# Molecular taxonomy of mosquitoes by dual-reaction end-point PCR

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| Mar 26, 2019 | Feb 27, 2025 | 7.0 | ARBO_Mosquito_Taxonomy_ENG.pdf | CC BY 4.0 DEED |



Surveillance of mosquito populations and the diseases they transmit is a key priority for global public health. Mosquito taxonomy through ITS2 rRNA (Internal Transcribed Spacer 2 ribosomal RNA) PCR is a valuable molecular tool for identifying and differentiating between mosquito species. The ITS2 region, located between the 5.8S and 28S rRNA genes, exhibits significant variation between species, making it an ideal marker for taxonomic classification. Amplification of ITS2 fragments through PCR allows for a robust, fast and easy distinction of closely related mosquito species which are often difficult to differentiate morphologically. Accurate species identification through ITS2 rRNA PCR is crucial for understanding the ecology, distribution, and vectorial capacity of mosquitoes, thereby improving strategies for disease control and prevention. This protocol describes the PCR components and conditions required for molecular taxonomical identification of mosquitoes. This two-reaction sequence specific primer (SSP-PCR) technique allows for the identification of *Anopheles*, *Aedes* and *Culex* genera, as well as species identification for *Aedes aegypti* and *Aedes albopictus* through a single end-point multiplex PCR reaction per mosquito using standard agarose gel electrophoresis.

## Oligonucleotide primer features

| Reaction | Name | Specificity | Sequence* | bp | %GC | Tm | Hair | HmD | Ref |
|---|---|---|---|---|---|---|---|---|---|
| 1 & 2 | Mosq-F | *Anopheles* *Aedes* *Culex* | 5'-TGTGAACTGCAGGACACAT-3' | 19 | 47.4 | 53.9 | -1.75 | **-10.24** | 1 |
| 1 & 2 | Mosq -R2 | | 5'-TTTAGGGGGTAGTCACACAT-3' | 20 | 45 | 52.6 | -0.02 | -1.47 | 2 |
| 1 | Mosq -RAE2 | *Aedes* | 5'-GAAAACRTCACBMYGCATACG-3' | 21 | 48.4 | 54.4 | 0.97 | **-14.23** | 2 |
| 2 | Mosq -RCX | *Culex* | 5'-GTCTTGAATGTTTTGCCAGC-3' | 20 | 45 | 52.8 | 1.04 | -3.14 | 2 |

***** Reverse oligonucleotide primer sequences given in this table are the reverse-complement of the sequence present in the alignments and as they should be ordered for synthesis. Hairpin (Hair) and homodimer (HmD) ΔG's are given in kcal/mol.

## Oligonucleotide heterodimer (HtD) ΔG analysis

| Reaction 1 | Mosq-F | Mosq-R2 | Mosq-RAE2 |
|---|---|---|---|
| Mosq -F | — | -6.82 | **-11.71** |
| Mosq -R2 | -6.82 | — | **-9.46** |
| Mosq -RAE2 | **-11.79** | **-8.02** | — |

| Reaction 2 | Mosq-F | Mosq-R2 | Mosq-RCx |
|---|---|---|---|
| Mosq -F | — | -6.82 | -5.09 |
| Mosq -R2 | -6.82 | — | -4.77 |
| Mosq -RCX | -5.09 | -4.77 | — |

***** Heterodimer (HtD) ΔG's are given in kcal/mol.

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# Vivantis Taq DNA Polymerase protocol

(Vivantis Technologies Sdn Bhd, Selangor, Malaysia Cat # PL1202)

