Measurement of gene amplifications related to drug resistance in *Plasmodium falciparum* using droplet digital PCR

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Methodology

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Abstract

Background: Copy number variations (CNVs) of the *Plasmodium falciparum multidrug resistance 1* (*pfmdr1*), *P. falciparum pfplasmepsin2* (*pfplasmepsin2*) and *P. falciparum GTP cyclohydrolase 1* (*pfgch1*) genes are associated with antimalarial drug resistance in *P. falciparum* malaria. Droplet digital PCR (ddPCR) assays have been developed for accurate assessment of CNVs in several human genes. The aim of the present study was to develop and validate ddPCR assays for detection of the CNVs of *P. falciparum* genes associated with resistance to antimalarial drugs.

Methods: The ddPCR assays were developed to detect the CNVs in the *pfmdr1*, *pfplasmepsin2* and *pfgch1* genes. The gene copy number (GCN) quantification limit, as well as the accuracy and precision of the ddPCR assays were determined and compared to conventional quantitative PCR (qPCR). In addition, the CNVs of genes of field samples collected from Thailand from 2015 to 2019 (n = 84) were assessed by ddPCR and results were compared to qPCR as the reference assay.

Results: Based on the obtained gene copy number quantification limit, the accuracy and percent relative standard deviation (%RSD) value of the multiplex ddPCR assay were 95% and 5%, respectively, for detection of the CNV of the *pfmdr1* gene, and 91% and 5% for detection of the CNV of the *pfplasmepsin2* gene. The accuracy and %RSD value of the duplex ddPCR assay were 94.88% and 3.71, respectively, regarding *pfgch1* GCN. In the *P. falciparum* field samples, *pfmdr1* and *pfplasmepsin2* GCNs were amplified in 15% and 27% of samples from Ubon Ratchathani, Thailand, while *pfgch1* GCN was amplified in 50% of samples from Yala, Thailand. There was 100% agreement between the GCN results obtained from the ddPCR and qPCR assays (κ = 1.00).

Conclusions: The developed ddPCR assays are simple, accurate, precise and cost-effective tools for detection of the CNVs in the *pfmdr1*, *pfplasmepsin2* and *pfgch1* genes of *P. falciparum*. The ddPCR assay is a useful additional tool for the surveillance of antimalarial drug resistance.

Background

Artemisinin-based combination therapies (ACTs) are recommended as front-line treatments for *Plasmodium falciparum* malaria, which remains an important infectious disease in tropical regions. However, the emergence and spread of resistance to ACTs and related drugs have resulted in poor curative rates, especially in Southeast Asia [1–5]. Molecular surveillance is needed not only for the detection of mutations to the *P. falciparum kelch* gene, which are associated with artemisinin resistance [3], but also molecular markers associated with the efficacy of other antimalarial drugs. An increase in the *P. falciparum multidrug resistance 1* (*pfmdr1*) GCN is associated with mefloquine resistance [6], while an increase in the *P. falciparum plasmepsin2* (*pfplasmepsin2*) GCN is associated with piperaquine resistance [7, 8]. Moreover, amplification of the *P. falciparum GTP cyclohydrolase 1* (*pfgch1*) GCN is linked to upregulation of the *Plasmodium falciparum dihydrofolate reductase* (*pfdhfr*) and *P. falciparum dihydropteroate synthase* (*pfdhps*) genes, which are associated with sulfadoxine-pyrimethamine resistance in Southeast Asia [9, 10].

Quantitative PCR (qPCR) assays are conventionally used to assess the copy number variations (CNVs) of genes related to drug resistance in *P. falciparum* malaria (i.e. *pfmdr1* [6], *pfplasmepsin2* [7], and *pfgch1* [11]). Alternatively, droplet digital PCR (ddPCR) technology was developed to measure CNVs and to provide highly precise measurements of the concentrations of target and reference genes in DNA samples [12, 13], as well as

tolerance to PCR inhibitors, such as heparin [14], and to generate calibration curves to determine the GCNs of target sequences [15, 16]. The ddPCR assay has been developed for accurate detection of CNVs in human genes associated with various human genetic diseases [17–19]. In addition, a ddPCR assay was developed and validated for the detection and quantification of *Plasmodium* species based on the *18S rRNA* gene sequence [20, 21], but this method has not yet been validated for the detection of the CNVs of genes associated with resistance to antimalarial drugs.

In the present study, ddPCR assays were developed and validated for quantification of the CNVs of the *pfmdr1*, *pfplasmepsin2* and *pfgch1* genes, as well as for the molecular surveillance of the efficacy of antimalarial drugs in field isolates. The ddPCR assays were used to detect the CNVs of the *pfmdr1*, *pfplasmepsin2* and *pfgch1* genes in field samples and validated against the results obtained with qPCR assays. A flowchart was generated including criteria for the detection of GCN with the novel ddPCR assays. Costs and time required for the ddPCR assays are discussed.

Material And Methods

DNA samples

Development and validation of the ddPCR assays used DNA extracted from *P. falciparum* laboratory strains of obtained from the Malaria Research and Reference Reagent Resource Center (American Type Culture Collection, Manassas, VA, USA). Parasite DNA from *P. falciparum* strain 3D7 (MRA-102), which carries single copies of the *pfmdr1* and *pfplasmespin2* genes, was used to develop and validate the ddPCR assays for the detection of the CNVs of these two genes. DNA samples of *P. falciparum* strain D6 originating from Sierra Leone, West Africa, which carries a single copy of the *pfgch1* gene [10], were used as single copy controls for the development and validation of ddPCR assays to detect the CNVs of the *pfgch1* gene. Two-fold serial dilutions of *P. falciparum* strains 3D7 and D6 were prepared and used to quantify the GCN, as well as to assess the accuracy and precision of the ddPCR assays.

P. falciparum strains 3D7, 7G8 and D6 (MRA-285G) obtained from the Malaria Research and Reference Reagent Resource Center (n = 7) were used to compare the CNVs obtained by the ddPCR and qPCR assays. In addition, the CNVs of the *pfmdr1*, *pfplasmepsin2* and *pfgch1* genes of field samples (n = 84) collected from patients with confirmed *P. falciparum* infections between 2015 and 2019 in Ubon Ratchathani (n = 60) and Yala (n = 24), Thailand, were determined. DNA samples were extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany). DNA concentrations were measured using a Nanodrop™ spectrophotometer (Thermo Scientific, Willington, DE, USA). The study protocol was approved by the Ethical Review Committee of the Faculty of Tropical Medicine, Mahidol University (Bangkok, Thailand) (approval no. MUTM 2012-045-05).

Development of the ddPCR assays

For the ddPCR assays, each 20-µL reaction contained 10 µL of ddPCR™ Supermix for Probes (Bio-Rad Laboratories, Hercules, CA, USA), 900 nM primers, 250 nM probes and 2 µL of DNA as a template. The primers and probes were previously designed for qPCR assays [6, 7, 11]. The *P. falciparum β tubulin* (*pfβtubulin)* gene was used as reference housekeeping gene. The ddPCR reaction was separated into 12,000–20,000 droplets using a QX200™ Droplet Generator (Bio-Rad Laboratories) and conducted using a T100™ Thermal Cycler (Bio-Rad Laboratories). During development of the ddPCR assays, a series of temperatures was tested to determine the optimal annealing temperature. Uniplex, duplex and multiplex ddPCR assays were developed to measure the

CNVs of the *pfmdr1*, *pfplasmepsin2* and *pfgch1* genes. The optimal annealing temperature for the uniplex ddPCR assay of the *pfmdr1*, *pfplasmepsin2*, *pfgch1* and *pfβtubulin* genes was determined to be 56 °C (Additional file 1), while that for the duplex ddPCR assay for detection of *pfmdr1/pfβtubulin* and *pfplasmepsin2/pfβtubulin* genes was 58 °C and for the duplex ddPCR assay of the *pfgch1/pfβtubulin* genes, the optimal annealing temperature was 60 °C. The optimal annealing temperature for the multiplex ddPCR assay of the *pfmdr1/ pfplasmepsin2/ pfβtubulin* genes was 60 °C. For validation, the ddPCR assays were performed in triplicate. After amplification, the ddPCR data were read with the use of a QX200™ Droplet Reader (Bio-Rad Laboratories) and analysed using QuantaSoft™ Software version 1.7.4 (Bio-Rad Laboratories). At least 12,000 accepted droplets were analysed [22, 23]. Manual thresholds were applied to distinguish between positive and negative droplets (Figs. 1 and 2). Positive and negative controls were included in each run. The GCN was calculated as the ratio of the concentration (copies/μL) of the target gene to that of the reference gene.

