can only be distinguished by molecular assays[3-9]. In addition, there are many downstream assays routinely conducted to aid epidemiological and population genetics studies. These include (1) a genotyping assay for speciation islands[10-12], (2) a genotyping assay recognizing non-synonymous SNPs in the 1014th amino acid codon position of *para* gene (the *knock-down resistance*, or *kdr,* SNP)[13-18], (3) parasite detection PCR[19-23], and (4) screening for host-specific DNA in mosquito midguts[24,25].

We developed a multi-detection assay (MDA) that combines all these assays into a single multiplex reaction with the goal of analyzing epidemiologically important characteristics of malaria vectors in Africa. The MDA assay includes multiple markers for detecting (1) species (*A. arabiensis, A. gambiae, A. coluzzii,* or other (none of the three)), (2) insecticide resistance represented in *kdr* SNPs (both L1014F *and L1014S),* (3) presence of two major malaria parasites*, Plasmodium falciparum* and *P. vivax*, and (4) blood source from one avian and six mammalian hosts.

Traditionally, these assays were conducted in separate polymerase chain reactions. When all these assays are done using a conventional PCR platform, it requires conducting 8-10 PCR reaction assays and accompanying gel electrophoresis steps. Each individual PCR reaction from preparation to documenting results takes 4-5 hr, while the MDA method presented here takes about 5 hr in totality. This is equivalent to a 90% savings in labor costs alone. The MDA presented here costs $5 per sample to genotype all 33 SNPs. This is considerably less expensive than the single agarose gel-based assays, which costs about $1.50 per sample. Assays detecting all the characteristics covered by the MDA would require a minimum of 8-10 separate agarose gel-based assays at a cost of $12-15 per sample. Moreover, the MDA greatly reduces the chance of generating a false positive or negative by utilizing at least three markers for each parasite or host source detection.

The platform we utilized is not limited to malaria vectors but can be used in a wide variety of applications such as medicine, veterinary medicine, and basic biology[26-28]. In-depth association studies or population genetics studies involving a large (in order of 100s) number of samples require cost-effective assays screening for multiple markers simultaneously. Most studies that utilize two or more separate PCR assays could implement the MDA for quicker results at a lower cost.

1. PCR Amplification

1. Mix all the PCR primers (See supplemental Table S1) in a 1.5ml microtube. Each SNP has two primers (forward and reverse). Ensure that the stock concentration of each PCR primer is kept at 100 µM. Make enough primer mix for 500-1,000 reactions to reduce pipetting error and time at the bench (See supplemental Table S2). If desired, prepare aliquots for 100-200 reactions per tube and store at -20°C.
2. Prepare PCR cocktail as described in the manufacturer's protocol (Table 1). The volume for 96 reactions includes an overhang of 20%. Vortex the tube containing the PCR cocktail lightly and centrifuge for 30 sec at 2,000 x g.
3. Dispense 3 µl of the PCR cocktail into each well of a non-skirted 96 well plate. A repeater pipette can save time for this process.
4. Add 2 µl of the appropriate genomic DNA of mosquitoes in each well.
    NOTE: Process of obtaining genomic DNA from mosquito tissues (head/thorax, abdomen or whole body) is described in other literatures[29-32]. Choose an appropriate DNA extraction protocol to differentiate those with *Plasmodium* in the blood (abdomen) from infective mosquitoes (*Plasmodium* in the head/thorax). We typically use DNA extracted from body parts (head/thorax or abdomen) of mosquitoes, so DNA concentration varies from 0.05-2ng/µl. Although the total DNA input is lower than the manufacturer's recommendation (5-10ng/rxn), we typically get robust SNP calls from 98% of DNA samples without whole genome amplification.
5. Seal the plate, gently mix or vortex, and centrifuge for 1 min at 370 x g.
6. Use the following thermocycler protocol to carry out PCR amplification: (1) 94°C for 2 min, (2) 94°C for 30 sec, (3) 56°C for 30 sec, (4) 72°C for 1 min, (5) repeat step (2)-(4) for 45 cycles, (6) 72°C for 1 min, and 4°C hold.

