Prevalence of PfDHFR and PfDHPS Mutations Associated with Drug Resistance Among Pregnant Women Receiving IPTp-SP at Msambweni County Referral Hospital, Kwale County, Kenya

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Abstract

Background: Prevention and treatment of malaria during pregnancy is crucial in dealing with maternal mortality and adverse fetal outcomes. WHO’s recommendation to treat all pregnant women with sulphadoxine-pyrimethamine (SP) through antenatal care structures was implemented in Kenya in the year 1998 but concerns about its effectiveness in preventing malaria in pregnancy has arisen due to the spread of parasites resistant to SP. We aimed to determine the prevalence of SP resistance markers in Plasmodium falciparum parasites isolated from pregnant women seeking antenatal care at Msambweni County Referral Hospital, located in coastal Kenya, between the year 2013 and 2015.

Methods: This hospital-based study included 106 malaria positive whole blood samples for analysis of SP resistance markers within the PfDHFR gene (codons 51, 59 & 108) and PfDHPS gene (codons 437 & 540). The venous blood collected from all pregnant women was tested for malaria via light microscopy, then thereafter separated into plasma and red cells and stored in a -86⁰ freezer for further studies. Archived red blood cells were processed for molecular characterization of SP resistance markers within the PfDHFR gene and PfDHPS using real time PCR platform.

Results: All samples had at least one mutation in the genes associated with drug resistance; polymorphism prevalence of PfDHFR51I, 59R and 108N was at 88.7%, 78.3% and 93.4%, respectively, while PfDHPS polymorphism accounted for 94.3% and 91.5% at 437G and 540K, respectively. Quintuple mutations (at all the five codons) conferring total SP resistance had the highest prevalence of 86%. Quadruple mutations were observed at a frequency of 10.4%, and 24.5% had a
heterogeneous outcome with both wildtype and mutant genotypes in the genes of interest.

Conclusion: The data suggest a high prevalence of Pf genetic variations conferring resistance to SP among pregnant women, which may explain reduced efficacy of IPTp treatment in Kenya. There is need for extensive SP resistance profiling in Kenya to inform IPTp drug choices for successful malaria prevention during pregnancy.

Background

Malaria is a significant public health problem in sub-Saharan Africa and remains a major contributor to morbidity and mortality in the African continent (1). The World Health Organization (WHO) has reported that Africa carries the highest burden of malaria: 92% of the malaria cases and 93% of malaria deaths worldwide (2). 99.7% of the cases are caused by Plasmodium falciparum with pregnant women being a particularly vulnerable population (3) especially those carrying first pregnancies at a young maternal age (4). Malaria in pregnancy contributes to maternal anemia leading to spontaneous abortion, stillbirth, premature birth and low birth weight (3). WHO has recommended an intermittent preventative treatment for pregnant women (IPTp) interventions using sulphadoxine-pyrimethamine (SP) drug, that Kenya implemented in the year 1998. Pregnant women then received at least two doses of SP given from the second trimester of pregnancy, which was later revised in 2009 to a monthly dose, to be administered during their antenatal clinic (ANC) visits (5). IPTp prophylactic treatment has quickly been countered by the rise of P. falciparum (Pf) parasites resistant to SP, resulting in a loss in sensitivity to the SP drug. This resistance is attributed to single nucleotide polymorphism (SNP) mutations within
the DHFR and DHPS genes that are target sites for the sulphadoxine and pyrimethamine active components of the drugs, which are most effective when working in synergy (6). In East Africa, the prevalence of these mutations is high, reaching near 100% in some regions (7, 8, 9, 10), thus raising concerns on the efficacy of the drug in preventing malaria in pregnancy. The SP drug is still considered by practitioners to be effective in clearing the parasites in pregnant women, despite the high levels of Pf resistance that have been reported. To clarify this issue, the WHO has recommended that more studies be carried out to investigate the prevalence of Pf SP resistance molecular markers in parasites in the context of IPTp (11). Malaria is the leading cause of morbidity in Kwale County with a prevalence of 37.7% in comparison to other disease morbidities such as diarrhea, influenza, and respiratory diseases among others, which account for 4.6, 16.4, 5, and 3.1 per cent of disease burden in the county, respectively (12). This study investigated the prevalence of SP resistance molecular markers in parasites isolated from blood samples collected from the pregnant women receiving IPTp-SP treatment between 2013 and 2015 at Msambweni County Referral Hospital in Kwale County, Kenya. Since SP is the recommended drug for IPTp, continuous monitoring of its efficacy and for Pf resistance molecular markers is key in addressing malaria control among pregnant women. To our knowledge, this study is the first to assess resistance markers among pregnant women in coastal Kenya, contributing invaluable data on the rising prevalence of SP resistant P. falciparum among pregnant women in Kenya.