## PCR components for reaction 1 (*Aedes* specific)

| | 1 | 8 | 16 | 24 | 32 | 40 | 48 | 56 | 64 | 72 | 80 | 88 | 96 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| dH2O | 9.58 | 86.22 | 172.4 | 249.1 | 325.7 | 402.4 | 479 | 565.22 | 641.86 | 718.5 | 795.14 | 871.78 | 948.42 |
| 10x Buffer | 1.5 | 13.5 | 27 | 39 | 51 | 63 | 75 | 88.5 | 100.5 | 112.5 | 124.5 | 136.5 | 148.5 |
| 50 mM MgCl2 | 0.45 | 4.05 | 8.1 | 11.7 | 15.3 | 18.9 | 22.5 | 26.55 | 30.15 | 33.75 | 37.35 | 40.95 | 44.55 |
| 10 mM dNTPs | 0.3 | 2.7 | 5.4 | 7.8 | 10.2 | 12.6 | 15 | 17.7 | 20.1 | 22.5 | 24.9 | 27.3 | 29.7 |
| 10 µM MOSQ-F | 0.45 | 4.05 | 8.1 | 11.7 | 15.3 | 18.9 | 22.5 | 26.55 | 30.15 | 33.75 | 37.35 | 40.95 | 44.55 |
| 10 µM MOSQ-R2 | 0.15 | 1.35 | 2.7 | 3.9 | 5.1 | 6.3 | 7.5 | 8.85 | 10.05 | 11.25 | 12.45 | 13.65 | 14.85 |
| 10 µM MOSQ-RAE2 | 0.45 | 4.05 | 8.1 | 11.7 | 15.3 | 18.9 | 22.5 | 26.55 | 30.15 | 33.75 | 37.35 | 40.95 | 44.55 |
| 5 IU/ µL Taq pol | 0.12 | 1.08 | 2.16 | 3.12 | 4.08 | 5.04 | 6 | 7.08 | 8.04 | 9 | 9.96 | 10.92 | 11.88 |
| 10-200 ng/µL DNA | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Mastermix Vf | 13 | 117 | 234 | 338 | 442 | 546 | 650 | 767 | 871 | 975 | 1079 | 1183 | 1287 |

## PCR components for reaction 2 (*Culex* specific)

| | 1 | 8 | 16 | 24 | 32 | 40 | 48 | 56 | 64 | 72 | 80 | 88 | 96 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| dH2O | 9.58 | 86.22 | 172.4 | 249.1 | 325.7 | 402.4 | 479 | 565.22 | 641.86 | 718.5 | 795.14 | 871.78 | 948.42 |
| 10x Buffer | 1.5 | 13.5 | 27 | 39 | 51 | 63 | 75 | 88.5 | 100.5 | 112.5 | 124.5 | 136.5 | 148.5 |
| 50 mM MgCl2 | 0.45 | 4.05 | 8.1 | 11.7 | 15.3 | 18.9 | 22.5 | 26.55 | 30.15 | 33.75 | 37.35 | 40.95 | 44.55 |
| 10 mM dNTPs | 0.3 | 2.7 | 5.4 | 7.8 | 10.2 | 12.6 | 15 | 17.7 | 20.1 | 22.5 | 24.9 | 27.3 | 29.7 |
| 10 µM MOSQ-F | 0.45 | 4.05 | 8.1 | 11.7 | 15.3 | 18.9 | 22.5 | 26.55 | 30.15 | 33.75 | 37.35 | 40.95 | 44.55 |
| 10 µM MOSQ-R2 | 0.15 | 1.35 | 2.7 | 3.9 | 5.1 | 6.3 | 7.5 | 8.85 | 10.05 | 11.25 | 12.45 | 13.65 | 14.85 |
| 10 µM MOSQ-RCX | 0.45 | 4.05 | 8.1 | 11.7 | 15.3 | 18.9 | 22.5 | 26.55 | 30.15 | 33.75 | 37.35 | 40.95 | 44.55 |
| 5 IU/ µL Taq pol | 0.12 | 1.08 | 2.16 | 3.12 | 4.08 | 5.04 | 6 | 7.08 | 8.04 | 9 | 9.96 | 10.92 | 11.88 |
| 10-200 ng/µL DNA | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Mastermix Vf | 13 | 117 | 234 | 338 | 442 | 546 | 650 | 767 | 871 | 975 | 1079 | 1183 | 1287 |

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# ABM Taq DNA polymerase protocol