2. SAP Treatment

1. Prepare the SAP enzyme solution in a 96 well plate (See Table 2). Process 96 samples in a plate. The volume for 96 reactions includes an overhang of 20%.
2. Vortex the tube of SAP enzyme solution lightly and centrifuge for 30 sec at 2,000 x g. Dispense 2 µl of the SAP enzyme solution into each well. Again, a repeater pipette can save time for this process.
3. Seal the plate, gently mix or vortex, and centrifuge for 1 min at 370 x g. Incubate the plate as follows: (1) 37°C for 40 min, (2) 85°C for 5 min to inactivate the enzyme, (3) 4°C hold. At completion of this step, store the plate at -20°C before continuing. Process the stored plate within 2 weeks.

3. SNP Extension

1. If the sample plate is frozen after SAP treatment, allow it to thaw to RT before proceeding.
2. Prepare the extension primer mix (Supplemental Table S2). Each SNP has one extension primer. Keep the stock concentration of each extension primer at 500 µM. Optionally, aliquot for 100-200 reactions per tube and store at -20°C.
3. Prepare extension mix as described in Table 3. The 96 reaction volume includes an overhang of 20%.
4. Vortex the tube of extension cocktail lightly and centrifuge for 30 sec at 2,000 x g. Dispense 2 µl of the extension cocktail into each well. Again, a repeater pipette can save time for this process.
5. Seal the plate, gently mix or vortex, and centrifuge for 1 min at 370 x g. Thermocycle the plate as shown in **Figure 1**. At this point, proceed to mass spectrometry or store the plate at -20°C up to 2 weeks.

4. Conditioning the SNP Extension Product to Optimize Mass Spectrometry Analysis

1. If the sample plate is frozen after SNP extension, allow it to thaw to RT before proceeding.
2. Transfer 15mg of resin from its container onto the dimple plate using an elongated spoon. Use the scraper to spread resin into the wells of the dimple plate. Sweep the scraper from side to side across the dimple plate to spread the resin. Make sure there is resin in each well.
3. Scrape excess resin off the dimple plate using the scraper. Let the resin stand in the dimple plate for at least 20 min. While letting the resin stand in the dimple plate, add 41 µl of HPLC grade water to each well of the sample plate.
4. Gently place the sample plate upside-down, onto the dimple plate. Make sure the sample plate resets against the small rounded post of the dimple plate, which aligns the wells in the sample plate with the wells in the dimple plate.
5. Holding the sample plate and the dimple plate together, gently flip them over so the resin falls out of the dimple plate into the wells of the sample plate. Tap the dimple plate so the resin falls out into the sample plate. Make sure all the resin in the dimple plate falls out into the sample plate wells. Centrifuge the plate at 3,200 x g for 30 sec.
6. Rotate the sample plate on a rotator for 5 min at RT. Rotator must rotate the sample plate 360° about the long axis.
7. Centrifuge the sample plate at 3,200 x g for 5 min. After this step, store the sample plate at -20°C up to 2 weeks before proceeding to the next step.

5. MALDI-TOF Mass Spectrometry

1. Transfer the conditioned SNP extension product onto a bioarray such as SpectroCHIP as per manufacturer's instructions. We use MassArray Nanodispenser for this step.
2. Once the transfer is complete, define how assays and plates are to be set up in the assay database. Name the assay and plates such that each plate has unique identifier that links to specific assay design (Supplemental Table S3).
3. After assay and plate have been defined, acquire spectra using a mass spectrometer.