Validation of ddPCR assays

Two-fold serial dilutions of *P. falciparum* strain 3D7 were prepared to quantify the CNVs of the *pfmdr1* and *pfplasmepsin2* genes, as well as validation of the accuracy and precision of the uniplex, duplex and multiplex ddPCR assays. Two-fold serial dilutions of *P. falciparum* strain D6 were prepared for validation of the uniplex and duplex ddPCR assays of the CNV of the *pfgch1* gene. The GCN was determined by three independent ddPCR runs. The accuracy of the ddPCR assays was calculated as %accuracy = 100 - %error and %error = the absolute difference between 1 and the GCN determined with the ddPCR assays. The precision of the ddPCR assays was calculated as the percent relative standard deviation (%RSD) = standard deviation/average × 100. Since the DNA samples might contain both *P. falciparum* and human DNA, the limit of the GCN, as determined with the ddPCR assays, was quantified based on the lambda (λ) value, which was calculated as λ = ln (number of negative droplets/number of accepted droplets). The limit of GCN quantification of the ddPCR assays is the range of the λ value providing a %RSD value of greater than 20% and %accuracy value of greater than 80% [23, 24]. In accordance with the guidelines of the Minimum Information for Publication of Quantitative Digital PCR Experiments [25], the GCN results of the *P. falciparum* reference strain were compared between the uniplex, duplex and multiplex ddPCR assays.

Assessment of *pfmdr1, pfplasmespsin2* and *pfgch1* CNVs of *P. falciparum* reference strains and field isolates from Thailand

DNA samples from the *P. falciparum* reference strain (n = 7) and the *P. falciparum* isolates from Thailand (n = 84) were used to evaluate the ddPCR assays. The CNV results obtained by the ddPCR assays were compared with the results of the qPCR assays, as previously described [6, 7, 11].

Statistical analysis

The GCN results of the *P. falciparum* reference strain, as determined with the uniplex, duplex and multiplex ddPCR assays, were compared using the independent samples median test with IBM SPSS Statistics for Windows, version 22.0 (IBM Corporation, Armonk, NY, USA). The kappa statistic was used to identify agreements between the GCN results obtained with the ddPCR assays and those obtained with the qPCR assays with the use of IBM SPSS Statistics for Windows, version 22.0.

Results

Development and validation of ddPCR assays for CNV measurements

Accuracy of the ddPCR assays

As shown in Fig. 3, the accuracies of the uniplex, duplex and multiplex ddPCR assays were 65–96%, 64–99% and 91–99%, respectively, for measurement of the *pfmdr1* GCN, and 76–96%, 85–97% and 87–99%, respectively, for measurement of the *pfplasmepsin2* GCN. Meanwhile, the accuracies of the uniplex and duplex ddPCR assays for measurement of the *pfgch1* GCN were 80–100% and 77–99%, respectively.

Precision of ddPCR assays

As shown in Fig. 3, the %RSD values of the uniplex, duplex and multiplex ddPCR assays were 2–36%, 0–35% and 1–41%, respectively, for detection of the *pfmdr1* GCN, and 3–28%, 2–39% and 2–21%, respectively, for detection of the *pfplasmepsin2* GCN. Meanwhile, the %RSD values of the uniplex and duplex ddPCR assays were 2–36% and 1–17% for detection of the *pfgch1* GCN.

Limitation of GCN quantification

As shown in Table 1, the accepted range of λ values of the uniplex, duplex and multiplex ddPCR assays were 0.011–0.987, 0.005–2.178 and 0.003–0.842, respectively, for the *pfmdr1* GCN, and 0.010–1.870, 0.002–1.890 and 0.003–0.941, respectively, for the *pfplasmepsin2* GCN. The accepted range of λ values of the uniplex and duplex ddPCR assays were 0.006–1.915 and 0.003–1.877, respectively, for the *pfgch1* GCN. Based on the limitation of GCN quantification, the average accuracy and %RSD value of the multiplex ddPCR assay were 95% and 5%, respectively, for measurement of the *pfmdr1* GCN, and 91% and 5%, respectively, for measurement of the *pfplasmepsin2* GCN. The accuracy and %RSD value of the duplex ddPCR were 95% and 4, respectively.

Table 1
Descriptive statistics of *P. falciparum mdr1, plasmepsin2*, and *gch1* CNVs based on the accepted lamda(λ) value of ddPCR assay of *pf-β-tubulin* gene.

Statistic	*pfmdr1* CNVs			*pfplasmepsin2* CNVs			*pfgch1* CNVs	
	Singleplex	Duplex	Multiplex	Singleplex	Duplex	Multiplex	Singleplex	Duplex
Range	0.253	0.177	0.225	0.315	0.276	0.214	0.324	0.236
Minimum	0.812	0.817	0.861	0.814	0.738	0.813	0.868	0.926
Maximum	1.065	0.994	1.086	1.129	1.014	1.027	1.192	1.162
Mean	0.922	0.919	0.964	0.943	0.895	0.906	1.016	1.039
SD	0.061	0.040	0.061	0.085	0.057	0.057	0.078	0.065
Variance	0.004	0.002	0.004	0.007	0.003	0.003	0.006	0.004
%RSD	6.954	3.273	4.755	8.675	5.947	5.385	5.282	3.705
%Accuracy	92.240	92.135	95.047	93.304	89.533	90.638	95.118	94.876
Accepted lamda(λ) range	0.011–0.976	0.005–2.173	0.003–0.839	0.010–1.860	0.002–1.890	0.003–0.938	0.006–1.909	0.003–1.874

Comparison between the uniplex, duplex and multiplex ddPCR assays

Two-fold serial dilutions of DNA from *P. falciparum* strain 3D7 (4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, 0.0078125, 0.00390625 and 0.001953125 ng/μL) were used to compare the CNVs of the *pfmdr1* and *pfplasmepsin2* genes obtained from the uniplex, duplex and multiplex ddPCR assays. In addition, two-fold serial dilutions of DNA from *P. falciparum* strain D6 (8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, 0.0078125 and 0.00390625 ng/μL) were used for comparison of the CNVs of the *pfgch1* gene obtained with the uniplex and duplex ddPCR assays. As shown in Fig. 4, there were no significant differences in the *pfmdr1* ($p = 0.363$) and *pfplasmepsin2* ($p = 0.330$) GCNs, as determined with the uniplex, duplex and multiplex ddPCR assays. In addition, there was no significant difference for detection of the *pfgch1* GCN between the uniplex and duplex ddPCR assays ($p = 0.276$).

Standardised analytical workflow of ddPCR analysis for quantification of GCN

A flowchart, including validation criteria, for a standardised analytical workflow of ddPCR analysis was designed based on the observed limit of quantification of the optimal accuracy and precision (Fig. 5). Control samples, as well as positive and negative controls, were included for each ddPCR assay. The accepted criteria used for the ddPCR assay are that the results of the negative control are negative, those of the positive single copy control are positive (ratio = 0.80–1.20) and those of the positive multiple copy control are positive (ratio > 1.20). Each ddPCR reaction contained at least 12,000 droplets. The GCN results were considered acceptable at a λ value of 0.003–0.800 for quantification of the *pfmdr1* and *pfplasmepsin2* genes with the multiplex ddPCR assay, and 0.003–1.900 for quantification of the *pfgch1* gene with the duplex ddPCR assay. GCNs were calculated as the ratio of the concentrations (copies/μL) of the target and references genes (single GCN ratio of 0.8–1.2 and multiple GCN ratio of > 1.20).

Agreement of GCN results between the ddPCR and qPCR assays

DNA samples from the *P. falciparum* reference strain (n = 7) and *P. falciparum* strains 3D7, 7G8, D10, DD2, HB3, W2 and D6 were collected to compare the *pfmdr1*, *pfplasmepsin2* and *pfgch1* GCNs determined with the ddPCR and qPCR assays. The results showed 100% agreement between the ddPCR and qPCR assays (κ = 1) (Additional file 2).

CNVs of the *pfmdr1, pfplasmespsin2* and *pfgch1* genes of *P. falciparum* isolates from Thailand

The extracted DNA samples were determined the CNVs in the *pfmdr1*, *pfplasmespsin2* and *pfgch1* genes following the a standardised analytical workflow obtained from this study. The results showed that the *pfmdr1* and *pfplasmepsin2* GCNs were amplified in 15% and 27% of samples from Ubon Ratchathani, Northeastern Thailand, whereas the *pfgch1* GCN was amplified in 50% of samples from Yala, Southern Thailand (Fig. 6, Additional file 3). Comparisons of the results of the ddPCR and qPCR assays were 100% in agreement for CNV assessments of the *pfmdr1*, *pfplasmepsin2* and *pfgch1* genes (Fig. 7)

Cost and turn-around time of ddPCR assays

The costs of the uniplex, duplex and multiplex ddPCR assays to determine the CNVs of the *pfmdr1*, *pfplasmepsin2* and *pfgch1* genes were 10.40, 5.50 and 5.70 USD per sample, respectively. The turn-around times

for the uniplex, duplex and multiplex ddPCR assays of 96 samples were12, 6 and 6 h, respectively.

Discussion

The qPCR assay is the most commonly used assay for the identification of genes associated with antimalarial drug resistance s [6, 7, 11]. Here, ddPCR assays were developed and validated for accurate assessment of the CNVs of several *P. falciparum* resistance genes. GCNs estimated by the qPCR assay were measured based on exponential curves, while estimates with the ddPCR assays were measured based on the ratio of the concentrations of the target and reference genes [13].

The results of the present study demonstrated that a higher or lower concentration of the target gene might affect the accuracy and precision of the ddPCR assays. As a consequence, optimal concentrations of the target genes are required for accurate detection of the GCNs. Here, an optimal DNA template was evaluated to accurately and precisely determine the GCN based on the λ value, which is estimated from the numbers of negative and accepted droplets generated by the ddPCR assays.

