Article DOI: https://doi.org/10.3201/eid3009.240444

Rapid, Cost-Effective, Colorimetric LAMP Assay to Detect Invasive Malaria Vector *Anopheles stephensi* Mosquitoes

Appendix

Colorimetric LAMP *Anopheles stephensi* identification assay (CLASS) protocol

This assay utilizes Loop Mediated Isothermal Amplification (LAMP) and a phenol-based color change to quickly screen and identify *An. stephensi*.

Procedure/Guidelines

1.0 Prepare MM for reactions as follows:

Volumes (µl) per Reaction Number			Component	Working Concentration	Primer Sequence (Rafferty et al, 2024)
1	**50**	**100**			
9	450	900	Ultra Pure Molecular grade water	___	_____
12.5	625	1250	WarmStart®Colorimetric LAMP 2X Master Mix,	5X	_____
0.5	25	50	F3	10µM	ATTGCACGGGGACTTCCA
0.5	25	50	B3	10µM	GCCTACAGACTCCACTGTCA
0.5	25	50	FIP	40µM	CGACTGCAACTGTATGCGAGGACGGGTC GAGTAACACTTGC
0.5	25	50	BIP	40µM	CCGTGTGGGTGAGTGAGGTTAGAATGAT GCGACGGGAGAAG
0.5	25	50	LF	20µM	AAGATACGAGCGCGTTGGG
24	**1200**	**2400**	Total (to each 24 µl reaction add 1 µl template DNA) or see note 3.2		

1.1 Incubate in a heat block or thermal cycler at 65 °C for 30 minutes.

1.2 Analyze samples visually against a white background for color change.

2.0 Interpretation

2.1 A sample is considered positive when the color changes from bright pink to bright yellow.

2.2 Samples with a light pink or orange color are considered negative but can be followed up for further confirmation.

3.0 Notes

3.1 All assays should include a positive control (confirmed *An. stephensi* DNA), a negative control (confirmed DNA from other *Anopheles* or *Aedes* mosquito), and a no DNA control to ensure results are valid.

3.2 A single leg can be used in lieu of DNA. If that is the case, add 25 µL of Mastermix to reaction, and incubate for 35 minutes.

3.3 To conserve reagents, it is possible to halve the reaction by using the following guidelines:

3.31 Ensure primers are at 10X concentration in the reaction.

3.3.2 Do not use single legs or un-extracted mosquito tissue as it will yield false positives.

3.4 Due to assay PH sensitivity, it is important to use high quality water and reagents for primer dilution and assay set up.

3.5 Positive samples should be followed up with Singh et al. (2023) assay and confirmed by sequencing.