6. Data Analysis

1. Perform data analysis using a real-time PCR analysis data interpretation software such as Typer Analyzer or TyperViewer (Sequenom). Obtain data in .xml format which contains the sample layout, mass spectrogram for each well, the marker information including name, expected mass for each variant of the corresponding SNP, and any error or warning messages associated with each reaction. See **Figure 2** for the resulting mass spectrogram.
2. Carry out SNP genotype clustering analysis by setting the detection threshold (magnitude or signal to noise ratio) and tolerance/variance allowed for each genotype. **Figure 3** shows an example of a 04707-KDR#2 SNP genotype clusters.
3. Export the resulting SNP genotype calls into a spreadsheet program.

Representative Results

Species identification:

The following 5 SNPs together identify three species (*A. arabiensis, A. coluzzii* and *A. gambiae*) (**Table 4**). If a sample is not one of the three species, the three SNPs (01073-213, 04679-157 and 10313-052) fail to amplify.

kdr genotype for inferring insecticide resistance:

The 1014th codon of the *para* voltage-gated sodium channel corresponds to *kdr*. Two SNPs on the 2nd and 3rd base of the 1014th codon determine the three possible amino acids. The possible combinations of the genotypes are listed in **Table 5**. This SNP works for three species of African malaria vectors: *A. arabiensis, A. coluzzii* and *A. gambiae*. These SNPs fail to amplify in *A. darlingi,* a Brazilian malaria vector. This is not surprising given that mitochondrial genome sequence similarity between *A. darlingi* and *A. gambiae* is only 88.9% while mitochondrial genome sequence similarity among three African malaria vectors are over 98%. Other species have not been tested. We were successful in amplification from samples collected in Mali, Cameroon, Tanzania and Zambia.

Plasmodium detection:

If intact *Plasmodium* DNA is present in the mosquito tissue, we expect to detect *P. falciparum* or *P. vivax* specific DNA among the DNA sample extracted from the mosquito. The species specific SNPs were identified from sequence alignment of the cytochrome b sequence of 123 *P. falciparum*, 97 *P. vivax*, 11 *P. malariae* and 18 *P. ovale* isolate sequences from Africa available on Genbank. We designed 6 SNPs which produce unique SNP genotypes to distinguish *P. falciparum* or *P. vivax* from other *Plasmodium* species (**Table 6**). We have tested this assay on mosquito samples collected in Mali, Cameroon, Tanzania, Zambia and Brazil and confirmed that this particular set of markers works regardless of mosquito species.

Bloodmeal detection PCR:

Cytochrome b sequences from 7 host species (human, chicken, cow, dog, goat, pig and sheep) were collected from Genbank and consensus sequences were generated. SNPs informative for each host were then selected for genotyping. We tested this with blood sources from 7 different animals (**Table 7**).

Because PCR fragments are short (80-120 bp), this works on somewhat degraded DNA or on partially digested bloodmeals from mosquito tissue. The probability of false positive calls is greatly reduced by having multiple markers per host type.

- 94° C for 30 seconds
- 94° C for 5 seconds ⎤
- 52° C for 5 seconds ⎢ 5 cycles
- 80° C for 5 seconds ⎦   40 cycles
- 72° C for 3 minutes
- 4° C forever

