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EXPERIMENTAL THERAPEUTICS

# Photo-Induced Electron Transfer Real-Time PCR for Detection of *Plasmodium falciparum plasmepsin 2* Gene Copy Number

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**ABSTRACT** Piperaquine is an important partner drug used in artemisinin-based combination therapies (ACTs). An increase in the *plasmepsin 2* and *3* gene copy numbers has been associated with decreased susceptibility of *Plasmodium falciparum* to piperaquine in Cambodia. Here, we developed a photo-induced electron transfer real-time PCR (PET-PCR) assay to quantify the copy number of the *P. falciparum plasmepsin 2* gene (*PfPM2*) that can be used in countries where *P. falciparum* is endemic to enhance molecular surveillance.

**KEYWORDS** PET-PCR, piperaquine, copy number, malaria

Malaria is one of the most common and serious tropical diseases in the world, with roughly 216 million cases and an estimated 445,000 malaria deaths globally (1). Malaria is caused by intracellular parasitic protozoa of the genus *Plasmodium*. *Plasmodium falciparum* is the most virulent among the human malarial parasites and causes disease with high morbidity and mortality (1, 2). The first-line therapy recommended by the World Health Organization for uncomplicated malaria caused by *P. falciparum* is artemisinin-based combination therapy (ACT) (4, 5), which consists of an artemisinin derivative combined with a partner drug of another class of antimalarial. However, resistance to artemisinin and partner drugs has been reported in Southeast Asia and confirmed by therapeutic efficacy studies (6–10). Dihydroartemisinin and piperaquine combination therapy is considered one of the remaining effective therapies against *P. falciparum* malaria; however, with the recent emergence of resistance to piperaquine, along with artemisinin in Southeast Asia, molecular surveillance to detect piperaquine-resistant parasites is now of utmost importance (7, 11–14). Recently, an increase in the copy number of the *plasmepsin 2–3* gene cluster in *P. falciparum* (*PfPM2–3*) was demonstrated to be associated with piperaquine resistance in Cambodia (11, 15, 16). This gene amplification was determined using genome-wide analysis of copy number variation and a singleplex TaqMan probe-based real-time-PCR (16). This real-time PCR method requires two separate singleplex PCRs (to amplify the single-copy housekeeping *P. falciparum* β-tubulin gene and the *PfPM2* gene of interest) and the use of a TaqMan probe or DNA intercalating dye. We previously demonstrated that the self-quenching photo-induced electron transfer (PET) fluorogenic primers used in the PET-PCR assay provide a more convenient to use and less expensive real-time PCR assay than conventional probe-based real-time PCR formats, as no internal probes (e.g., TaqMan probes) or intercalating dyes are required (17, 18). We designed and evaluated a multiplex PET-PCR assay (results obtained using a single PCR) to determine the number of copies of *PfPM2* gene in *P. falciparum*. This novel assay was compared with the previously published singleplex TaqMan probe-based real-time PCR assay (16).

The *PfPM2* and *P. falciparum* β-tubulin gene primers used in this study were previously reported by Witkowski and collaborators (16). In our multiplex PET-PCR

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**TABLE 1** Oligonucleotide primer sequences used in the PET-PCR *PfPM2* copy number assay[a]

| PET-PCR primer | PET-PCR sequence (5′ to 3′) | $T_m$ (°C) | Product size (bp) | Melting temp range (°C) |
|---|---|---|---|---|
| PfPM2_F | TGGTGATGCAGAAGTTGGAG | 59.8 | 79 | 76.8–77.2 |
| PfPM2_R_FAM | **aggcgcatagcgcctgg**TGGGACCCATAAATTAGCAGA | 59.4 | | |
| Pf β-tubulin_F | TGATGTGCGCAAGTGATCC | 61.9 | 87 | 79.0–79.2 |
| Pf β-tubulin_R_HEX | **aggcgcatagcgcctgg**TCCTTTGTGGACATTCTTCCTC | 60.5 | | |

[a]The PET-tag (lowercase letters in bold) attached to the 5′ end of the target-specific sequences does not show any homology to *Plasmodium* species. The *PfPM2* (gene accession no. PF3D7_1408000-811659) and β-tubulin gene (gene accession no. PF3D7_1008700) primers were previously reported in the singleplex probe-based real-time PCR assay (16).