To reduce the cost and turn-around time, multiplex ddPCR assays were developed for detection of both the *pfmdr1* and *pfplasmepsin2* genes in a single reaction. The results showed that there were no significant differences in the GCN assessments between the assays, favouring the multiplex, rather than the uniplex, ddPCR assay as the preferred method [25]. In, addition, a duplex ddPCR assay was also developed for the detection of the *pfgch1* GCN instead of the uniplex ddPCR assay.

Compared to the uniplex ddPCR assay, use of the duplex ddPCR assay can reduce costs by 47% from 10.40 to 5.47 USD and reduce the required assay time by 50% from 12 to 6 h. Moreover, the use of the multiplex ddPCR PCR assay to detect the *pfmdr1* and *pfplasmepsin2* GCNs reduced costs by 72% from 20.80 to 5.73 USD and reduced the required time by 75% from 24 to 6 h.

The CNVs of the *pfmdr1*, *pfplasmepsin2* and *pfgch1* genes of the *P. falciparum* reference strains and the samples collected from Thailand were measured. The GCN results obtained with the ddPCR and qPCR assays were in 100% agreement. The GCN results of *pfmdr1* and *pfgch1* of the reference strain were compared with previously published methods [10, 11, 26]. The results of *pfplasmepsin2* gene amplification associated with piperaquine resistance were compared with the results of a previous publication, which showed amplification of the *pfplasmepsin2* gene in Northeast Thailand from 2011 to 2018 [27]. The results of the present study revealed substantial amplification of the *pfgch1* gene in Yala, Thailand, associated with sulfadoxine-pyrimethamine resistance, as described in a previous surveillance conducted from 2014 to 2016 [28]. Moreover, the prevalence of the *pfmdr1*, *pfplasmepsin2* and *pfgch1* GCNs obtained from this study might be useful for surveillance of the efficacy of antimalarial drugs.

Conclusion

Uniplex, duplex and multiplex ddPCR assays for detection of the CNVs of the *P. falciparum mdr1*, *plasmepsin2* and *gch1* genes were developed and validated. The results confirmed the accuracy and precision of the proposed assays, which reduced the cost and turn-around time for surveillance of the efficacy of antimalarial drugs. The assay is a valuable additional tool for genetic surveillance of antimalarial drug resistance.

List Of Abbreviations

DNA Deoxyribonucleic acid

ddPCR Droplet digital PCR

GCN Gene copy number

PCR Polymerase Chain Reaction

18S rRNA *18 s ribosomal RNA* gene

pfmdr1 P. falciparum multidrug resistance 1 gene

pfgch1 P. falciparum GTP cyclohydrolase 1 gene

pfplasmepsin2 P. falciparum plasmepsin2 gene

nM Nanomolar

µL Microliter

Declarations

Ethics approval and consent to participate

Ethical approvals for the study were obtained from the ethical review committees of the Faculty of Tropical Medicine, Mahidol University (MUTM 2012-045-05). Informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SS and MI contributed to study design. RS collected samples. SS and KS undertook laboratory work. SS and MI analysed data. SS, AD and MI drafted the manuscript. All authors read and approved the final manuscript.

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Figures

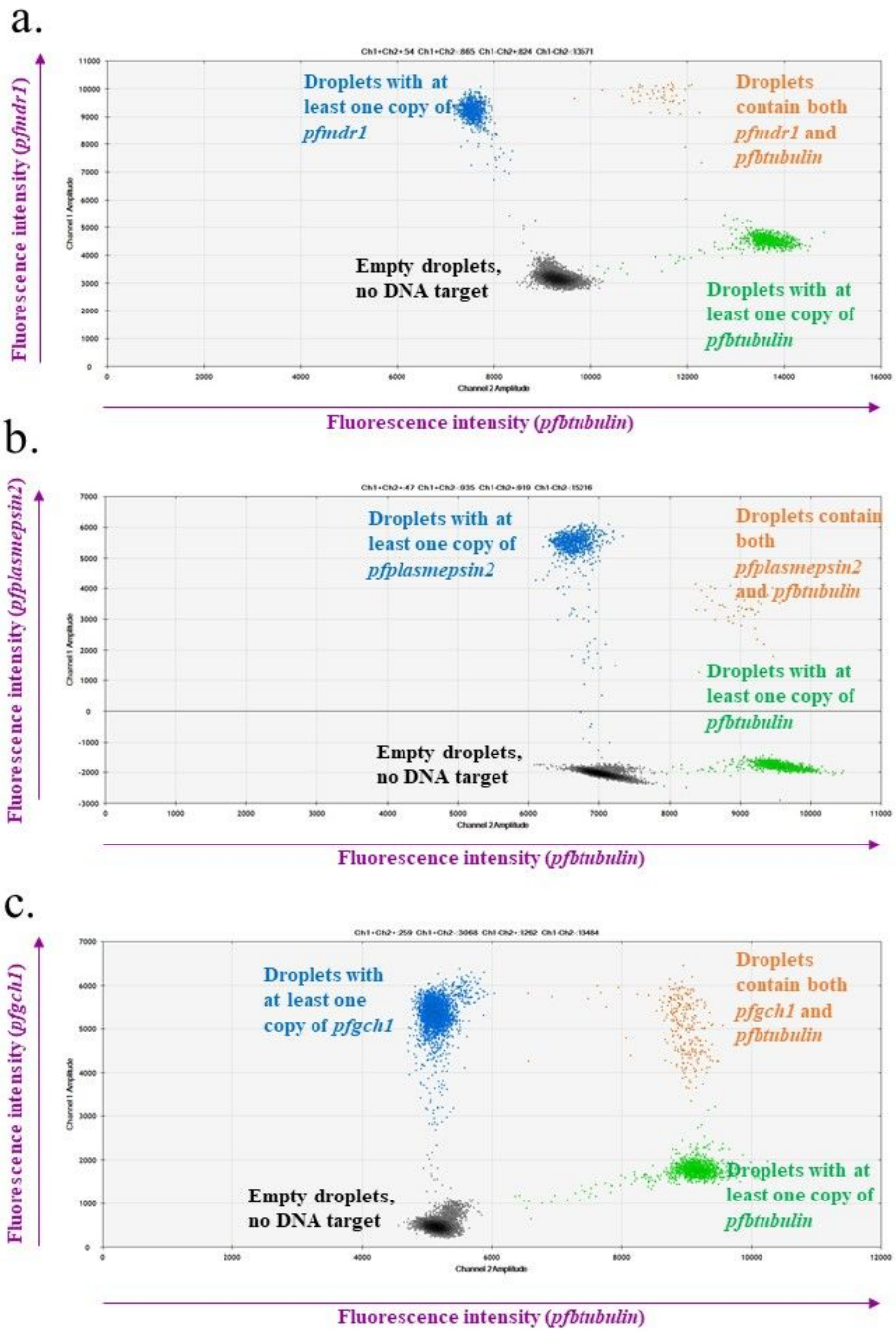
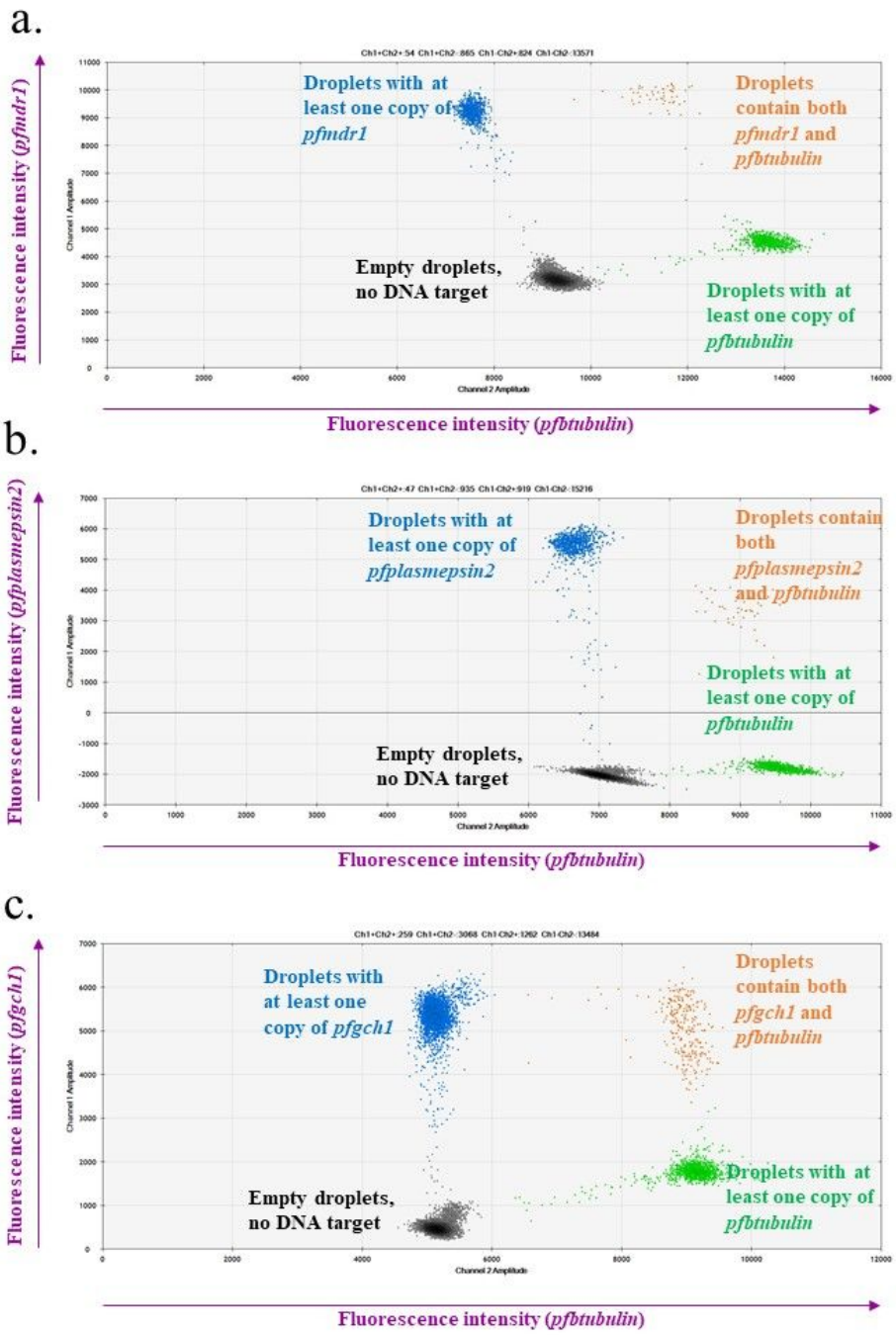