Figure 1. Thermocycler program for SNP extension step. The amplification cycle is total of 200 (5x40).
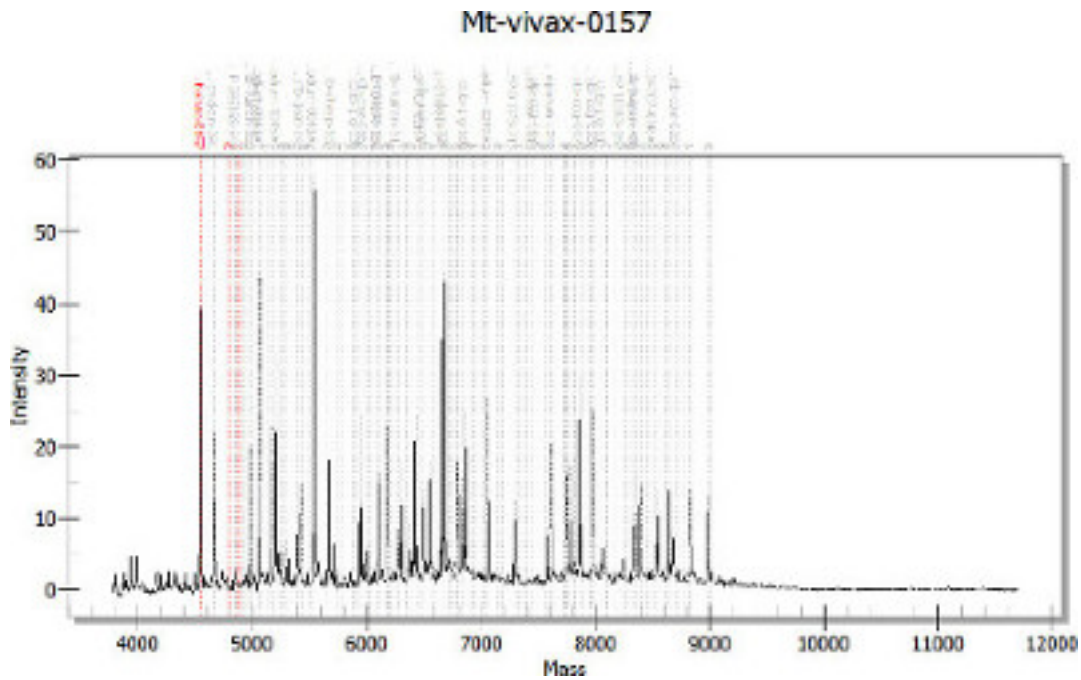
Figure 2. Mass-spectrogram for the MDA. The X axis is mass (Da) of SNP extension products and the Y axis is signal intensity. The Red lines indicate the expected mass of Unincorporated Extension primer, SNP allele 1 and allele 2 for the selected marker. Gray lines indicate expected mass of other markers in the MDA. This plot is generated from data analysis software (Typer Analyzer or Typer Viewer) from the output data file after Step 5. Please click here to view a larger version of this figure.
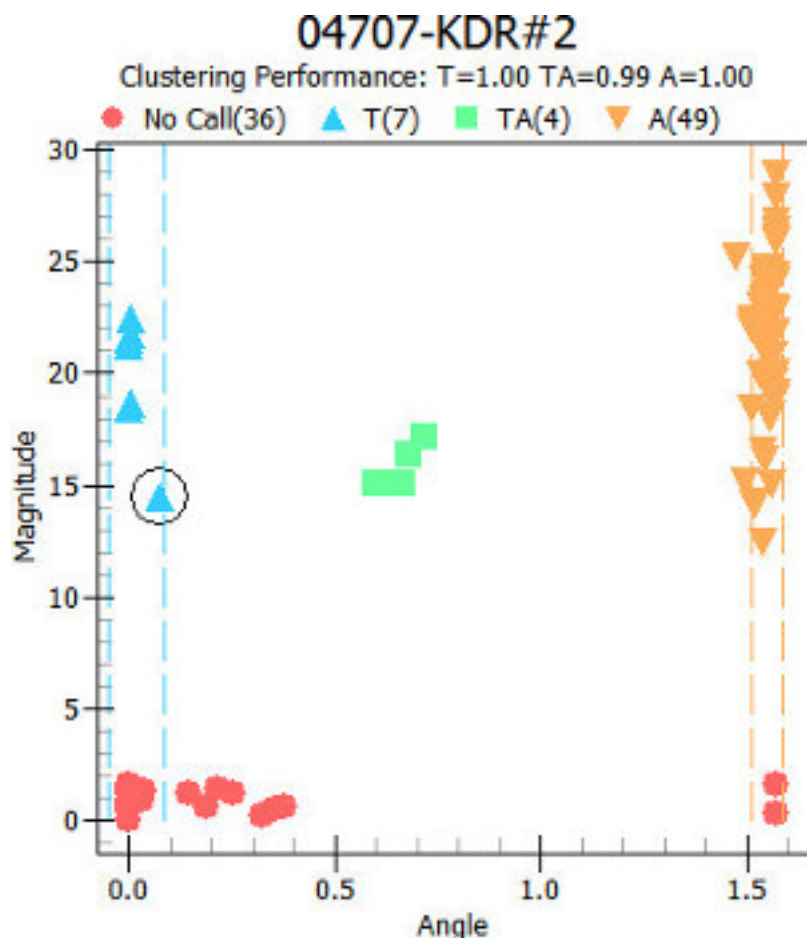
Figure 3. Individual SNP genotype cluster view The X axis is the arctangent value (labeled as "Angle") of the signal intensities of SNP allele 1 and SNP allele 2. The Y axis is the magnitude of genotype call signal. This plot is provided in the data analysis software (Typer Analyzer or Typer Viewer). Any particular data can be selected with a click of a mouse button and selected sample data is indicated by circle. Clustering analysis provided in the software clusters genotypes with a user-defined variance threshold and automatically colors three genotypes of a biallelic SNP. Example shown here indicates homozygous T (TT) individuals as blue triangles, heterozygote (TA) as green squares and homozygous A (AA) individuals as orange inverted triangles. Red circles represent No call (no amplification). Please click here to view a larger version of this figure.