(Applied Biological Materials Inc, British Columbia Canada Cat# G009)

## PCR components for reaction 1 (*Aedes* specific)

| | 1 | 8 | 16 | 24 | 32 | 40 | 48 | 56 | 64 | 72 | 80 | 88 | 96 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| dH2O | 8.4 | 75.6 | 151.2 | 218.4 | 285.6 | 352.8 | 420 | 495.6 | 562.8 | 630 | 697.2 | 764.4 | 831.6 |
| 5x Buffer | 3 | 27 | 54 | 78 | 102 | 126 | 150 | 177 | 201 | 225 | 249 | 273 | 297 |
| 10 mM dNTPs | 0.25 | 2.25 | 4.5 | 6.5 | 8.5 | 10.5 | 12.5 | 14.75 | 16.75 | 18.75 | 20.75 | 22.75 | 24.75 |
| 10 µM MOSQ-F | 0.45 | 4.05 | 8.1 | 11.7 | 15.3 | 18.9 | 22.5 | 26.55 | 30.15 | 33.75 | 37.35 | 40.95 | 44.55 |
| 10 µM MOSQ-R2 | 0.15 | 1.35 | 2.7 | 3.9 | 5.1 | 6.3 | 7.5 | 8.85 | 10.05 | 11.25 | 12.45 | 13.65 | 14.85 |
| 10 µM MOSQ-RAE2 | 0.45 | 4.05 | 8.1 | 11.7 | 15.3 | 18.9 | 22.5 | 26.55 | 30.15 | 33.75 | 37.35 | 40.95 | 44.55 |
| Taq pol | 0.3 | 2.7 | 5.4 | 7.8 | 10.2 | 12.6 | 15 | 17.7 | 20.1 | 22.5 | 24.9 | 27.3 | 29.7 |
| 10-200 ng/µL DNA | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mastermix Vf | 13 | 117 | 234 | 338 | 442 | 546 | 650 | 767 | 871 | 975 | 1079 | 1183 | 1287 |

## PCR components for reaction 2 (*Culex* specific)

| | 1 | 8 | 16 | 24 | 32 | 40 | 48 | 56 | 64 | 72 | 80 | 88 | 96 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| dH2O | 8.4 | 75.6 | 151.2 | 218.4 | 285.6 | 352.8 | 420 | 495.6 | 562.8 | 630 | 697.2 | 764.4 | 831.6 |
| 5x Buffer | 3 | 27 | 54 | 78 | 102 | 126 | 150 | 177 | 201 | 225 | 249 | 273 | 297 |
| 10 mM dNTPs | 0.25 | 2.25 | 4.5 | 6.5 | 8.5 | 10.5 | 12.5 | 14.75 | 16.75 | 18.75 | 20.75 | 22.75 | 24.75 |
| 10 µM MOSQ-F | 0.45 | 4.05 | 8.1 | 11.7 | 15.3 | 18.9 | 22.5 | 26.55 | 30.15 | 33.75 | 37.35 | 40.95 | 44.55 |
| 10 µM MOSQ-R2 | 0.15 | 1.35 | 2.7 | 3.9 | 5.1 | 6.3 | 7.5 | 8.85 | 10.05 | 11.25 | 12.45 | 13.65 | 14.85 |
| 10 µM MOSQ-RCX | 0.45 | 4.05 | 8.1 | 11.7 | 15.3 | 18.9 | 22.5 | 26.55 | 30.15 | 33.75 | 37.35 | 40.95 | 44.55 |
| Taq pol | 0.3 | 2.7 | 5.4 | 7.8 | 10.2 | 12.6 | 15 | 17.7 | 20.1 | 22.5 | 24.9 | 27.3 | 29.7 |
| 10-200 ng/µL DNA | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mastermix Vf | 13 | 117 | 234 | 338 | 442 | 546 | 650 | 767 | 871 | 975 | 1079 | 1183 | 1287 |

## PCR conditions

Use Applied Biosystems MiniAmp Plus thermal cyclers 1 through 4 (total run time of 1hr 34 min).