Methods

Study area and sample collection
This descriptive cross-sectional study involved pregnant women seeking antenatal care at the Msambweni County Referral Hospital between the year 2013 and 2015. There were a total of 763 pregnant women enrolled in the study who visited the antenatal clinic, and all were tested for malaria using light microscopy (13). Whatman filter paper was used to prepare dried blood spots (DBS) from the archived red blood cell samples which were then individually reserved in coded plastic bags with silica desiccant beads. The DBS were transported at room temperature to Centre for Biotechnology Research and Development (CBRD) within the Kenya Medical Research Institute (KEMRI) in Nairobi, Kenya for further molecular analysis.

**Molecular genotyping**

Genomic DNA was extracted from the DBS using a QIAamp DNA mini blood kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. The malaria microscopy results were then confirmed for 

\[ Pf \]

using a Singleplex real-time qPCR assay carried out on an Applied Biosystems™ 7500 Fast Real-time PCR machine using a primer and probe set previously published (14) with each sample denatured at 95°C for 10 seconds and cycled 45 times with each cycle consisting of 95°C for 15 seconds and 55°C for 60 seconds (15)

A modified Multiplex real time PCR assay described elsewhere (15) was performed with the same thermocyclic conditions using two hydrolysis probes for each codon. The wildtype strain was detected by a FAM labeled probe while the mutant strain was detected by a HEX labeled probe and each probe was tagged to a Black hole quencher. These probes differentiated single nucleotide polymorphisms (SNPs) within the \( PfDHFR \) gene and \( PfDHPS \) genes that are associated with SP resistance, targeting three SNPs within the \( PfDHFR \) gene at codon 51I, 59R and 108N that confer
resistance to pyrimethamine and two SNPs within the PfDHPS gene at codon 437G and 540E that confer resistance to sulphadoxine.

PCR was carried out in a 25µl final volume containing 12.5µl of Agpath-ID™ One-Step RT-PCR Kit, 5µl of DNA template, forward and reverse primers at various concentrations shown in table 4 and both mutant and wildtype probes at a final concentration of 0.2uM (15) P. falciparum strain 3D7 and Dd2 were used as controls for wildtype and mutant strains respectively.

Statistical analysis

Characteristics of women were presented as means with standard deviations (SD) and as proportions. The prevalence of malaria parasites and mutations were expressed as a proportion with their respective 95% confidence interval (95% CI), while parasite load as median and its interquartile range (IQR). Comparisons of different factors for significant difference was done using t-test for quantitative variables while Chi square was used for binary variables, with a p-value of <0.05 considered significant. Statistical analysis was conducted using Stata version 12.0 software (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845 USA).

Results

The majority of 763 pregnant women screened for malaria in this study were married (72%) and had up to primary level of education (81%). The mean (± SD) age and gestation were 26 (± 6.4) years and 23 (± 5.2) weeks respectively. The median (IQR) number of visits with dispensed folic tablets and SP-IPTp were three (3–4) and three (2–4) respectively. The majority of women (98%) had at least one dose of IPTp, while 88% received the WHO recommended IPTp doses (≥2). Malaria parasites were detected in 135 pregnant women, yielding a prevalence of 17.7 %
(95% CI: 15.1–20.6). Young age, marital status and first pregnancy were significantly associated with malaria parasite infection (Table 1). SP-IPTp was not associated with decreased odds of malaria infection (p = 0.251). Of the 132 single women in the study, 32 (24%) were infected with malaria compared to 88 (16%) of married women. Therefore, single women (who were also likely to be younger) had increased odds of malaria infection compared to married women (OR = 1.7; 95%CI: 1.1–2.7). Similarly, primigravidae were 1.7 times as likely to be detected with malaria parasites compared to multigravidae (OR = 1.7; 95%CI: 1.1–2.5).