assay, the reverse primers of both *PfPM2* and the *P. falciparum* β-tubulin gene were modified with the PET tag and labeled with 6-carboxyfluorescein (FAM; *PfPM2*) and HEX (*P. falciparum* β-tubulin gene) fluorophores, as previously described by Lucchi et al. (17) (Table 1). The PET-PCR was carried out in a 20-μl volume containing 2× TaqMan Environmental master mix 2.0 (Applied Biosystems, Foster City, CA, USA), 0.5 μM each forward and reverse primer for *PfPM2*, 0.25 μM for the *P. falciparum* β-tubulin gene primers, and 3 μl of template DNA. The amplifications were performed under the following conditions: 95°C for 15 min, 45 cycles of 95°C for 15 s, 58°C for 1 min, and 72°C for 20 s. All samples were tested in triplicate, and the assays were performed using Agilent Mx3005pro thermocyclers (Agilent Technologies, Santa Clara, CA, USA). Evaluation of PET-PCR amplification efficiency and reproducibility was performed using a 10-fold dilution of a *P. falciparum* control strain (3D7), with parasite densities of 2,000 parasites/μl to 2 parasites/μl. The amplification efficiencies of the *PfPM2* and the *P. falciparum* β-tubulin gene PET-primers were 97% and 100%, respectively (Fig. 1A and B). The *PfPM2* copy number was determined by the $2-\Delta\Delta C_T$ method ($\Delta C_T = C_T$ *PfPM2* $- C_T$ *P. falciparum* β-tubulin gene; $C_T$, threshold cycle) using the 3D7 *P. falciparum* strain, known to have a single copy of the *PfMD2* gene, as a calibrator and two additional controls (control 2 [Ctrl-2] and Ctrl-3 obtained from Cambodia with one copy and three copies, respectively). To validate the accuracy of the PET-PCR for copy number variation determination, six additional clinical samples, all obtained from Cambodia, with known *PfMD2* copy numbers were tested in a blinded fashion using both the novel PET-PCR and the previously reported singleplex TaqMan probe-based assay. These specimens were kindly provided by R. M. Fairhurst's (samples MB-PPQ-001 to MB-PPQ-004) and D. Fidock's (samples MB-PPQ-005 and MB-PPQ-006) laboratories and were previously evaluated for *PfMD2* copy numbers using probe-based real-time PCR assays and/or whole-genome sequencing. The PET-PCR multiplex *PfPM2* copy number results had 100% agreement with the previously published results of the singleplex TaqMan probe-based assay (16) (Table 2).

Simple molecular tools are valuable for conducting molecular surveillance of drug

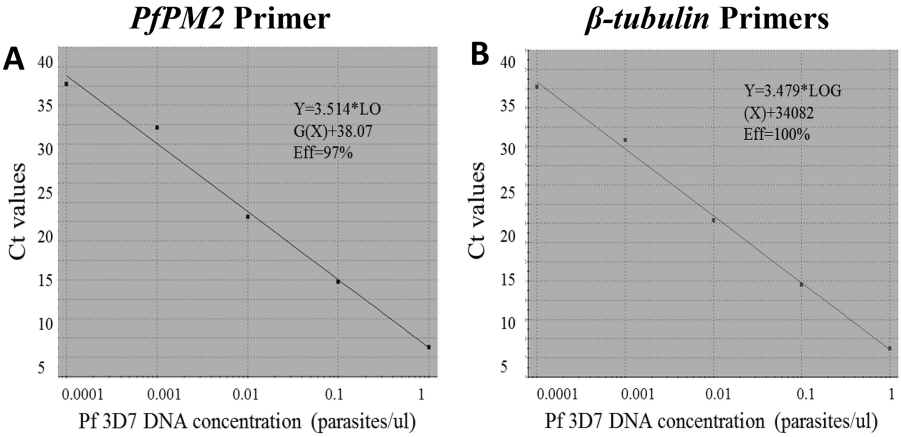


**FIG 1** Amplification efficiencies of the novel PET-PCR primers. The amplification efficiency (Eff) of the *PfPM2* primers were 97% (A), and that of the *P. falciparum* β-tubulin gene primers was 100% (B).

**TABLE 2** Comparison between PET-PCR and TaqMan probe-based real-time PCR assay[a]

| Sample ID[b] | Observed no. of *PfPM2* copies | |
|---|---|---|
| | PET-PCR | TaqMan-based real-time PCR |
| Ctrl-1 (strain 3D7) | 1 | 1 |
| Ctrl-2 | 1 | 1 |
| Ctrl-3 | 3 | 3 |
| MB-PPQ-001 | 1 | 1 |
| MB-PPQ-002 | 1 | 1 |
| MB-PPQ-003 | 1 | 1 |
| MB-PPQ-004 | 3 | 3 |
| MB-PPQ-005 | 2 | 2 |
| MB-PPQ-006 | 3 | 3 |

[a]The PET-PCR multiplex *PfPM2* copy number results had 100% agreement with previously published singleplex real-time PCR assay results (16). Ctrl, control samples; MB-PPQ-001 to -006, clinical samples that were kindly provided by R. M. Fairhurst and D. Fidock.
[b]ID, identification number.

resistance markers. The PET-PCR is a molecular diagnostic assay with performance characteristics that are similar to those of commonly used real-time PCR methods, but the PET-PCR is less expensive, easy to use, and can be used for large-scale surveillance studies, even in developing country settings (17). The novel multiplex PET-PCR assay for *PfPM2* copy number determination has performance characteristics similar to those of a singleplex TaqMan probe-based assay but without the need for running two independent PCRs. The novel assay provides a useful cost-effective alternative for the evaluation of the *PfPM2* copy number. As monitoring for piperaquine resistance is gaining momentum and urgency, it is hoped that this new method will help enhance molecular surveillance in a timely manner even in reference laboratories in countries endemic for the disease. In addition, the PET-PCR format described here provides an alternative real-time PCR format that can be utilized for the determination of any gene amplification of interest.

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