Reagent	Concentration in 5 µl	Volume	Volume
		(1 rxn)	(96 rxns)
Water (HPLC grade)	NA	0.8 µl	92.2 µl
10x PCR Buffer with 20 mM MgCl2	1x (2 mM MgCl2)	0.5 µl	57.6 µl
MgCl2 (25 mM) **	2 mM	0.4 µl	46.1 µl
dNTP mix (25 mM each) ***	500 µM	0.1 µl	11.5 µl
Primer mix (500 nM each)	100 nM	1.0 µl	115.2 µl
PCR Enzyme (5 U/µl)	1.0 U/rxn	0.2 µl	23.0 µl
Total Volume:		3.0 µl	345.6 µl

Table 1. Multiplex PCR cocktail recipe. The volume of each reagent for the PCR extension step for one reaction and 96 reactions with a 20% overhang.

Reagent	Volume (1 rxn)	Volume (96 rxn)
Water (HPLC grade)	1.53 µl	176.3 µl
SAP Buffer (10x)	0.17 µl	19.6 µl
SAP enzyme (1.7 U/µl)	0.30 µl	34.6 µl
Total Volume	2.00 µl	230.4 µl

Table 2. SAP enzyme solution. The volume of each reagent for the Shrimp Alkaline Phosphatase treatment for one reaction and 96 reactions with a 20% overhang.

Reagent	Conc. In 9 µl	Volume (1rxn)	Volume (96 rxns)
Water (HPLC grade)	NA	0.6190 µl	71.3 µl
iPLEX Buffer Plus (10x)	0.222x	0.2000 µl	23.0 µl
iPLEX Termination mix	1x	0.2000 µl	23.0 µl
Extension Primer mix		0.9400 µl	108.3 µl
iPLEX enzyme	1x	0.0410 µl	4.7 µl
Total Volume		2.0000 µl	230.4 µl

Table 3. SNP Extension cocktail The volume of each reagent for the SNP extension step for one reaction and 96 reactions with a 20% overhang.