Figure 1

Two dimensional ddPCR amplitude plots of duplex ddPCR assays. The duplex ddPCR assay of pfmdr1/pf-β-tubulin plot (a.), duplex ddPCR assay of pfplasmepsin2/pf-β-tubulin plot (b.), and duplex ddPCR assay of pfgch1/pf-β-tubulin plot (c.) shows droplets with at least one copy of target genes (blue), reference gene (green), droplets contain both target and reference gene (orange), and empty droplets (grey).



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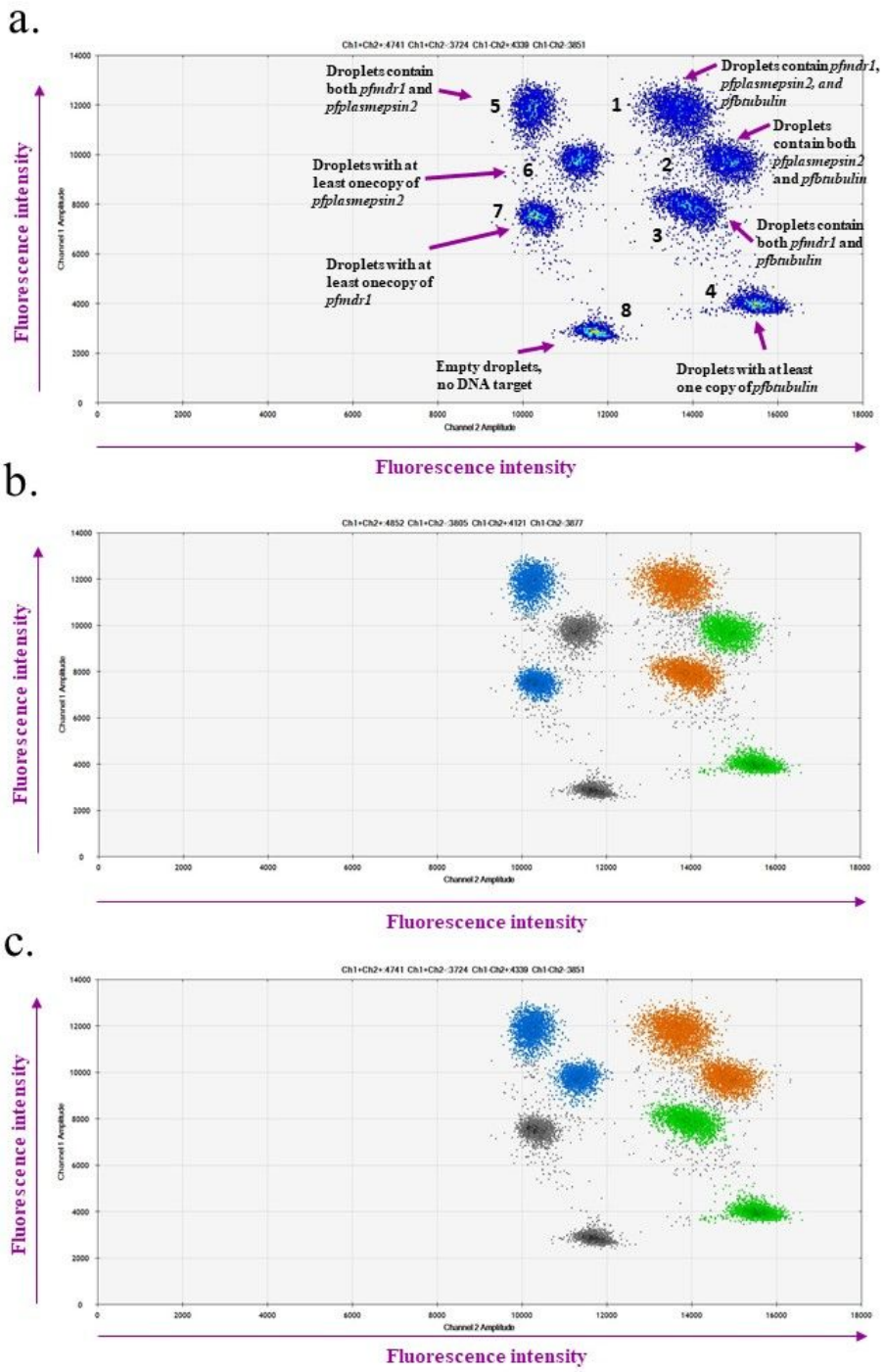
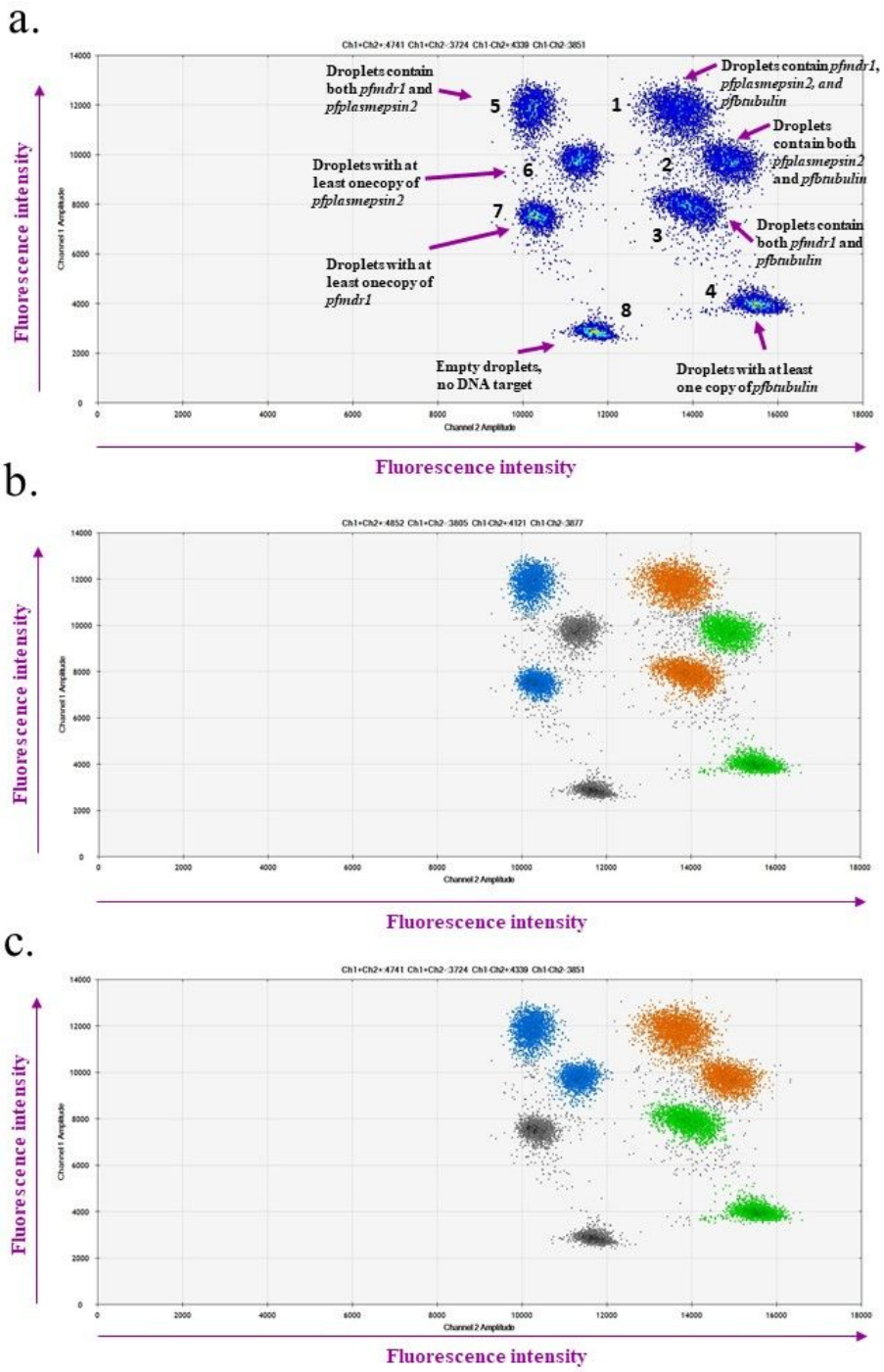


