Rapid Assay for Identification of Blood Meals in *Aedes albopictus*

Introduction

This assay decreases the amount of time and costs associated with sequencing and cloning for blood meal identification in *Aedes albopictus* by successfully amplifying the target (mammalian mitochondrial DNA—Cytochrome B locus) without amplifying DNA of the mosquito itself or of human nuclear copies (due to a pseudogene-blocking primer). Due to differential sizing of human vs. non-human mammal amplicons, human blood meals can quickly be detected when the PCR product is run on a gel, as can opossum blood meals, and mixed blood meals of human and non-human sources. In our tests the assay has successfully amplified the following species: human, eastern grey squirrel, domestic dog, domestic cat, white-footed mouse, eastern cottontail, Virginia opossum, and white-tailed deer.

Materials Required

1. Freezers –Blooded *Ae. albopictus* need to be placed in a -80C freezer as soon as possible after collection to protect the integrity of the DNA. In our experience blood meal DNA degrades very quickly especially under field conditions (dampness, etc.) After DNA extraction, the DNA can be kept in a -20C freezer.
2. Extracted blood meal DNA – We first dissect out the abdomen of blooded *Ae. albopictus* and extract DNA only from the blood or the abdomen full of blood using a protocol for DNA extraction from blood. After resuspending in 100ul this gives a DNA yield of approximately 10-100 ng/ul depending on size and condition of the blood meal. Our lab uses Qiagen DNeasy Blood & Tissue Kits[1] however any DNA extraction method suited for blood should be successful.
3. PCR reagents – Required are Nuclease free water, Taq Polymerase, PCR Buffer (10mM Tris-HCl, pH 8.3, and 50mM KCl), MgCl2, and dNTP's. For the Inner PCR reaction a special type of Taq is required—the Stoffel fragment, which lacks 3'-5' exonuclease activity allowing its successful use with the blocking primer.
4. Primers – See Table 1. Stock primers are suspended in T.E Buffer (10 mM Tris-HCl and 0.1 mM EDTA, see recipe at end of document) for a working concentration of 10mM.
5. Micropipettes and tips – We use Rainin brand pipettes in sizes 10ul, 20ul and 200ul, and tips in sizes 20ul and 200ul. For very large PCR master mixes (e.g. 96 well plates) a 1000ul pipette and tips is required.

[1] Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA or other involved parties.

6. PCR reaction tubes – strip tubes for smaller numbers of reactions or 96 well optical reaction plates for whole plate reactions.
7. Microcentrifuge tubes – 1.5ml and 0.5ml, to hold the master mix and primer stocks.
8. PCR machine – We use Applied Biosystems Veriti 96 Well Thermal Cycler but any PCR machine will do of course.
9. Gel electrophoresis machine and boxes – Including base, tray, lid, and combs (Fisher Scientific).
10. Chemicals for gel electrophoresis – Ethidium bromide (EtBr), TBE running buffer, 100bp DNA ladder, Gel loading dye, Agarose
11. UV transilluminator – for visualizing DNA bands on gel. We use a Syngene UGenius system with a built-in camera and software for photographing and annotating gels.

Table 1. Sequences of primers used to amplify a segment of cytochrome b in an assay developed for blood meal identification in *Aedes albopictus*. CytbVertR1 and UnvRev1C are used to produce a 571bp product in the external PCR amplification, followed by the internal multiplexed PCR with Human741F, NonHumanF1C, PossumSp1, UnvRev1C and NewPseudBlckdT. Table from Egizi et al. (2013) *Infection, Genetics and Evolution* 16: 122-128.

Primer name	Sequence (5'-3')	Product size w/UnvRev1C	w/ NonHumanF1C
CytbVertR1	GGACGAGGACTATACTACGG	571	--
Human741F	GGCTTACTTCTCTTCATTCTCTCCT	175	--
NonHumanF1C	GGGMTTCTCMGTWGAYAAAG	368	--
PossumR1	TGTGAGATGGGTCGGAATGC	--	468
UnvRev1C	CTAGTTTGTTAGGGATGGAKCG	--	368
NewPseudBlckdT	CGTAAGATTGCRTATGCAAACAA	--	--

Protocol

1. Prepare External PCR master mix by adding the following amounts of reagents and primers (see Table 2A) for a total volume of 20ul.
2. Add 18ul of master mix to each tube followed by 2ul of extracted DNA. Be sure to run negative (water or buffer) and positive (known mammalian DNA, e.g. human or cat) controls in each reaction.
3. Run on PCR machine using the following protocol: Initial denaturing temperature of 95 °C for 3 min 50 s, followed by 20 cycles of a denaturing step of 95 °C for 40 s, an annealing step of 59 °C for 50 s, and an extension step of 72 °C for 40 s, with a final extension of 72 °C for 5 min.
4. Prepare Internal PCR master mix (see Table 2B) and add 19ul of master mix to each tube followed by 1ul of External PCR product from step 3 above.