Chromosome	X	X	X	2	3
species	28S IGS-540	28S IGS-649	01073-213	04679-157	10313-052
A. arabiensis	TT	TT	GG	TT	AA
A. gambiae	CC	TT	AA	TT	AA
A. coluzzii	TT	AA	GG	CC	GG
coluzzii x gambiae (F1)	TC	AT	AG	TC	AG
Admixed *A. coluzzii*	TT	AA	GG	TT	GG
	TT	AA	GG	TC	GG
A. darlingi 1	No Call	No Call	No Call	No Call	No Call
A. darlingi 2	TT	TT	No Call	No Call	No Call
A. darlingi 3	CC	TT	No Call	No Call	No Call

Table 4. SNP genotypes for each species. 28S IGS-540, 28S IGS-649 and 01073-213 are located on the X chromosome, 04679-157 is on chromosome 2 and 10313-052 is on chromosome 3. Hybrid genotype (heterozygote) is highlighted in yellow. Fixed variant in *A. coluzzii* is marked in light blue and fixed variant in *A. gambiae* is marked in dark blue.

kdr genotype	Amino acid	1st base	KDR#1 (2nd base)	KDR#2 (3rd base)
Susceptible (+)	L	T	TT	AA
L1014F	F	T	TT	TT
L1014S	S	T	CC	AA
L1014F/+	L/F	T	TT	AT
L1014F/L1014S	S/F	T	CT	AT
L1014S/+	S/L	T	CT	AA

Table 5. SNP genotypes for kdr. Two SNPs KDR#1 and KDR#2 constitute a genotype for the 1014th codon of para gene. The first base is invariable so it is not included in the assay. Susceptible homozygous genotypes are marked in blue. The most resistant L1014F homozygote genotype is marked in dark blue. Yellow color indicates heterozygote. The L1014S, intermediately resistant genotype, is shown in orange.

kdr genotype	Amino acid	1st base	KDR#1 (2nd base)	KDR#2 (3rd base)
Susceptible (+)	L	T	TT	AA
L1014F	F	T	TT	TT
L1014S	S	T	CC	AA
L1014F/+	L/F	T	TT	AT
L1014F/L1014S	S/F	T	CT	AT
L1014S/+	S/L	T	CT	AA

Table 6. SNP genotypes for P. falciparum and P. vivax. Total 6 SNPs are used to determine the presence of malaria parasite. PI-0041, PI-1269 and PI-1549 contain P. falciparum specific alleles (G, T and T respectively). Simultaneous amplification of the three alleles indicates presence of relatively intact P. falciparum DNA in sample DNA. Amplification of alternative alleles (A for PI-0041 and PI-1269) indicates presence of other Plasmodium species (e.g., P. vivax, P. ovale or P. malariae) in the sample. Pf-1549 is located in regions with many more P. falciparum specific flanking regions thus this SNP is only amplified when P. falciparum is present. PI-0071, PI-1245 and PI-0157 contain P. vivax specific alleles (G, G and T respectively). Amplification of alternative alleles (A, A and C respectively) indicates presence of other Plasmodium DNA in the sample. Uninfected mosquitoes will have no amplification on all 6 markers.

Host	Human-311	Human-479	Human-953	Chicken-091	Chicken-341	Chicken-734	Cow-0267	Cow-0867	Cow-1013	Dog-0108	Dog-0829	Dog-1026	Goat-142	Goat-486	Goat-783	Pig-111	Pig-239	Pig-690	Sheep-041	Sheep-633
Chicken	NC	NC	NC	CC	CC	TT	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Cow	CC	NC	NC	NC	NC	NC	TT	TT	CC	NC	NC	NC	CC	CC	NC	NC	NC	AA	CT	NC
Dog	NC	NC	NC	NC	NC	AA	NC	CC	NC	TT	TT	CC	NC	NC	NC	NC	NC	NC	NC	CC
Goat	CC	NC	NC	NC	NC	AA	TT	NC	NC	NC	NC	NC	TT	TT	GG	NC	CC	AA	CC	CC
Human	CC	GG	CC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	CC
Pig	CC	NC	NC	NC	NC	AA	NC	NC	NC	NC	NC	NC	NC	NC	NC	GG	TT	CC	CC	NC
Sheep	NC	NC	TT	NC	NC	AA	NC	NC	NC	NC	NC	NC	NC	NC	AA	NC	NC	AA	TT	TT

Table 7. SNP genotypes for 7 hosts: Human, Chicken, Cow, Dog, Goat, Pig and Sheep. "NC" stands for "No Call" (no amplification) in the following table. Note that some non-specific amplification may occur at some loci, but not for all the SNPs of a particular host. Please click here to view a larger version of this figure.