Figure 2

Two dimensional ddPCR amplitude plots of multiplex ddPCR assays. Multiplex ddPCR. Heat map shows 8 clusters of droplets (a.) including, droplets contain pfmdr1, pfplasmepsin2, and pfbtubulin (cluster 1), droplets contain both pfplasmepsin2 and pf-β-tubulin (cluster 2), droplets contain both pfmdr1 and pf-β-tubulin (cluster 3), droplets with at least one copy of pf-β-tubulin (cluster 4), droplets contain both pfmdr1 and pf-β-tubulin (cluster 5), droplets with at least one copy of pfplasmepsin2 (cluster 6), droplets with at least one copy of pfmdr1 (cluster 7), Empty droplets, no DNA target (cluster 8). Classification cluster of droplets for pfmdr1 copy number detection (b.). Classification cluster of droplets for pfplasmepsin2 copy number detection (c.).



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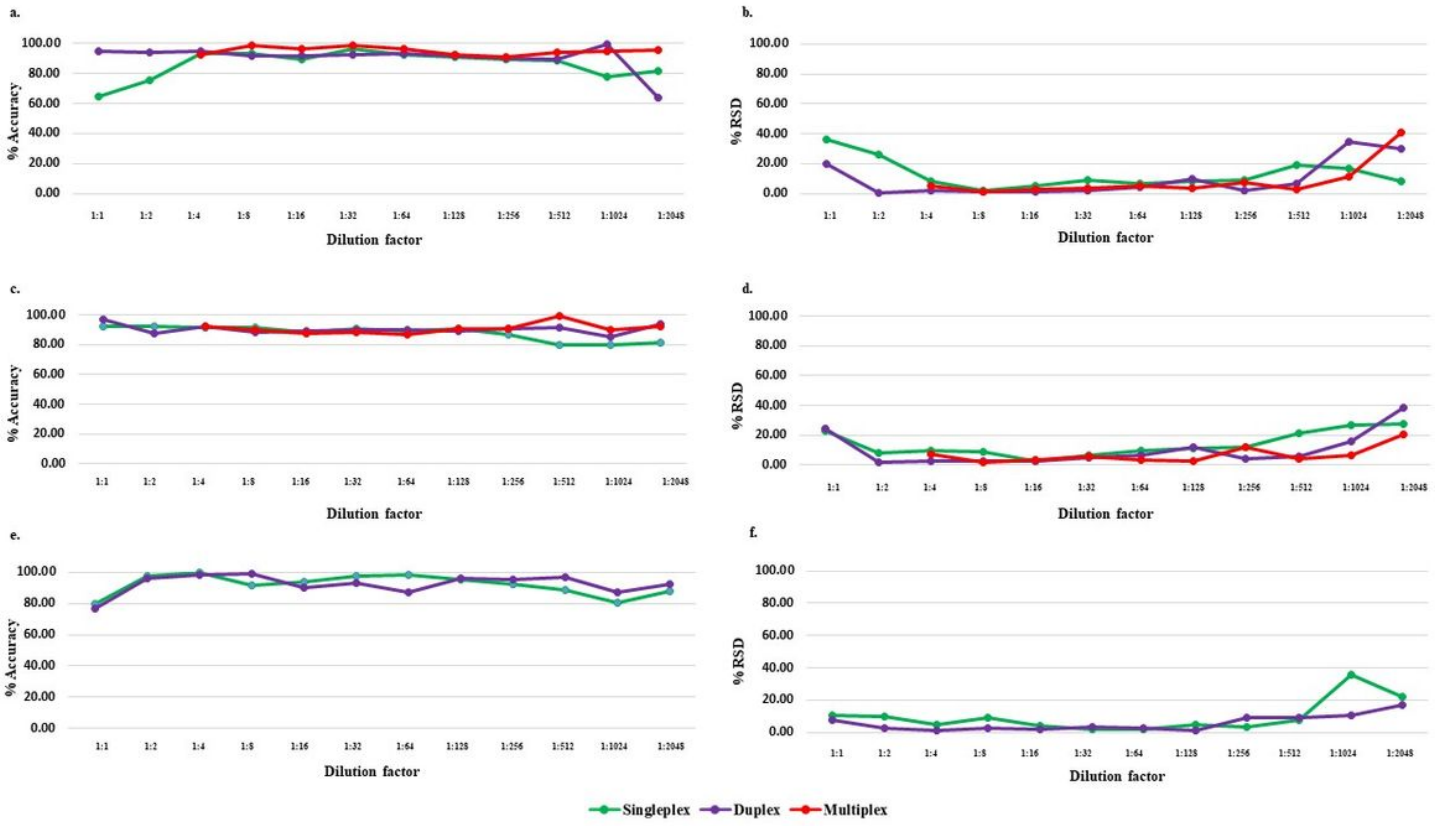
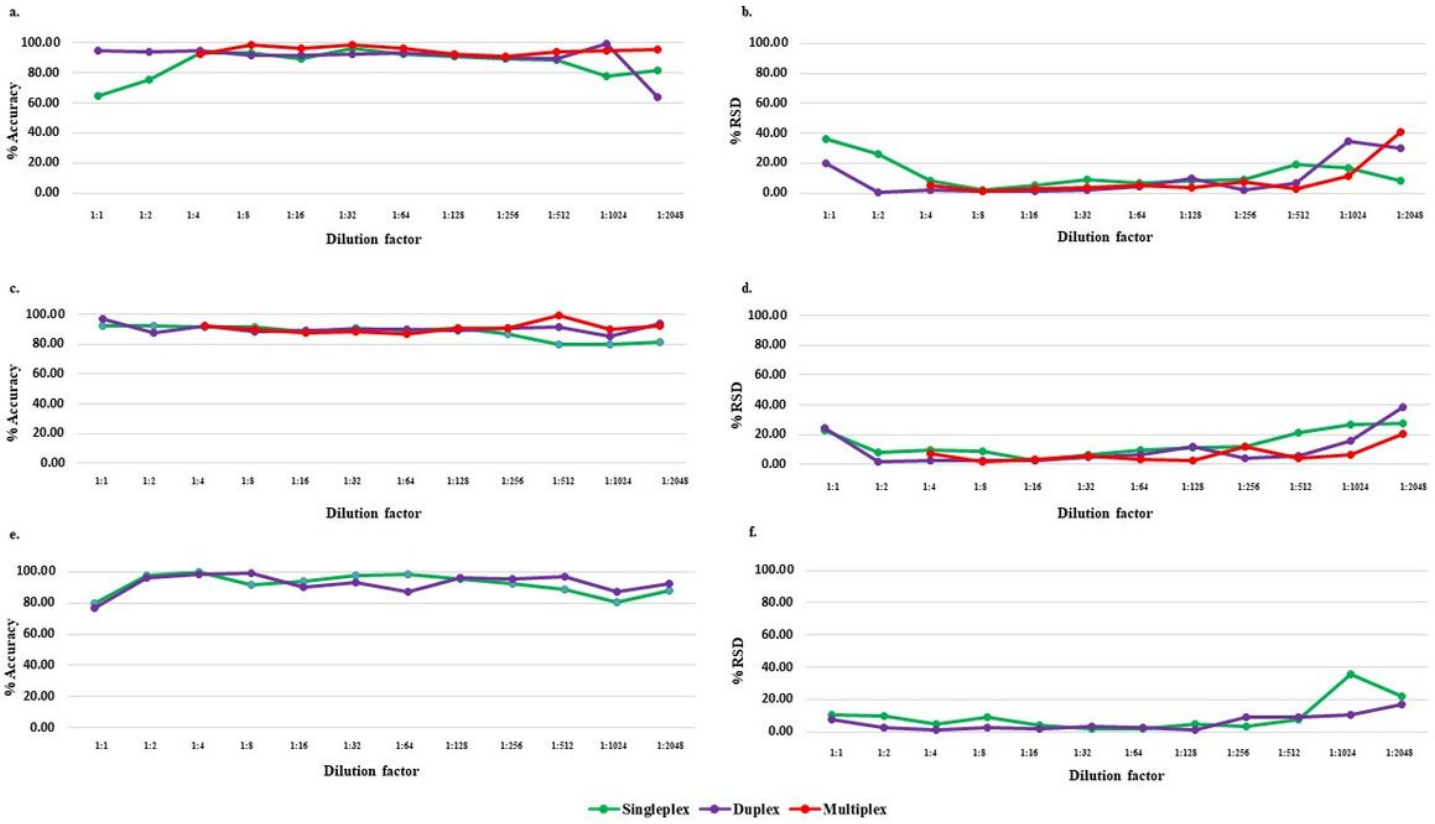


Figure 3

Limitation of gene quantification, accuracy and precision of ddPCR assays for pfmdr1 copy number detection (a.), pfplasmepsin2 copy number detection (b.), and pfgch1 copy number detection (c.).



