Short technical report

Quantification of female and male *Plasmodium falciparum* gametocytes by reverse transcriptase quantitative PCR

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ABSTRACT

The transmission of malaria parasites depends on the presence of sexual stages (gametocytes) in the blood, making the ratio and densities of female and male gametocytes important determinants of parasite fitness. This manuscript describes the development of reverse transcriptase quantitative PCR (RT-qPCR) assays to separately quantify mature female and male gametocytes of the human malaria parasite *Plasmodium falciparum*, and reveals that *Pfs25* mRNA is expressed only in female gametocytes. The female (*Pfs25*) and male (*Pfs230p*) gametocyte specific RT-qPCR assays have lower detection limits of 0.3 female and 1.8 male gametocytes per microlitre of blood, respectively, making them more sensitive than microscopy. Accurate quantification of the ratio and densities of female and male gametocytes will increase understanding of *P. falciparum* transmission and improve the evaluation of transmission blocking interventions.

For malaria (*Plasmodium*) parasites, transmission between vertebrate hosts requires a mosquito vector. Throughout infections in the vertebrate host, *Plasmodium* produces female and male sexual stages (gametocytes). Upon ingestion by a blood-feeding mosquito, gametocytes differentiate into gametes, mate, and after several developmental transitions, the progeny are able to infect another vertebrate host. The densities of female and male gametocytes in the blood and the sex ratio (proportion of gametocytes that are

male) influence infectivity to mosquitoes, and so, onwards transmission to new hosts [1–3].

Techniques to determine the sex of *P. falciparum* gametocytes are laborious. They include microscopical examination of Giemsa-stained thin blood smears and immunofluorescence assays using antibodies recognising female- and/or male-specific antigens [4]. Furthermore, the sensitivity of these methods is low: transmission to mosquitoes readily occurs at densities below the microscopic detection limit of 8–16 gametocytes/μl [3,5]. This makes it very difficult to obtain accurate estimates of sex-specific gametocyte densities, and sex ratios, from as many as 50% of infections capable of transmitting to mosquitoes [3] because in some cases as few as 10 gametocytes are sexed [6]. However, separately following the densities of female and male gametocytes during natural infections is necessary to understand how their dynamics and sex ratios influence disease transmission and to evaluate transmission blocking interventions [2,7].

To facilitate the study of *P. falciparum* female and male gametocyte dynamics and gametocyte sex ratios, high-throughput, sensitive molecular methods for the quantification of mature

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Table 1

P. falciparum female (*Pfs25*) or male (*Pfs230p*) gametocyte-specific RT-qPCRs. Sequences for forward primers (FW), reverse primers (RV) and TaqMan probe (P) are shown with their location, based on genbank accession numbers AE014185.2 and AE001362.1 for the *Pfs25* (*PF3D7_1031000*) and *Pfs230p* (*PF3D7_0208900*) assays.

Name	Sequence	Location
Pfs25-FW:	CCATGTGGAGATTTTTCCAAATGTA	1253852–1253828
Pfs25-RV:	catttaccgttaccacaagttaCATTC	1253710–1253736
Pfs25-P1:	6FAM-ccgtttcatacgcttgtaa-MGB	1253809–1253791
Pfs230p-FW3:	CCCAACTAATCGAAGGGATGAA	363440–363461
Pfs230p-RV4:	AGTACGTTTAGGAGCATTTTTTGGTAA	363635–363609

PCR conditions

Pfs25 assay: 900 nM of forward and reverse primers (FW), 250 nM probe (MGB-TaqMan probes, Life Technologies), 500 nM ROX reference dye and 1x PCR reaction buffer 3 mM MgCl$_2$, 0.4 mM dNTPs, 1 unit taq (FastStart Taq dNTPack, Roche); *Pfs230p* assay: 900 nM of forward and reverse primers, 1x GoTaq® qPCR mastermix including BRYT Green® (Promega). Both assays can be run simultaneously within the same PCR plate since they use the same PCR programme (95 °C 10 min; 45 cycles of: 95 °C 15 s, 60 °C 1 min) and despite different detection chemistries both assays require detection in the FAM channel. For details on sample processing see Supplementary data, section 3).

female and male gametocytes were developed. The female-specific assay quantifies mRNA of the gene encoding *Pfs25* (*PF3D7_1031000*). *Pfs25* is expressed in late stage gametocytes, shows limited polymorphism and is currently used to quantify gametocytes from field isolates [3,8].