| Temp | Time | Cycles |
|---|---|---|
| 94 ºC | 2 min | 1 |
| 94 ºC | 30 sec | 30 cycles |
| 52 ºC | 40 sec | |
| 72 ºC | 45 sec | |
| 72 ºC | 5 min | 1 |
| 25 °C | 5 min | 1 |

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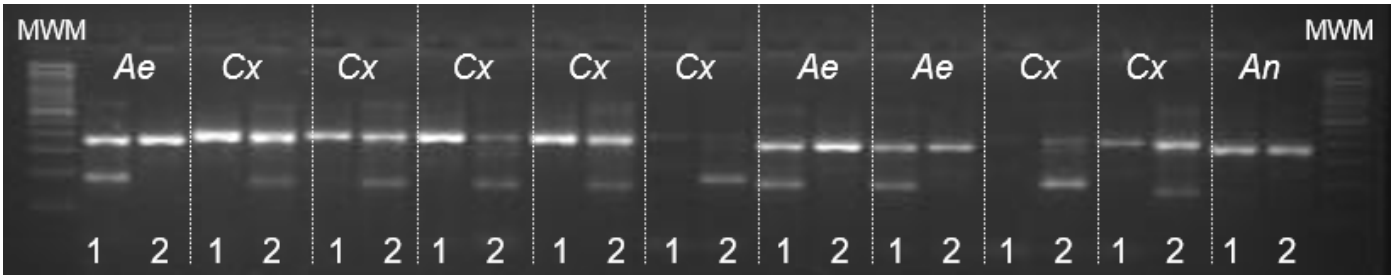
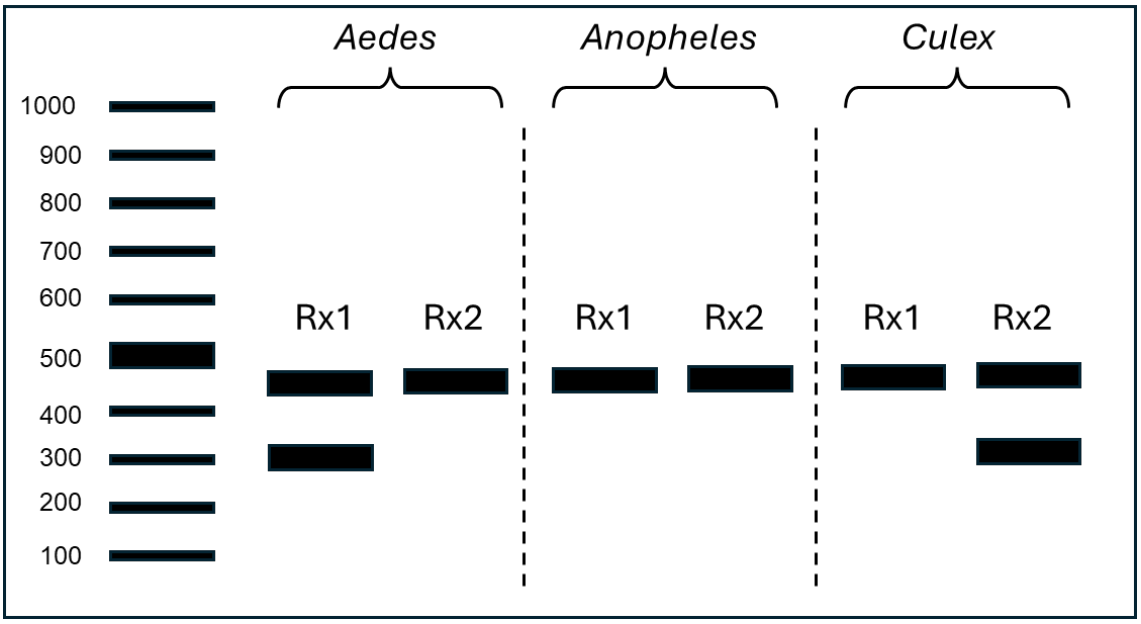
## Electrophoresis conditions

Add 5 µL of orange loading buffer to each PCR reaction corresponding to individual mosquitos and load 25 µL into a 3% agarose gel and run for 90 minutes at 90 VDC (7.2 volts/cm) followed by 50 minutes at 40 VDC (3.2 volts/cm) to achieve maximum resolution.