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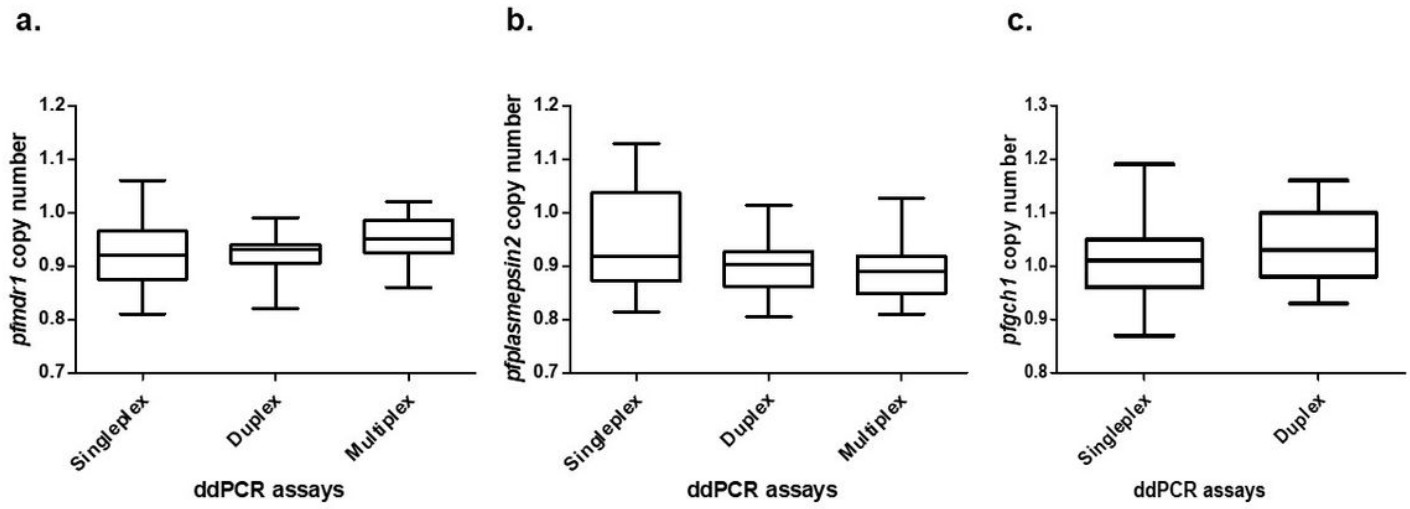
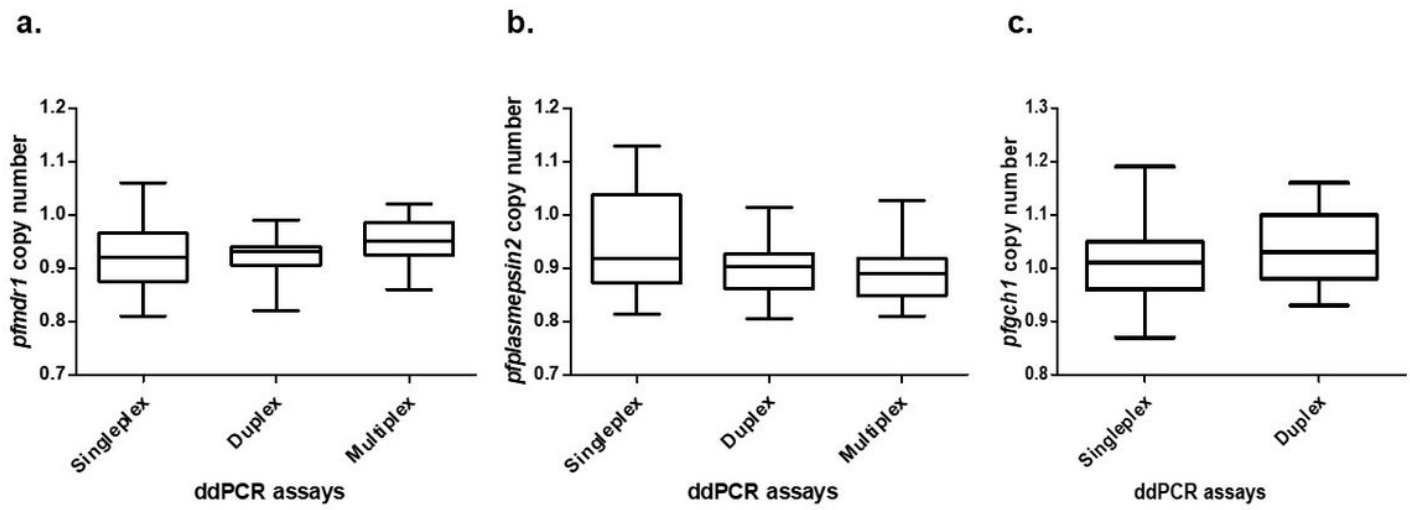


Figure 4

Whisker plots show median, maximum, and minimum of estimated pfmdr1 (a.), pfplasmepsin2 (b.), and pfgch1 (c.) copy number.