The sex-specificity of *Pfs25* mRNA expression is tested in this manuscript, and confirmed to be female-specific, like mRNA of its orthologue in the rodent malaria *P. berghei* (Pbs21) [9,10].

Only a few *P. falciparum* male-specific proteins have been described. The most well-known are alpha-tubulin II and the Pfs230 paralogue, Pfs230p [11,12]. Low levels of α-tubulin II protein have been demonstrated in asexual blood stages and early stage female gametocytes [12]. Therefore, the male-specific assay quantifies *Pfs230p* (*PF3D7_0208900*) mRNA [11], and its specific expression in late stage male gametocytes is verified. The design, optimisation, and validation (including the assessment of specificity and sensitivity) of assays to quantify female or male *P. falciparum* mature gametocytes, are presented below.

Primers and probe (Table 1) for the *Pfs25* and *Pfs230p* assays are *P. falciparum* specific (BLAST). No significant single nucleotide polymorphisms were detected in the *Pfs25* and *Pfs230p* primer and probe regions among almost 2000 *P. falciparum* samples collected from all over the world, (The Pf3K Project (2014): pilot data release 2. www.malariagen.net/data/pf3k-2) [13]. Therefore, the primers and probe are suitable for use on field samples that potentially

contain multiple parasite genotypes. To provide large amounts of material from the target genes for assay optimisation and calibration, independent of the need to repeatedly culture *P. falciparum* parasites, constructs were made to produce DNA, RNA and cDNA *in vitro* (Supplementary data, section 1). Because the target genes have single exons, DNA sequences of the target regions (*in vitro*-produced DNA: ivDNA) and cDNA are identical, so ivDNA was used to optimise assay conditions (Table 1). PCR efficiencies for the *Pfs25* and *Pfs230p* qPCRs were 93.6% with a standard error (se) of 1.5%, and 98.5 (se 2.4%), respectively. Amplification was not observed in the no-template controls, and the lower limit of detection was similar at 9 and 2 molecules/25 µl reaction for the *Pfs25* and *Pfs230p* qPCRs, respectively (Fig. 1). The concentration of *Pfs25* and *Pfs230p* ivDNA was highly correlated to PCR quantification cycles (Cq; adjusted $r^2 = 0.98$, $p < 0.001$ for both assays) and the small standard errors indicate high repeatability between replicates (Fig. 1; for details on statistical analysis see Supplementary data, section 2). In summary, the assays are sensitive and quantify their respective ivDNA accurately.

Next, the *Pfs25* and *Pfs230p* assays were assessed for species-specificity. The *Pfs25* and *Pfs230p* assays do not amplify nucleic acids from uninfected human blood samples that were collected from volunteer donors (n = 10, with written informed consent). Orthologues for the *Pfs25* and *Pfs230p* genes are present in *P. vivax*, *P. knowlesi*, and in the rodent malaria species *P. berghei* and *P. chabaudi* (PlasmoDB.org). Sequence similarity between the *P. falciparum* sequences and the different orthologues is comparable (44–47% for *Pfs25*, 41–42% for *Pfs230p*). Therefore, and given the difficulty in obtaining samples for single-species infections of *P. knowlesi* or *P. vivax*, DNA from *P. chabaudi* (CW) and *P. berghei* (ANKA) (from experiments carried out in accordance with the UK Animals Scientific Procedures Act 1986) was used to empirically test *P. falciparum* species-specificity of the *Pfs25* and *Pfs230p* qPCR assays. No amplicons were detected, confirming the *in silico* analysis.