Table 2. Amounts and concentrations of reagents to add for (A) External and (B) Internal PCR master mixes.

A. Reagents (Exernal)	**Final Concentration**	**Volume per sample (for 20ul rxn)**
ddH2O	--	13.2ul
PCR Buffer	1x	2ul
MgCl2	2.5 mM	2ul
dNTP	200uM each	0.4ul
Primers	50nM each	0.1ul each of CytbVertR1 and UnvRev1C
Taq Polymerase	1 unit	0.2ul
Template DNA	varies	2ul
B. Reagents (Internal)	**Final Concentration**	**Volume per sample (for 20ul rxn)**
ddH2O	--	9.8ul
PCR Buffer	1x	2ul
MgCl2	2.5 mM	2ul
dNTP	200uM each	0.4ul
Primers	150nM each of Human741F and PossumR1, 500nM of NonHumanF1C, 300nM of UnvRev1C and 1200nM of NewPseudBlckdT	0.3ul each of Human741F and PossumSp1, 1ul of NonHumanF1C, 0.6ul of UnvRev1C and 2.4ul of NewPseudBlckdT
Stoffel Taq Polymerase	2 units	0.2ul
PCR product DNA	varies	1ul

5. Run on PCR machine using the following protocol: Initial denaturing temperature of 95 °C for 5 min, followed by 35 cycles of a denaturing step of 95 °C for 40 s, an annealing step of 59 °C for 40 s and an extension step of 72 °C for 40 s, with a final extension of 72 °C for 7 min.
6. Prepare 1% agarose gel by mixing 1g of Agarose with 100ul of TBE and heating until the agarose is completely dissolved. Next add 2ul of EtBr and mix. Pour melted gel into prepared gel box with tray and sample combs, then let sit until gel is firm (approximately 20min at room temp).
7. Once gel is firm, remove sample combs and rotate gel tray to correct orientation. Add TBE buffer to fill line. Then mix 8ul of each sample with 2ul of loading dye and inject into the wells. At the end of each row of samples add 5ul of 100bp ladder mixed with loading dye.
8. Slide lid onto gel box, plug lid into electrophoresis machine and run at 80volts for 40 minutes.
9. Turn off Electrophoresis machine and carefully relocate gel to transilluminator. Make sure protective eyewear is in place before turning on UV light. Once UV light is on, take a picture of gel or make notation of band sizes for each sample. Human bands will be the smallest, 175bp, nonhuman mammal band is 368bp, and the possum specific band is 468bp. Multiple bands in the same well indicate a blood meal of mixed sources (See Fig.1)

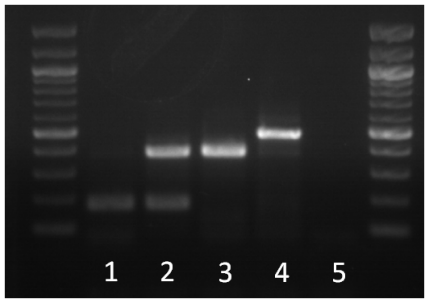

Figure 1. Gel visualization of assay on control DNA samples. Lane 1 is human; lane 2 is a mix between human and dog; lane 3 is squirrel; lane 4 is opossum; and lane 5 is the negative control. Outside lanes are 100bp ladder. Figure from Egizi et al. (2013) *Infection, Genetics and Evolution* 16: 122-128.

10. For human and opossum blood meals, you are finished. For nonhuman blood meals, the samples must be sent for sequencing to determine their blood source. Prepare for sequencing by purifying PCR products, for example we use ExoSap-IT (Affymetrix) to remove unused Primers and dNTPs. Next prepare strip tubes with your sample, nuclease free water, and sequencing primer (NonHumanF1B) in the amounts desired by your sequencing facility. When sequences are received, enter them into a NCBI BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to uncover which mammalian species your sequence matches most closely. A 98% or greater match is good evidence that your unknown blood meal came from that species.

Recipe for T.E buffer (10 mM Tris, 0.1 mM EDTA)

Chemical	Amount
Tris-HCl	500ul of 1M, pH 7.5
EDTA	10ul of 0.5M, pH 7.5
ddH2O	To 50 ml