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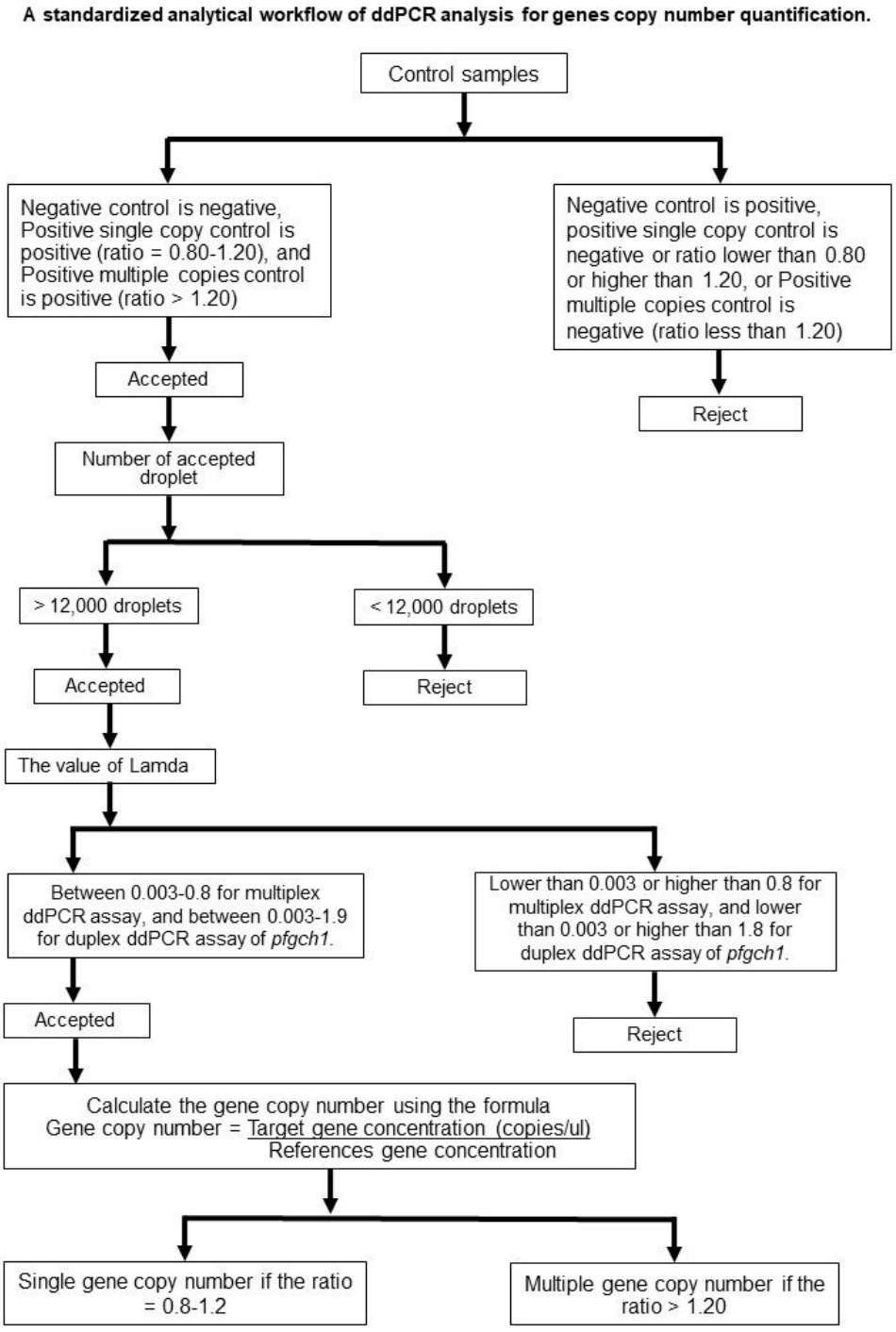
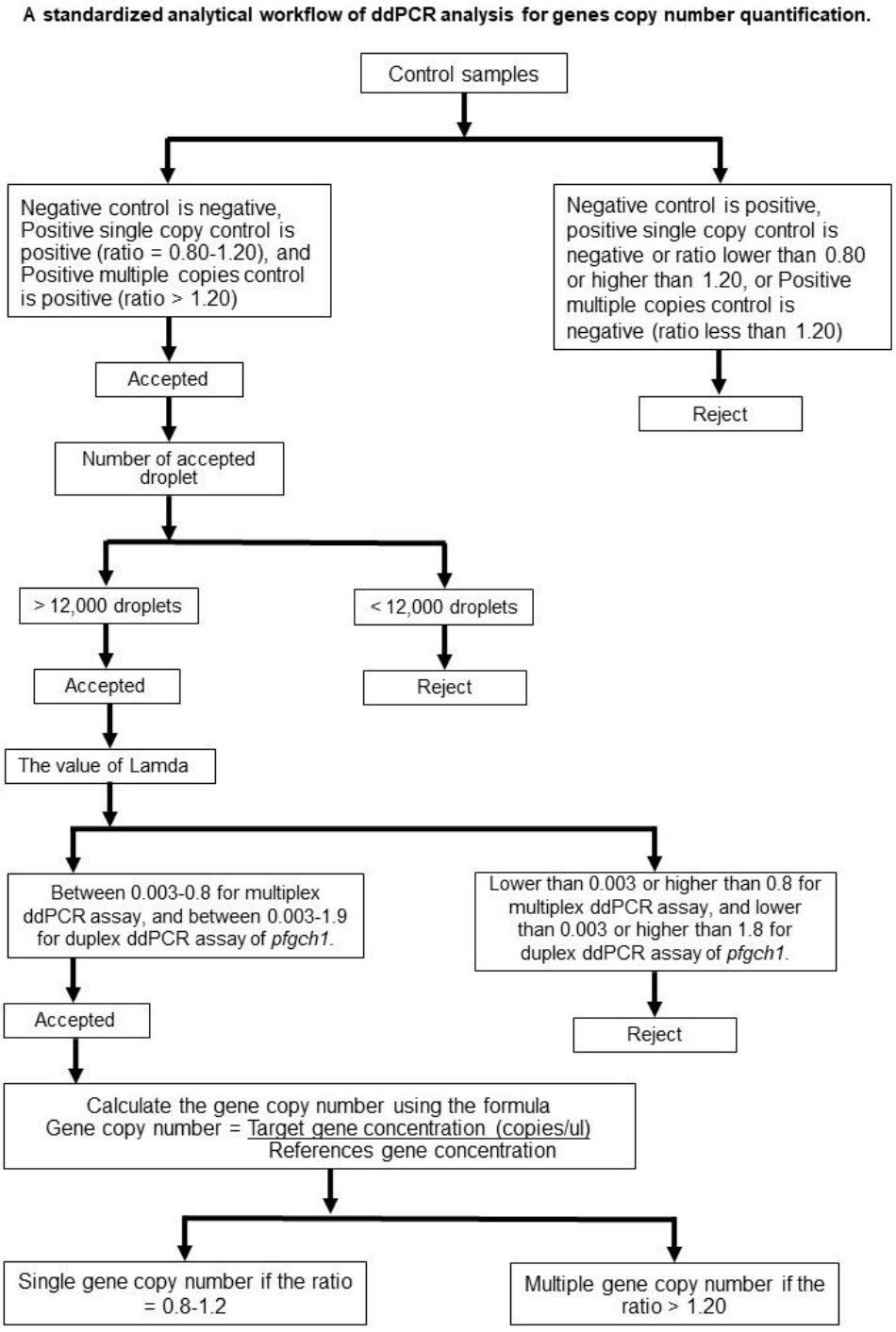


Figure 5

A standardized analytical workflow of ddPCR analysis used for genes copy number quantification.



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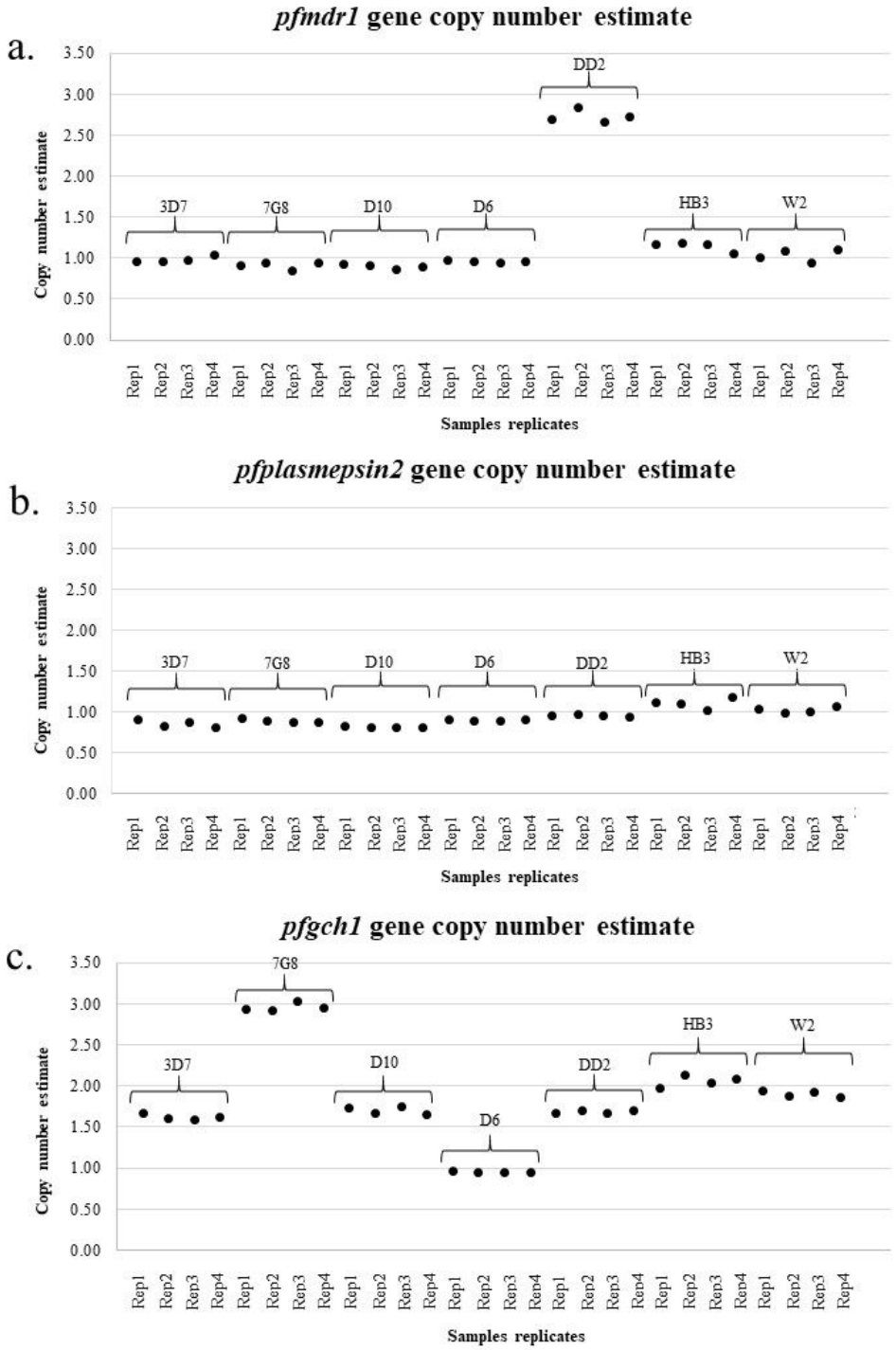
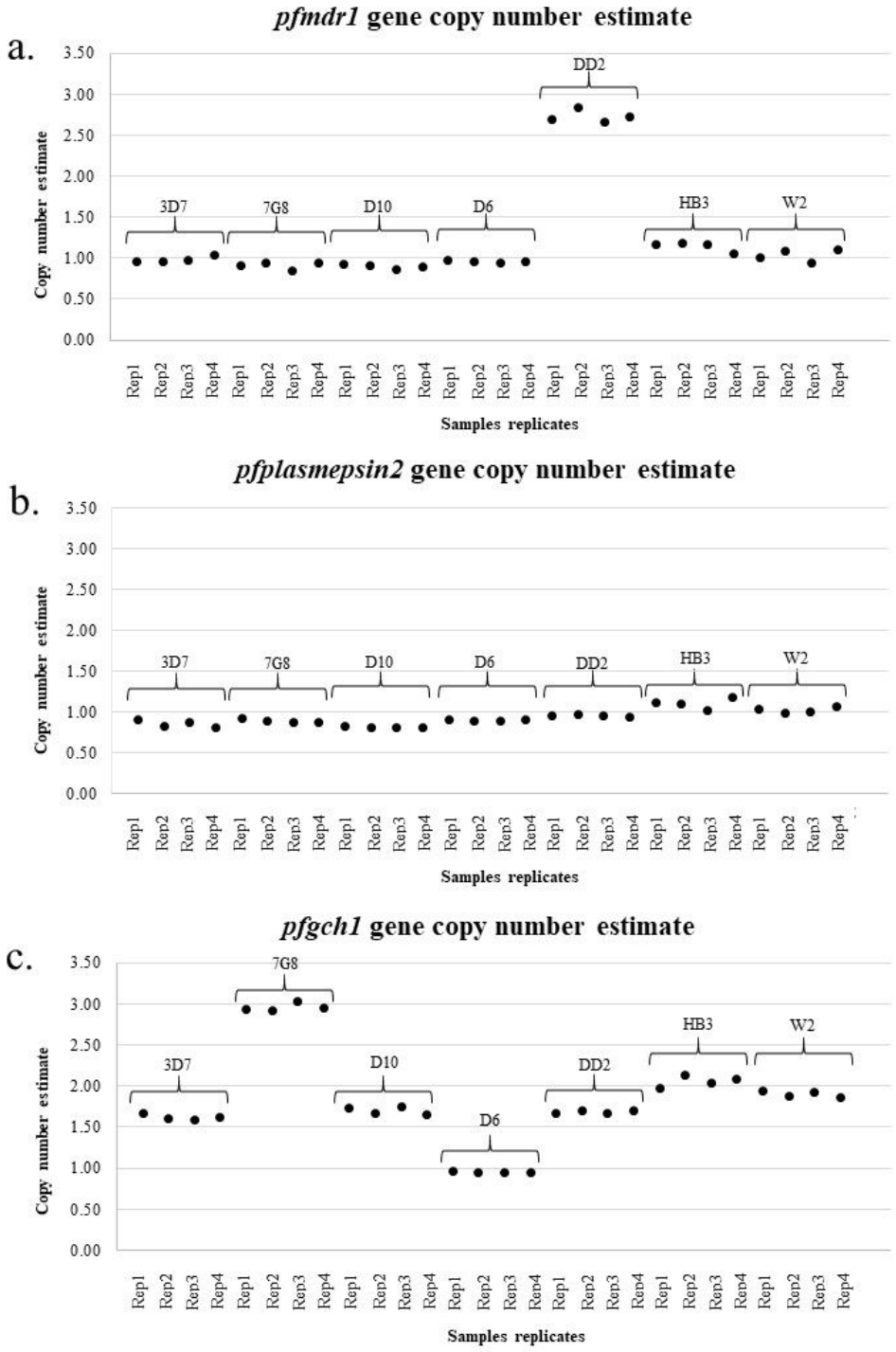