For the assessment of stage- and sex-specificity and to determine sensitivity of the assays, a variety of *P. falciparum* samples were used. These samples include: (1) "Asexual stages": sorbitol-synchronised asexual ring stage parasites (clone 3D7), freed of gametocytes through magnetic separation [14,15]; (2) "Mixed sex gametocytes" free of asexual stages after treatment with N-acetyl glucosamine [15], including (a) sample 3D7-1: gametocytes of clone 3D7 at development stage IV, with a sex ratio (proportion of gametocytes that are males) of 0.05 as determined by morphological examination of Giemsa stained thin films; (b) sample 3D7-2:
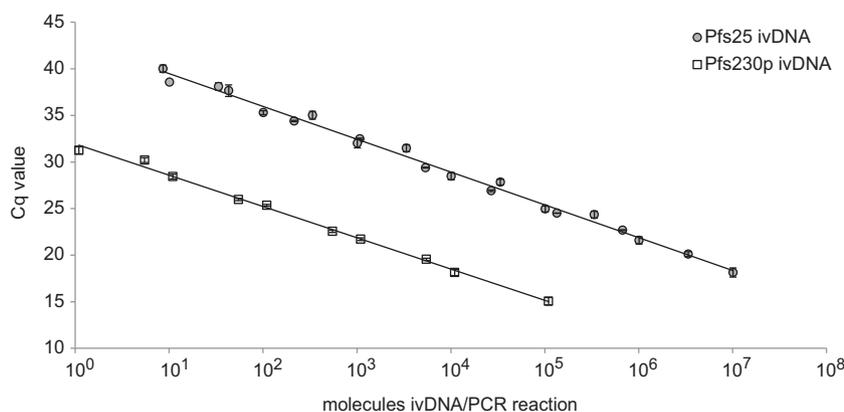

Fig. 1. The *Pfs25* TaqMan and *Pfs230p* BRYT Green® qPCRs are sensitive and accurate for the quantification of their respective DNA target sequences (ivDNA, Supplementary data, section 1). Average Cq values with standard errors are presented for the quantification of 13 and 4 dilution series of respectively *Pfs25* ivDNA and *Pfs230p* ivDNA. Regression equations are *Pfs25*: Cq value = $-3.49 \times 10 \log Pfs25$ ivDNA/PCR + 41.39, $r^2 = 0.98$, $p < 0.001$; *Pfs230p*: Cq value = $-3.36 \times 10 \log Pfs230p$ ivDNA/PCR + 31.95, $r^2 = 0.98$, $p < 0.001$.
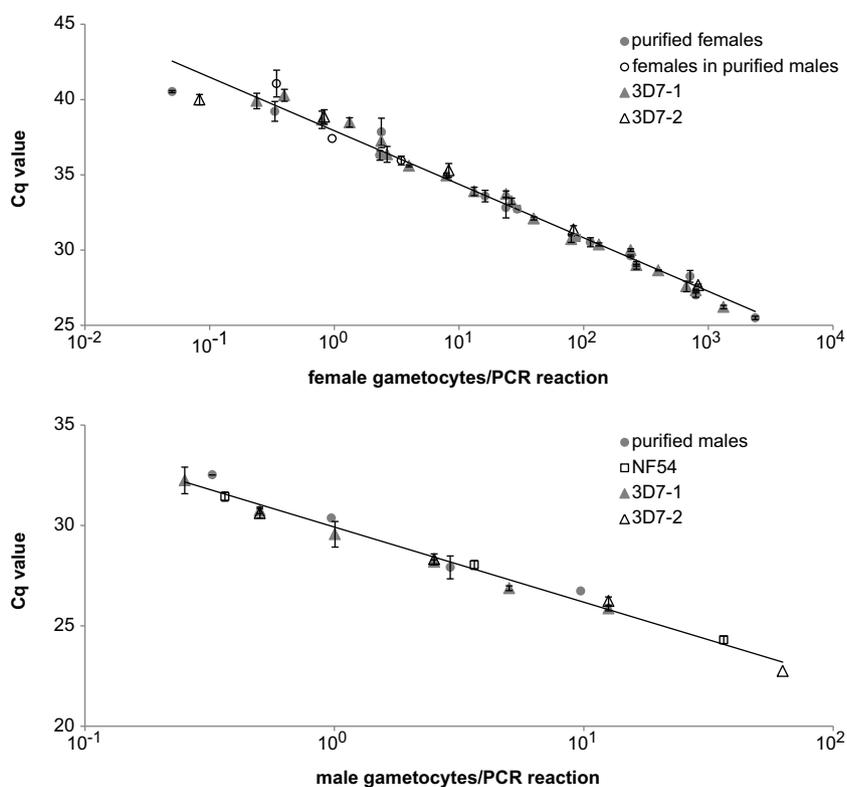
Fig. 2. Gametocyte quantification by sex-specific RT-qPCR. The *Pfs25* TaqMan (2A) and *Pfs230p* BRYT Green® (2B) RT-qPCRs are sensitive and accurate for the quantification of respectively female and male gametocytes. To enable direct comparison of various sample processing methods (see Supplementary data, section 3) average Cq values with standard errors are presented for the number of female (2A) or male (2B) gametocytes/PCR reaction. *Pfs25* RT-qPCR: data originate from cDNA of 2 RNA extractions each for "Purified females", "Purified males", "Mixed sex gametocytes" 3D7-1 and 3D7-2 respectively, with each sample measured in 2–7 replicates. Regression equation: Cq value = -3.62×10 log female gametocytes/PCR + 41.18, $r^2 = 0.97$, $p < 0.001$ (2A); *Pfs230p* RT-qPCR: data originate from cDNA of 1, 3, 2 and 3 RNA extractions for "Purified females", "Mixed sex gametocytes" 3D7-1, 3D7-2 and NF54 respectively, with each sample measured in 2–8 replicates. Regression equation: Cq value = -3.53×10 log male gametocytes/PCR + 29.78, $r^2 = 0.91$, $p < 0.001$ (2B).