Typical SSP-PCR banding patterns for Aedes (Ae), Anopheles (An) and Culex (Cx) mosquitos.

## Result interpretation



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# SSP-PCR amplicon lengths

| | | Amplicon size (bp) | | |
|---|---|---|---|---|
| Genus | Species | Mosq-F + Mosq-R2 | Mosq-F + Mosq-RAE2 | Mosq-F + Mosq-RCX |
| Aedes | abserratus | ~ 354 | ~ 178 | – |
| | aegypti | 328 | 146 | – |
| | albopictus | 529 | 161 | – |
| | atlanticus | 391 | 178 | – |
| | atropalpus | * Unknown | ~ 179 | – |
| | aurifer | * Unknown | ~ 178 | – |
| | camptorhynchus | 341 | 167 | – |
| | cantator | * Unknown | ~ 178 | – |
| | cataphylla | ~ 384 | 178 | – |
| | cinereus | 347 | 155 | – |
| | dorsalis | * Unknown | ~ 178 | – |
| | excrucians | * Unknown | ~ 178 | – |
| | intrudens | * Unknown | ~ 178 | – |
| | japonicus | ~ 411 | ~ 221 | – |
| | notoscriptus | 416 | 191 / 342 | – |
| | polynesiensis | 434 | 143 | – |
| | sticticus | * Unknown | ~ 178 | – |
| | taeniorhynchus | * Unknown | ~ 210 | – |
| | triseriatus | 375 | 179 | – |
| | vexans | 378 | 168 | – |
| Anopheles | albimanus | 502 | 392 | – |
| | apicimacula | 473 | – | – |
| | crucians | 431 | – | – |
| | darlingi | 536 | – | – |
| | gambiae | 557 | 262 | – |
| | pseudopunctipennis | 502 | – | – |
| | punctimacula | 386 | – | – |
| | quadrimaculatus | 466 | – | – |
| Culex | annulirostris | 401 | – | 189 |
| | erraticus | 359 | 155 | 186 |
| | erythrothorax | 411 | – | 180 |
| | nigripalpus | 399 | – | 175 |
| | pipiens | 462 | – | 168 |
| | quinquefasciatus | 460 | – | 169 |
| | restuans | 409 | – | 185 |
| | salinarius | 417 | – | 194 |
| | tarsalis | 415 | – | 191 |
| Psorophora | ferox | 362 | ~ 192 | – |

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# Oligonucleotide primer specificity