Discussion

The MDA is composed of five major steps: PCR amplification, shrimp alkaline phosphatase (SAP) reaction, SNP extension, extension product conditioning, and matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrometry[33-37]. The first PCR amplification step amplifies DNA flanking each SNP so that enough template DNA will be available at the SNP extension step. The SAP reaction neutralizes unused dNTPs which can interfere with the following SNP extension step. SNP extension involves a single extension primer per SNP locus

and mass-modified terminator nucleotides. The 3'-end modified nucleotides prevent subsequent nucleotides from being incorporated into the extension primer. Thus the mass of the single base indicates the genotype of the corresponding SNP.

The most critical step in ensuring the success of this approach is having a good dataset of existing polymorphisms in target organisms. We used well characterized SNPs for species identification (N>900) [10,12,38-40] and *kdr* (N>1,000) [15,18]. The species specific SNPs for *Plasmodium* were identified from sequence alignment of the cytochrome b sequences of 123 *P. falciparum*, 97 *P. vivax*, 11 *P. malariae* and 18 *P. ovale* isolate sequences from Africa which were available on Genbank. Cytochrome b sequences from 7 host species (human, chicken, cow, dog, goat, pig and sheep) were collected from Genbank for host-specific SNP identification. Based on this large body of sequence data, we were able to design a robust diagnostic assay using an assay designer software.

The number of markers each reaction can accommodate is determined by the biochemical compatibility of the primer mix given a tolerance to primer dimer potential (0.9 of 0-1 scale). Authors used the default value (1; most strict) for false priming potential. Relaxation of this parameter can potentially increase the number of SNPs that can be assayed in a single reaction in addition to the SNPs included in this study.

The PCR amplification steps are robust and typically do not require adjustment from the initial assay design. The SNP extension mix (**Table 3**), however, may need modification in relative quantity of each primer using mass spectrometry to ensure a sufficient signal to noise ratio is achieved for each primer. The provided SNP extension primer recipe is the result of these adjustments. Compatibility among extension primers may limit the total number of SNPs that can be multiplex in a single reaction. Since the reaction volume is small (5-8 µl), the sample plate needs to be properly sealed to prevent evaporation during amplification steps.

This platform can simultaneously score up to 35 SNPs per reaction, while other SNP genotyping platforms can accommodate over one thousand SNPs per reaction [41]. With the advances in genome sequencing technology, one can acquire millions of SNPs using next-generation sequencing [29,42,43]. However, the technique used here provides an ideal application for studies that require genotyping a relatively small number of markers for many (100s-1,000s) individuals. Additional discussion of the design constraints of different SNP genotyping platform is covered in previous studies [41].

The MDA is aimed at characterizing epidemiologically important traits of malaria vectors and provides an ideal protocol for medium-throughput SNP genotyping assays analyzing thousands of mosquitoes collected from natural populations. The MDA presented here provides (1) over a 90% reduction in labor time, (2) a 60% reduction in reagent costs, and (3) reduction in false positives/negatives by utilizing multiple markers. The proposed assay can enhance epidemiological studies by greatly facilitating data collection. Also, it can improve genome-wide association studies by characterizing the mosquito samples with respect to population origin, insecticide resistance, parasite susceptibility and host choice. Finally, this MDA may provide inspiration and a template for designing other multiplex assays using a MALDI-TOF based SNP genotyping platform.

Disclosures

The authors have nothing to disclose.

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