3D7 stage IV gametocytes depleted of uninfected red blood cells by magnetic purification [14] with a sex ratio of 0.05 determined by morphological examination of Giemsa stained thin films; (c) sample NF54: stage IV-V gametocytes of isolate NF54. NF54 parasites cultured using the methods as described in [15] consistently have a sex ratio of 0.2 as determined by morphological examination of Giemsa stained thin films; (3) "Purified females": stage IV-V gametocytes of line p47GFP (derived from NF54) that expresses green fluorescent protein specifically in female gametocytes, purified using flow cytometry, free from asexual stages and containing 98% females (assessed by re-sorting aliquots by flow cytometry [16]); and (4) "Purified males": stage IV-V gametocytes of line Dyn-GFP (derived from NF54) that expresses green fluorescent protein specifically in male gametocytes, purified using flow cytometry, free of asexual stages and containing >98% males (assessed by flow cytometry [16]. Samples were stored in RNA-preserving reagents (for details on sample processing see Supplementary data, section 3) and stored at −70 °C. RNA from all samples were extracted on a semi-automatic MagMAX™ Express Particle Processor (Life Technologies) using the MagMAX™-96 total RNA isolation kit according to manufacturer's protocol, with the exception of RNA from the "Mixed sex gametocytes NF54" sample, which was processed in a different laboratory (Supplementary data, section 3) and was extracted using the MagNAPure system at protocol HS500 and the MagNAPure LC Total Nucleic Acid Kit–High Performance. All samples were DNAse I treated (RQ1, Promega) and confirmed to be DNA free by 18S qPCR [17] before cDNA was synthesised by reverse transcriptase PCR (High capacity cDNA Reverse Transcription Kit, Life Technologies). The female-specific *Pfs25* qPCR followed by the male-specific *Pfs230p* qPCR are presented the following paragraphs.