| | | | Mosq-F (generic) | | | Mosq-RAE2 (Aedes) | | | Mosq-RCX (Culex) | | | Mosq-R2 (generic) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mewara A, 2018 | | | Jurado-Sánchez N, 2022 | | | Jurado-Sánchez N, 2022 | | | Jurado-Sánchez N, 2022 | | |
| Genus | Species | TotSeq | BC[1] | Seq[2] | Exten[4] | BC | n/% | Exten[4] | BC | n/% | Exten[4] | BC | n/% | Exten[4] |
| Aedes | abserratus | 2 | Unknown[3] | | | All bp | All seq | Yes | None | All seq | No | All bp | All seq | Yes |
| Aedes | aegypti | 19 | All bp | Some seq[3] | Yes | All bp | All seq | Yes | None | All seq | No | All bp | All seq[3] | Yes |
| Aedes | albopictus | 166 | Mostl bp[3] | All seq[3] | Yes | All bp | All seq | Yes | None | All seq | No | Most bp[3] | All seq[3] | Yes |
| Aedes | atlanticus | 2 | Mostl bp[3] | All seq[3] | Yes | All bp | All seq | Yes | None | All seq | No | All bp | All seq | Yes |
| Aedes | atropalpus | 1 | Unknown[3] | | | All bp | All seq | Yes | None | All seq | No | Unknown[3] | | |
| Aedes | atropos | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | aurifer | 1 | Unknown[3] | | | All bp | All seq | Yes | None | All seq | No | All bp | All seq | Yes |
| Aedes | campestris | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | camptorhynchus | 8 | Mostl bp[3] | All seq[3] | Yes | All bp | All seq | Yes | None | All seq | No | All bp | All seq | Yes |
| Aedes | canadensis | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | cantator | 1 | Unknown[3] | | | All bp | All seq | Yes | None | All seq | No | Unknown[3] | | |
| Aedes | cataphylla | 3 | Unknown[3] | | | All bp | All seq | Yes | None | All seq | No | Some bp[3] | All seq[3] | Likely[3] |
| Aedes | cinereus | 4 | All bp | Some seq[3] | Yes | All bp | All seq | Yes | None | All seq | No | All bp | All seq[3] | Yes |
| Aedes | dorsalis | 1 | Unknown[3] | | | All bp | All seq | Yes | None | All seq | No | Unknown[3] | | |
| Aedes | earlei | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | excrucians | 1 | Unknown[3] | | | All bp | All seq | Yes | None | All seq | No | Unknown[3] | | |
| Aedes | flavescens | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | fulvus | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | grossbecki | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | hensilli | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | hespernotius | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | infirmatus | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | intrudens | 1 | Unknown[3] | | | All bp | All seq | Yes | None | All seq | No | Unknown[3] | | |
| Aedes | japonicus | 5 | Unknown[3] | | | All bp | All seq | Yes | None | All seq | No | Most bp 3 | Most seq 3 | Likely[3] |
| Aedes | kochi | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | melaminon | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | mitchellae | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | nigromaculis | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | notoscriptus | 25 | All bp | All seq[3] | Yes | All bp | All seq | Yes | None | All seq | No | All bp | All seq | Yes |
| Aedes | polynesiensis | 2 | All bp | Most seq[3] | Yes | All bp | All seq | Yes | None | All seq | No | Some bp[3] | All seq[3] | Likely[3] |
| Aedes | solicitans | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | squamiger | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | sticticus | 3 | Unknown[3] | | | All bp | All seq | Yes | None | All seq | No | Unknown[3] | | |
| Aedes | taeniorhynchus | 1 | Unknown[3] | | | All bp | All seq | Yes | None | All seq | No | Unknown[3] | | |
| Aedes | territans | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | triseriatus | 2 | All bp | Most seq[3] | Yes | All bp | All seq | Yes | None | All seq | No | All bp | Most seq 3 | Yes |
| Aedes | trivitattus | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Aedes | vexans | 5 | Mostl bp[3] | Most seq[3] | Yes | All bp | All seq | Yes | None | All seq | No | All bp[3] | Most seq[3] | Yes |
| Aedes | vigilax | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Anopheles | albimanus | 8 | Mostl bp[3] | Most seq[3] | Yes | None | All seq | No | None | All seq | No | All bp | All seq[3] | Yes |
| Anopheles | apicimacula | 10 | Mostl bp[3] | All seq[3] | Yes | None | All seq | No | None | All seq | No | All bp | All seq[3] | Yes |
| Anopheles | crucians | 3 | All bp | All seq | Yes | None | All seq | No | None | All seq | No | All bp | All seq | Yes |
| Anopheles | darlingi | 26 | Mostl bp[3] | Most seq[3] | Yes | None | All seq | No | None | All seq | No | All bp | All seq[3] | Yes |
| Anopheles | gambiae | 14 | All bp | All seq[3] | Yes | Most bp | All seq | Likely | None | All seq | No | All bp | All seq[3] | Yes |
| Anopheles | pseudopunctipennis | 2 | All bp | Most seq[3] | Yes | None | All seq | No | None | All seq | No | All bp | All seq[3] | Yes |
| Anopheles | punctimacula | 11 | All bp | All seq | Yes | None | All seq | No | None | All seq | No | All bp | All seq[3] | Yes |
| Anopheles | quadrimaculatus | 5 | All bp | All seq | Yes | None | All seq | No | None | All seq | No | All bp | All seq | Yes |
| Coquillettidia | perturbans | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Culex | annulirostris | 11 | All bp | All seq | Yes | None | All seq | No | Most bp | All seq | Yes | All bp | All seq | Yes |
| Culex | erraticus | 69 | All bp | Some seq[3] | Yes | All bp | All seq | Yes | Some bp | All seq | Unlikely | All bp | All seq | Yes |
| Culex | erythrothorax | 2 | All bp | All seq | Yes | None | All seq | No | All bp | All seq | Yes | All bp | All seq | Yes |
| Culex | nigripalpus | 6 | Unknown[3] | | | None | All seq | No | Most bp | All seq | Yes | All bp | All seq | Yes |
| Culex | pipiens | 23 | All bp | Most seq[3] | Yes | None | All seq | No | All bp | All seq | Yes | All bp | All seq | Yes |
| Culex | quinquefasciatus | 59 | All bp | Most seq[3] | Yes | None | All seq | No | All bp | All seq | Yes | All bp | All seq | Yes |
| Culex | restuans | 6 | All bp | All seq | Yes | None | All seq | No | Most bp | All seq | Yes | All bp | All seq | Yes |
| Culex | salinarius | 6 | All bp | All seq | Yes | None | All seq | No | All bp | All seq | Yes | All bp | All seq | Yes |
| Culex | tarsalis | 4 | All bp | All seq | Yes | None | All seq | No | Most bp | All seq | Yes | All bp | All seq | Yes |
| Culiseta | inornata | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Culiseta | melanura | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Culiseta | morsitans | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Deinocerites | cancer | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Deinocerites | mathesoni | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Deinocerites | pseudes | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Mansonia | dyari | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Mansonia | signipennis | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Mansonia | titillans | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Psorophora | ciliata | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Psorophora | ferox | 1 | Unknown[3] | | | All bp | All seq | Yes | None | All seq | No | Most bp 3 | All seq | Yes |
| Uranotaenia | lowii | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Uranotaenia | sapphirina | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Wyeomyia | mitchellae | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Wyeomyia | smithii | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| Wyeomyia | vanduzeei | 0 | – | – | – | – | – | – | – | – | – | – | – | – |