Figure 6

Genes copy number of P. falciparum mdr1 (a.), plasmepsin2 (b.), and gch1 of reference strains estimated by ddPCR in replicates.



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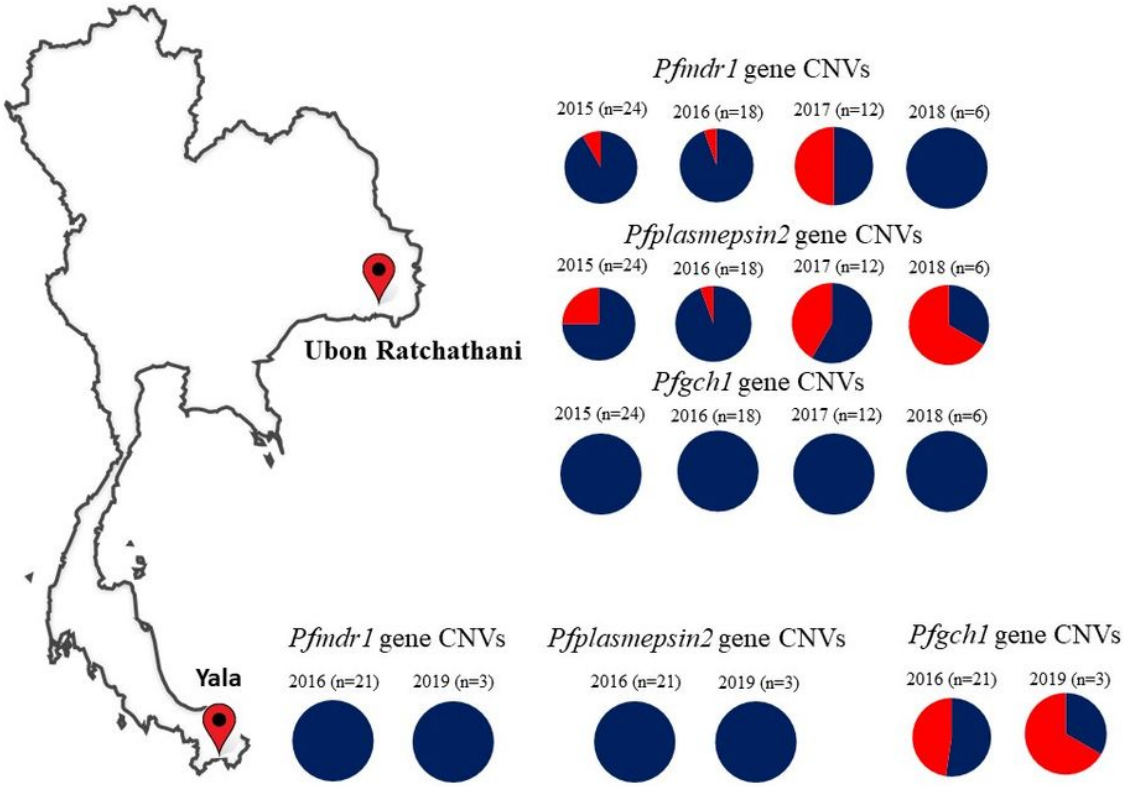
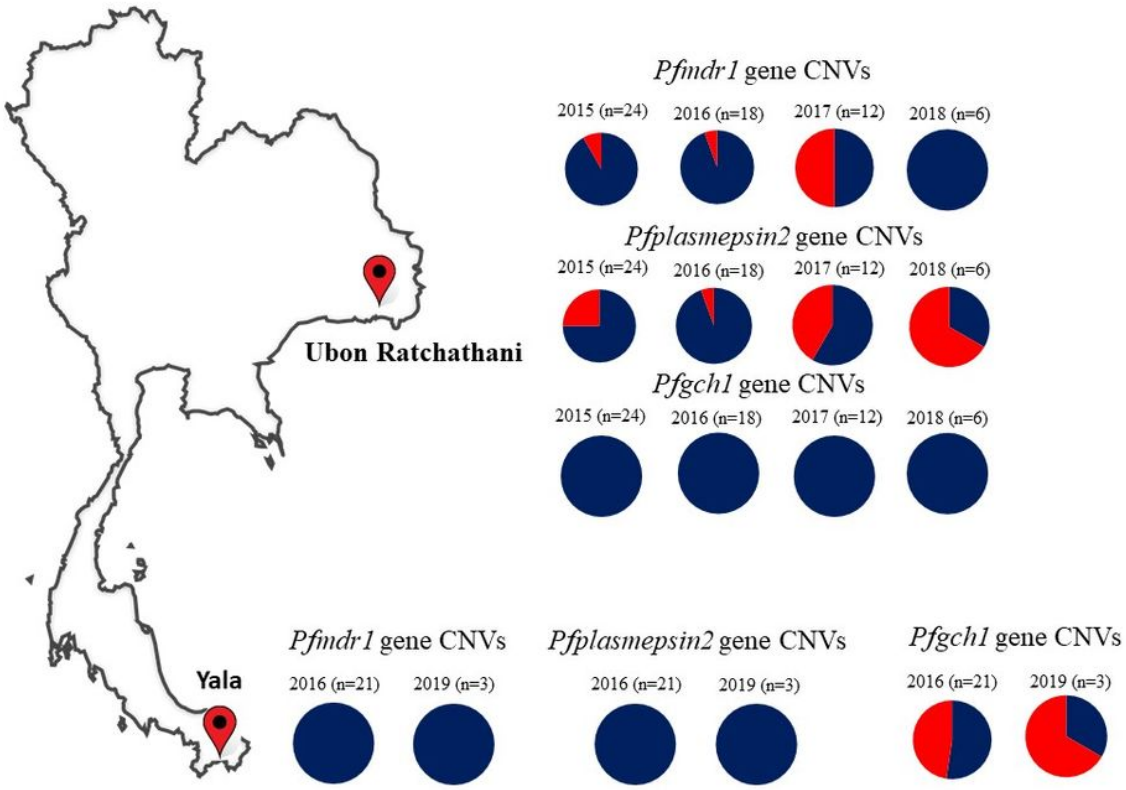


Figure 7

Prevalence of pfmdr1, pfplasmepsin2, and pfgch1 gene amplification isolated from Ubon Ratchathani and Yala.



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Prevalence of pfmdr1, pfplasmepsin2, and pfgch1 gene amplification isolated from Ubon Ratchathani and Yala.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.xlsx
- Additionalfile1.xlsx
- Additionalfile2.xlsx
- Additionalfile2.xlsx
- Additionalfile3.xlsx
- Additionalfile3.xlsx