First, to test whether *Pfs25* mRNA expression is female gametocyte-specific, like mRNA of its orthologue in the rodent malaria *P. berghei* (Pbs21) [9,10], qPCR was performed using cDNA from "Asexual stages" (8.9×10^5 parasites/μl blood) and the "Purified males" (6.7×10^3 gametocytes/μl blood, <2% females). No amplicons were obtained with samples from "Asexual stages", confirming gametocyte-specificity. However, low levels of *Pfs25* amplicons were detected from the "Purified males". The expression of *Pfs25* mRNA for each gametocyte from the "Purified males" was significantly lower than in the "Purified females", and "Mixed sex gametocytes" samples 3D7-1 and 3D7-2 ($F_{3,178} = 253.11$, $p < 0.001$). *Pfs25* mRNA expression was 274-fold higher in the "Purified females", which is consistent with the "Purified males" (purity expected to be >98% males) containing a 0.36% contamination of female gametocytes. When the proportion of female gametocytes in each sample (0.36%, 95% and 98% for the "Purified males", "Purified females", "Mixed sex gametocytes" 3D7-1 and 3D7-2, respectively) is taken into account, there are no significant differences in *Pfs25* mRNA expression per female gametocyte across these sex ratios ($F_{3,178} = 2.63$, $p = 0.052$; Fig. 2A). Extremely low expression of *Pfs25* mRNA in male gametocytes cannot be ruled out, but even in this case, the 274-fold lower mRNA expression in male *versus* female gametocytes will not significantly reduce the ability of the *Pfs25* assay to accurately quantify female gametocytes. Therefore, the *Pfs25* RT-qPCR, and the *Pfs25* QT-NASBA currently used for field surveys [3], can be considered to quantify female gametocytes only.

The *Pfs25* qPCR assay's sensitivity, PCR efficiency, and accuracy of quantification were assessed using 4 samples: "Mixed sex gametocytes" samples (clone 3D7, 2 samples each with 95% females;

spanning cDNA densities equivalent to $0.17–9.37 \times 10^3$ (3D7-1) and $0.34–5.86 \times 10^3$ (3D7-2) female gametocytes/µl blood); the "Purified females" (line p47GFP, 98% females; spanning cDNA densities equivalent to $0.33–1.68 \times 10^4$ female gametocytes/µl blood); and the females present in the "Purified males" (line DynGFP, 0.36% females; spanning cDNA densities equivalent to $0.24–24.3$ female gametocytes/µl blood). The lower limit of quantification, *i.e.* the lowest tested dilution at which all replicates were reliably quantified, was equivalent to 0.3 female gametocytes/µl blood ("Purified females", corresponding to 0.04 female gametocytes/PCR reaction; Fig. 2A). PCR efficiency was 89.0% (se 1.6%) and not significantly different between the 4 tested samples ($F_{3,175} = 1.48$, $p = 0.22$). Female gametocyte numbers and Cq values were significantly correlated (adjusted $r^2 = 0.97$, $p < 0.001$), and the small standard errors demonstrate high repeatability (Fig. 2A). Pfs25 RT-qPCR efficiency ($F_{1,11} = 1.96$, $p = 0.19$) and Cq values ($F_{1,12} = 0.68$, $p = 0.42$) were not affected by the presence of cDNA from "Asexual parasites" (7.8×10^4 asexual parasites/µl blood) nor did these vary across the percentage of parasites that were female gametocytes (0.0007–100%) or gametocyte densities (0.6–611 females gametocytes/µl blood) similar to those found in natural *P. falciparum* infections. This shows that the Pfs25 RT-qPCR is reliable and sensitive for quantification of female gametocytes, also in the presence large quantities of non-target nucleic acids, and therefore neither gametocytaemia (proportion parasites that is gametocytes) nor sex ratio (proportion of gametocytes that is male) affect the accuracy with which female gametocytes are quantified.

