

# Molecular Tools for Early Detection of Invasive Malaria Vector *Anopheles stephensi* Mosquitoes

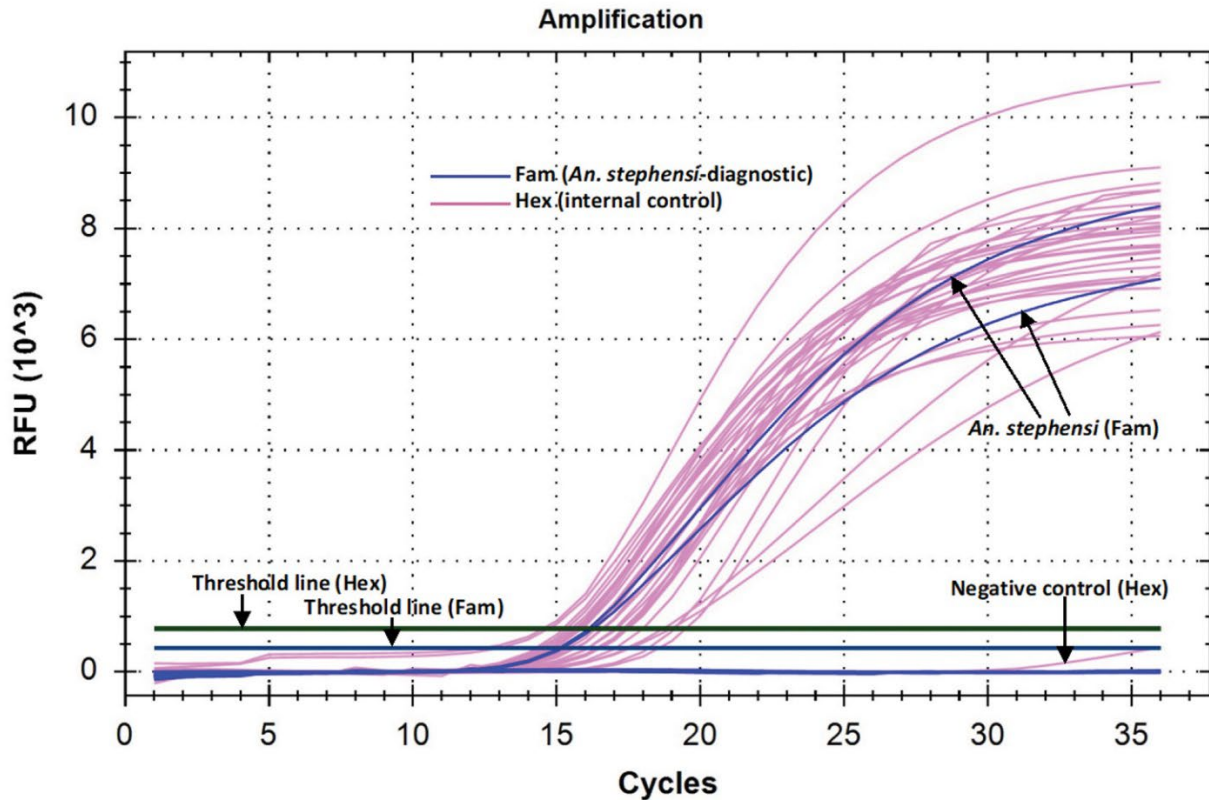
## Appendix

### **DNA Sequencing of Mosquito Samples Detected False Positive for *Anopheles stephensi* in Real-time PCR with Late Ct Value**