## Notes on SSP-PCR amplicon lengths:

1. ~ Amplicon length inferred from alignments to other mosquito sequences of the same genus provided for sequences not spanning oligonucleotide binding sites.

2. ⊢ No amplification possible given oligonucleotide specificity.

3. * Amplicon length estimation not possible.

*Aedes notoscriptus* has two complementary sites for the extensible 3' end (last 7 bases) of Mosq-RAE2

## Notes on oligonucleotide primer specificity table:

1. Base complementarity, number of oligonucleotide primer bases annealing to mosquito sequences (All if ≥90% bp, Most if 75%-90% bp, Some if 50%-75% bp, Few if 25%-50%, None if <25%).

2. Number of mosquito sequences (Seq) annealing with described BC to oligonucleotide sequences (All if ≥90% seq, Most if 75%-90% seq, Some if 50%-75% seq, Few if 25%-50% seq, None if <25% seq).

3. Uninformative NCBI GenBank nucleotide sequence.

4. Likelihood of oligonucleotide primer being extended (Yes, No, Likely, Unlikely, Probably).

5. *Cullex erraticus* will paradoxically amplify with Mosq-RAE2 but not with Mosq-RCX.

## References

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## Revision history

1.0 Original document.

2.0 Updated protocol,

3.0 Update protocol for 25 uL vf PCR's and Thermo Scientific MiniAmp Plus thermalcyclers.

4.0 Protocol updated for new oligonucleotide primers (MOSQ-RAE2 & MOSQ-R2).

5.0 Protocol updated for two PCR reaction setup.

6.0 Optimized RAE2 concentration, Tm temperature and electrophoretic parameters.

7.0 Optimized Mosq-R2 concentration