For the male-specific PCR, gametocyte-specificity was confirmed by the absence of Pfs230p mRNA expression in the *P. falciparum* "Asexual stages" sample (equivalent to 8.9×10^5 parasites/µl blood). The expression of Pfs230p mRNA is different for gametocytes from the "Purified females", the NF54, 3D7-1 and 3D7-2 "Mixed sex gametocytes" ($F_{3,119} = 67.21$, $p < 0.001$), with Pfs230p mRNA expression lowest in the "Purified females" (1.7×10^4 female gametocytes/µl blood) and highest in the samples with largest proportion of males. When taking into account the presence of 2%, 5%, 5% and 20% male gametocytes in the samples of "Purified females", "3D7 Mixed sex gametocytes 3D7-1 and 3D7-2" and "NF54 Mixed sex gametocytes", respectively, the expression level of Pfs230p mRNA per male gametocyte is not significantly different between the 4 samples ($F_{3,119} = 1.40$, $p = 0.25$, Fig. 2B). This confirms the previously published male-specificity of Pfs230p mRNA expression [11].

The performance of the Pfs230p RT-qPCR assay was further assessed as described for the Pfs25 RT-qPCR, using 4 samples: "Mixed sex gametocytes" clone 3D7, 2 samples each with 5% males; spanning cDNA densities equivalent to 0.89–88.84 (3D7-1) and 1.18–444.21 (3D7-2) male gametocytes/µl blood); "Mixed sex gametocytes" sample NF54 (20% males; cDNA densities equivalent to 0.2–200.2 male gametocytes/µl blood); and the males present in the "Purified females" (line p47GFP, 2% males; spanning cDNA densities equivalent to 0.76–68.72 male gametocytes/µl blood). The lower limit of quantification was equivalent to 1.8 male gametocytes/µl blood ("Mixed sex gametocytes" 3D7-1, corresponding to 0.3 male gametocytes/PCR reaction; Fig. 2B). Detection of Pfs230p mRNA was possible below 0.3 male gametocytes/PCR reaction, but occasional false negatives occurred and quantification is unreliable due to a non-linear relationship between male gametocyte densities and Cq values. Pfs230p RT-qPCR efficiency was 92.0% (se 3.6%) and was not significantly different for male gametocytes from the 3D7-1, 3D7-2 and NF54 "Mixed sex gametocytes" and from the "Purified females" ($F_{3,106} = 1.26$, $p = 0.29$). Above the lower limit of quantification, male gametocyte numbers were significantly correlated to Cq values and quantification was repeatable (adjusted $r^2 = 0.91$, $p < 0.001$; Fig. 2B). Pfs230p RT-qPCR efficiency ($F_{1,22} = 0.0002$, $p = 0.99$) and quantification of male

gametocytes ($F_{1,23} = 0.005$, $p = 0.94$) were not affected by the presence of cDNA from "Asexual parasites" (8.9×10^5 asexual parasites/µl blood), the percentage of parasites that were male gametocytes (0.002–100%) or the range of gametocyte densities (1–324 male gametocytes/µl blood) similar to those found in natural infections. Because Pfs230p RT-qPCR was not affected by large quantities of non-target nucleic acids, the accuracy of male gametocyte quantification is not affected by gametocytaemia or sex ratio.

In conclusion, both sex-specific RT-qPCRs are accurate and more sensitive than microscopy [5], and can quantify mature female and male *P. falciparum* gametocytes, including submicroscopic gametocyte densities as low as 0.3 female and 1.8 male gametocytes/µl of blood. The female-specificity of the Pfs25 assay means that the Pfs25 QT-NASBA, currently used for field surveys, quantifies female gametocytes only [3]. Although this should be taken into account when interpreting data, female density reflects an important aspect of transmission because it places the upper limit on ookinete (offspring) numbers. The sex-specific RT-qPCRs facilitate the study of why the densities of mature female and male gametocytes, and the sex ratio (proportion of gametocytes that are male) vary during infections and between hosts, and their influence on transmission. Another application of the assays is the separate assessment of female and male gametocyte clearance after drug treatment. Male gametocytes are thought to be limiting for transmission in general [2] and this could be exacerbated after treatment with gametocytocidal drugs because males are more sensitive to a variety of drugs compared to female gametocytes [7,18].

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2015.03.006.

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