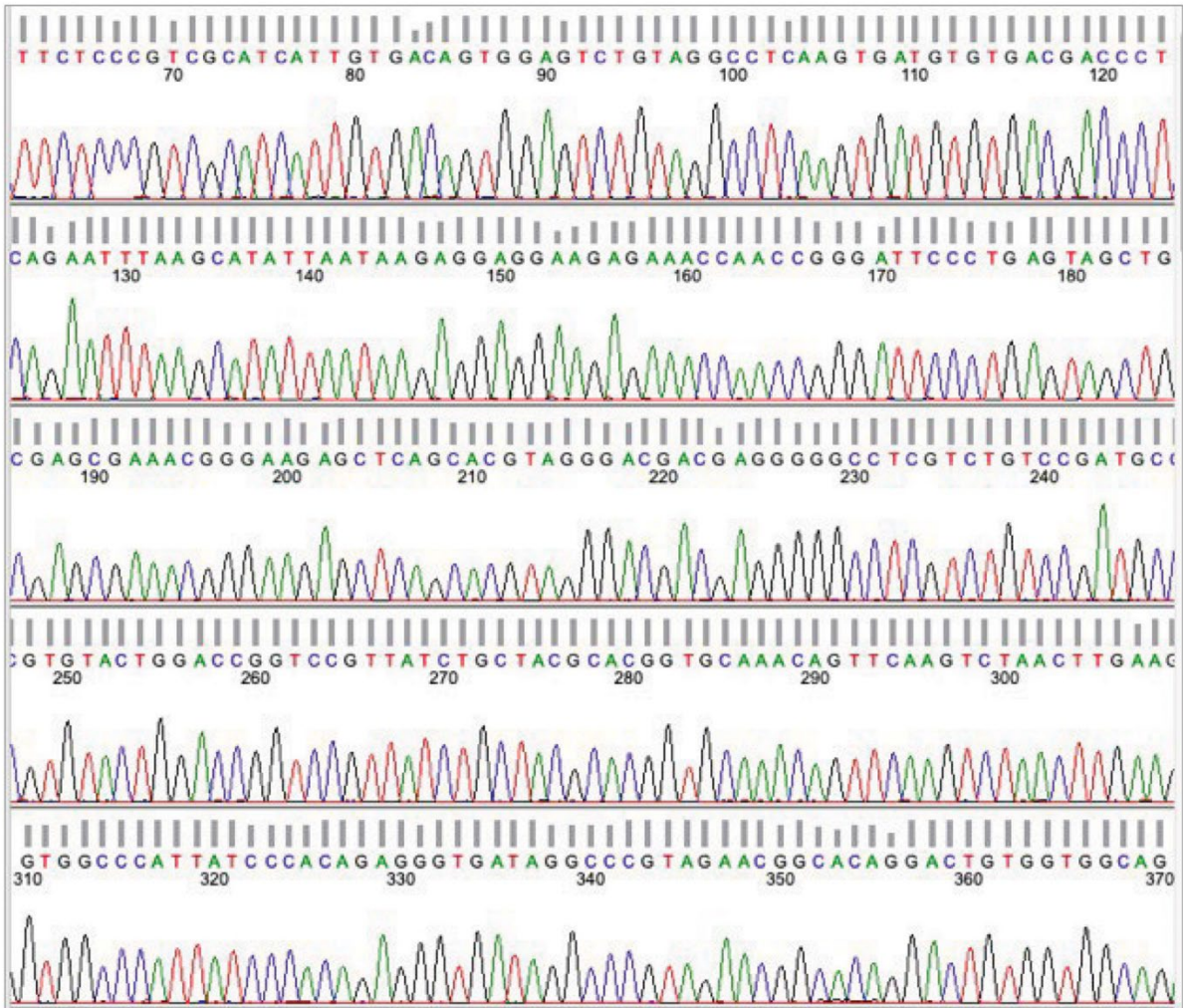
Two pre-isolated DNA of mosquitoes, one each of *Aedes aegypti* and *Anopheles subpictus*, were detected false-positive as *Anopheles stephensi* by real-time PCR with late Ct values (32.95 and 33.09, respectively). To check if false positivity is due to the contamination of DNA from *An. stephensi*, these samples were subjected to DNA sequencing following methods described in the main article under section “DNA sequencing strategy for the confirmation of PCR-based identification of *An. stephensi* in pooled samples” with modification where number of PCR cycles were increased to 45. Both samples showed ≈450 bp amplicon (Appendix Figure 3). The PCR products were sequenced from both directions of the strand. NCBI-blast search revealed 100% homology of all sequences with *An. stephensi*, confirming contamination of samples with DNA from *An. stephensi*.

Appendix Table. List of biologic material used to evaluate specificity of *An. stephensi*-diagnostic PCR assays

