
Technical Appendix

Sanger Sequencing

*pfmdr1* polymorphisms

*pfmdr1* gene was divided into two separate fragments (*pfmdr1_f1* and *pfmdr1_f2*). The *pfmdr1_f1* and *pfmdr1_f2* fragments were amplified separately in 25μl reactions including 5μl of template DNA, 0.5μM of each forward and reverse specific primer set for *pfmdr1_f1* and *pfmdr1_f2* fragments (Technical Appendix Table) and 1xHOT FirePol Master Mix (Solis BioDyne; Riaa, Tartu, Estonia), reaction volume was raised by PCR-grade water. In a thermocycler, both the fragment amplification followed the same PCR conditions, the template DNA was denatured at 95°C for 15min, followed by 35 cycles of amplification (95°C for 1min, 60°C for 1min, and 72°C for 1min 30s) and a final extension at 72°C for 10min. PCR products were run on 2% agarose (Invitrogen, Carlsbad, CA, USA) gels in 1× TBE buffer (Invitrogen, Carlsbad, CA, USA) to determine the presence and size of the amplified DNA and PCR products were visualized using a UV trans-illuminator. The expected size of PCR products for *pfmdr1_f1* and *pfmdr1_f2* fragments was 610bp and 1295bp respectively. Whenever enough amplified products were not obtained, re-PCR were performed using the same primer sets and PCR conditions mentioned above for both *pfmdr1_f1* and *pfmdr1_f2* fragments, 5μl of PCR products were used as DNA templates for re-PCRs. To sequence the f1 and f2 fragments of *pfmdr1* gene, two new pairs of sequencing primers were used (Technical Appendix Table). The sequencing primer pair, specific for *pfmdr1_f1* fragment, covers 45 – 209 aa and *pfmdr1_f2* specific sequencing primer pair covered 984 – 1277 aa of PfMDR1 protein. The specificity of all primer sets was tested against human genomic DNA and no DNA template control.
**pfldhps polymorphisms**

For *pfldhps* gene amplification, a single PCR based assay was designed. In brief, amplification was performed in 25μl reaction including 5μl of template DNA, 0.5μM of each forward and reverse specific primer set for *pfldhps* (Technical Appendix Table) and 1x HOT FirePol Master Mix, reaction volume was raised by PCR-grade water. The template DNA was denatured at 95°C for 15min in a thermocycler, followed by 32 cycles of amplification (95°C for 1min, 54°C for 1min, and 72°C for 1min) and a final extension at 72°C for 10 min. The expected size of the PCR product was 875bp was visualized as mentioned above covering 357- 647 aa of PfDHPS protein. PCR products were sequenced using a forward primer of PCR and new reverse sequencing primer (Technical Appendix Table) covering 357 – 477 aa of PfDHPS protein. Positive and negative (water instead of template DNA) controls were added in every PCRs and plates. The specificity of all primer sets was tested against human genomic DNA and no DNA template control.

For bi-directional sequencing, ≈1200ng of PCR products (quantified using EPOCH Biotech system) were sent to Genewiz (Takeley, United Kingdom), following safety instructions for the accurate shipment of PCR amplicons. The variations in the test sequences of *K13, pfcrt, pfmdr1* and *pfldhps* were identified by sequence alignment against PF3D7_1343700, PF3D7_0709000, PF3D7_0523000 and PF3D7_0810800 reference sequence of 3D7 respectively, retrieved from PlasmoDB.

**pfldhps - Restriction fragment length polymorphism**

To detect the K540E polymorphism in *pfldhps*, the restriction fragment length polymorphism (RFLP) was performed. The PCR products of 875bp (described above) of *Pfdhps* gene were digested with FokI enzyme (New England Biolabs, Ipswich, MA, USA) as per manufacture instruction’s which generated 538bp, 315bp and 22bp fragments whenever mutant allele was present, and two fragments of 853bp and 22bp were seen for wild type allele.

**pfmdr1 and pfpm2 gene copy number - qPCR**

The quantitative PCR (qRT-PCR) was used to assess variations in the copy number (CN) of *pfpm2* and *pfmdr1* genes as described elsewhere (1) with minor changes. In brief, 96-well plates were prepared containing separate 20μl reaction mixtures for *pfpm2, pfmdr1* and *pfβ-tubulin* genes. *Pfβ-tubulin* gene was used as an endogenous control. Each reaction mixture had
2X Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Warrington, WA, UK), 0.25 μM of each forward and reverse primers and 5μl of template DNA, reaction volume was makeup by PCR-grade water. Amplifications were performed with a holding for 3mins at 50°C, initial denaturation for 10mins at 95°C, followed by 40 cycles of 95°C for 15s and 58°C for 45s, extra dissociation stage was added to ensure the specificity of primer pairs. The 20μl reaction mixtures in a 96-well plate were amplified in a 7500 HT Real-Time System (Applied Biosystem, Foster City, USA). For each run, the \textit{pfpm2} and \textit{pfmdr1} copy numbers of each sample were measured in triplicate. The PCR efficiencies of the \textit{pfpm2}, \textit{pfmdr1} and \textit{pfβ-tubulin} genes were measured using ten-fold dilutions of 3D7 DNA. The specificity of three primer pairs against human genomic DNA was also determined. Along with no template control, we also included one positive control with the known 3-4 copies of \textit{pfpm2} gene provided by Dr. Didier Ménard (Pasteur Institute, Cambodia) and another positive control for \textit{pfmdr1} gene, genomic DNA of DD2 parasite line with known 3-4 copies of \textit{pfmdr1}. All samples with C\textsubscript{t} >33 for \textit{pfpm2}, \textit{pfmdr1} and \textit{pfβ-tubulin} were not considered for the CN analysis. The \textit{pfmdr1} and \textit{pfpm2} genes CNs were estimated as described previously (1). All samples with estimated CN values above 1.5 were confirmed two more times.

Reference


**Technical Appendix Table.** The sequence of the oligonucleotides used for PCR and sequencing, PCR annealing temperature (Tm), several cycles, the size of amplicons and covered amino acids

<table>
<thead>
<tr>
<th>Gene</th>
<th>Conventional PCR</th>
<th>Sequencing</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PCR primers</td>
<td>Annealing Temp (°C)</td>
</tr>
<tr>
<td>\textit{K13}</td>
<td>OuterF:cggagtgacccaatctggga</td>
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</tr>
<tr>
<td></td>
<td>OuterR:gggaatctggattgtaacgc</td>
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<tr>
<td></td>
<td>NestedF:gcaaggctgccattttg</td>
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<td>NestedR:gggaatctggattgtaacgc</td>
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<td>\textit{pfcrt}</td>
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<td>\textit{pfmdr1_F2}</td>
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<tr>
<td></td>
<td>R:tgctgataggtgatagcttt</td>
<td>984–1277</td>
</tr>
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</table>
Technical Appendix Figure. Assessment of the detection limit by Sanger sequencing in artificially mixed DNA samples, containing various proportions of pfcrt (K76T) and pfmdr1 (Y184F and S1034C) alleles. Panel A: K76T polymorphism of pfcrt gene; Panel B and C is for Y184F and S1034C polymorphisms of pfmdr1 gene, respectively.

Additional Text References