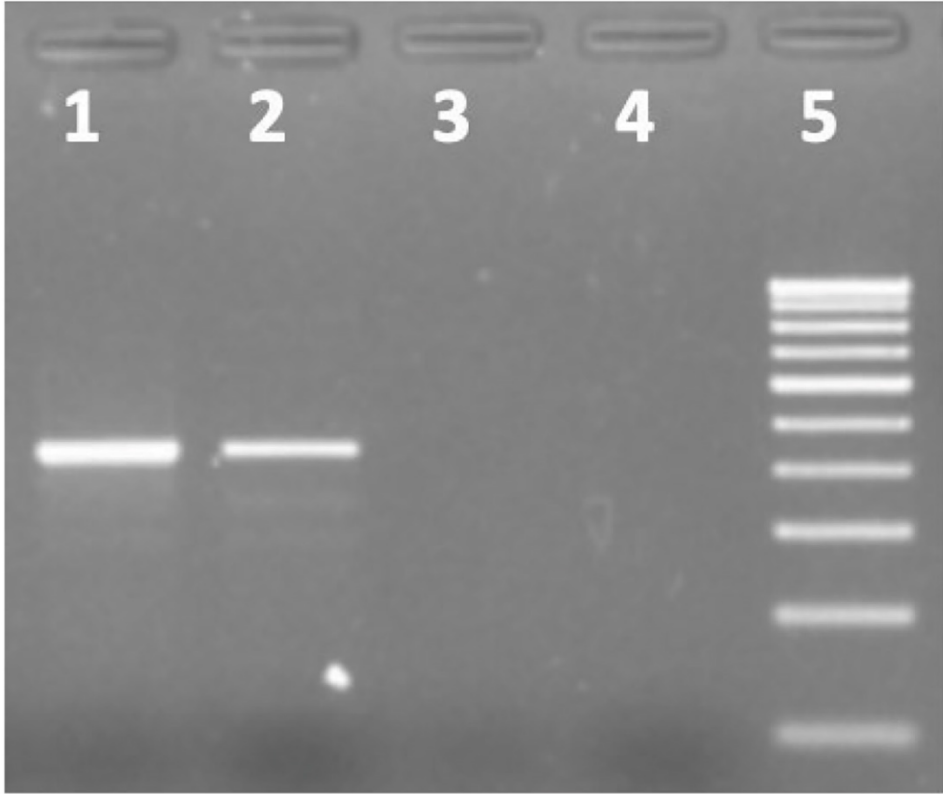
Description of species	Source and nature of material (origin)
Anopheline species	
<i>An. albimanus</i> , strain STECLA	BEI Resources, USA, frozen adult (Santa Tecla, El Salvador)
<i>An. atroparvus</i> , strain EBRO	BEI Resources, USA, frozen adult (Tarragona, Amposta village, Ebro delta, Spain)
<i>An. dirus</i> , strain WRAIR2	BEI Resources, USA, frozen adult (Thailand)
<i>An. farauti</i> , strain FAR1	BEI Resources, USA, frozen adult (Papua, New Guinea)
<i>An. freeborni</i> , strain F-1	BEI Resources, USA, frozen adult (USA Marysville, California)
<i>An. funestus</i> , strain FUM0Z	BEI Resources, USA, frozen adult (Matolo Province, southern Mozambique)
<i>An. gambiae</i> , strain G3	BEI Resources, USA, frozen adult (Pimperena, Mali)
<i>An. merus</i> , strain OPHANSI	BEI Resources, USA, frozen adult (South Africa)
<i>An. quadrimaculatus</i> , strain ORLANDO	BEI Resources, USA, frozen adult (USA)
<i>An. stephensi</i> , strain STE2	BEI Resources, USA, frozen adult (India)
<i>An. stephensi</i> type form, strain DEL	Lab colony, adults and larvae (Delhi)
<i>An. culicifacies</i> species A, strain NIMR	Lab colony, adults (Delhi)
<i>An. culicifacies</i> species B	Pre-isolated DNA from adults (Raipur, India)
<i>An. fluviatilis</i> species S	Pre-isolated DNA from adults (Sundergarh, India)
<i>An. fluviatilis</i> species T	Pre-isolated DNA from adults (Hardwar, India)
<i>An. subpictus</i> molecular form A	Wild caught adults (Delhi, India)
<i>An. subpictus</i> molecular form A	Pre-isolated DNA from adults (Raipur, India)
<i>An. subpictus</i> molecular form B	Pre-isolated DNA from adults (Chilka, Odisha, India)
<i>An. sondaicus</i> cytoform D	Pre-isolated DNA from adults (A&N Island, India)
<i>An. stephensi</i> intermediate form	Wild caught adults (Nuh, Haryana, India)
<i>An. stephensi</i> var. <i>mysorensis</i>	Wild caught adults (Nuh, Haryana, India)
Culicines species	
<i>Ae. aegypti</i> , NIMR strain	Lab colony, adults (Delhi)
<i>Ae. aegypti</i>	Pre-isolated DNA from adults (Bengaluru)
<i>Ae. albopictus</i>	Wild caught adults (Delhi, India)
<i>Cx. quinquefasciatus</i>	Wild caught adults (Delhi, India)
Pool of mixed species ( <i>An. culicifacies</i> s.l., <i>An. subpictus</i> s.l., <i>An. fluviatilis</i> s.l. and <i>Cx. quinquefasciatus</i> )	Wild caught adults (Dadri, Uttar Pradesh, India)



**Appendix Figure 1.** Amplification curve of real-time PCR on *An. stephensi* and other mosquitoes (anophelines and culicine) showing threshold lines for Fam and Hex as determine by the software. Amplification crossing threshold value for Fam was seen in *An. stephensi* only and for Hex was seen in all mosquitoes tested (*An. stephensi*, *An. albimanus*, *An. quadrimaculatus*, *An. dirus*, *An. farauti*, *An. gambiae*, *An. freeborni*, *An. funestus*, *An. atroparvus*, *An. culicifacies* complex, *An. subpictus* complex, *An. merus*, *An. fluviatilis* complex, *Cx. quinquefasciatus*).



**Appendix Figure 2.** DNA sequence chromatogram (partial ITS2 and 28S) of *An. stephensi* showing the quality of sequence derived from DNA isolated from a pool of 500 mosquitoes containing single *An. stephensi* and rest *An. culicifacies*.



**Appendix Figure 3.** Gel photograph showing amplification of *An. stephensi*-specific PCR product in DNA isolated from *Ae. aegypti* (lane 1) and *An. subpictus* (lane 2) which were detected false positive for *An. stephensi* in real-time PCR with late Ct values. Lanes 3 and 4 are negative controls; Lane 5: 100 bp DNA ladder.