Placentas from malaria infected women had a significantly lower mean weight (518.3±96.74g) compare to that of women negative for malaria (550.3±98.86g), p<0.01. However, malaria infection was not associated with mean placenta length, width or height (Table 1). Of the 568 women with hemoglobin levels, the mean Hb was 9.8 (± 1.78) g/dL and 425 (75%) were anemic (Hb ≤11.0 g/dL). Among 277 women with anemia, 30 (11%) had hookworm infection while only three of 99 women without anemia were infected. Therefore, hookworm infection was significantly associated with a near 4-fold increased odds of anemia among these pregnant women (OR = 3.9; 95%CI: 1.2–13.0), but not infection with malaria parasites (p = 0.699). Women with hookworm infection had a significantly lower mean Hb (9.3±1.38 g/dL) compared to those negative for hookworm (10.0±1.78 g/dL, p<0.01).

Of the 135 pregnant women who tested positive for malaria, only 84 had archived blood samples available and these were included in *P. falciparum* genetic analysis. These women had a mean age: 23.7±6.14 years and 32 (38%) were primigravidae. The mean baseline Hb was 9.5 (± 2.31) g/dL (Table 2). From these women, 106 of the blood samples (70 blood samples collected at first antenatal care visit and 36
collected at delivery) were all confirmed to contain *P. falciparum* parasites by real
time PCR assay and were included in the mutation analyses. The median parasite
load was 2760 (1200–7133) parasites/µL of blood.

All samples were successfully genotyped yielding prevalences of *PfDHFR* gene and
*PfDHPS* SNP mutations that ranged from 83–100% (Table 3a). Of the 106 genotypes,
94 (88.7%) harbored *PfDHFR* gene mutant allele 51I, thus conferring a change in
amino acid from asparagine (N) to isoleucine (I). Five samples (4.7%) had a wildtype
allele at codon 51N and six samples (5.6%) had mixed outcomes of both wild-type
and mutant alleles at this codon (Table 3a). In 83 samples (78.3%), mutant allele
59R was detected resulting in expression of amino acid arginine (R) from cysteine
(C). Two samples (1.9%) had the wildtype allele 59C and 21 samples (19.8%) had
mixed outcomes with both wildtype and mutant alleles at codon 59. Almost all
samples genotyped for the 108N mutation at 94.3% with a SNP of C to T base pair
leading to expression of asparaginase (N) from serine (S). None of the 106 samples
had the 108S wildtype allele but six samples (5.6%) had mixed outcomes of both
wild-type and mutant alleles at this codon. The overall frequency of parasites with
the *PfDHFR* triple mutantI₅₁R₅₉N₁₀₈ genotype was 87.4%.

In estimating the prevalence of mutations observed in the *PfDHPS* gene, 94.3% of
the 106 samples harbored the mutant allele 437G thus expressing amino acid
glycine (G) from the wild-type alanine (A). Two samples (1.9%) exhibited the
wildtype haplotype and four samples (3.8%) had mixed outcome of both wild-type
and mutant alleles. A polymorphism frequency of 91.5% was observed at codon 540
for all the samples genotyped with the mutant allele 540E where glutamic acid (G)
replaced the wild-type amino acid lysine (K). Five samples (4.7%) exhibited wildtype
haplotypes at codon 540 with only one sample having mixed outcome of both wild-
type and mutant alleles. The overall frequency of mutations in the \textit{PfDHPS} gene $G_{437}E_{540}$ was at 94.4%.

In combination of \textit{PfDHFR} and \textit{PfDHPS} haplotypes (Table 3b), quintuple mutant genotype was the most prevalent 85.8% (91/106). Quadruple mutations were observed at 10.4% (11/106) where five of the samples had the wildtype allele at codon 51N and two sample at codon 540K. Triple mutation was the least prevalent at 3.8% (4/106) where mutant polymorphisms were in the \textit{PfDHFR} gene while wildtype alleles were present in the \textit{PfDHPS} gene. While two samples were wildtype at \textit{PfDHPS} gene, no sample was fully wildtype at the \textit{PfDHFR} gene. Clinically, 72 (86%) of the 84 pregnant women had quintuple genotype (i.e. had pure or mixed mutations at all the five loci) suggesting total resistance to SP among these women. Only eight and four women had quadruple and triple genotypes, respectively.

**Discussion**

Malaria prevalence of 18% found in this study was higher than 10% and 13% observed among pregnant women in another Kenyan coastal region (16) and the lake region in Tanzania (17), respectively, but significantly lower than 31% documented in the Kenyan lake regions (16). Therefore, the high burden of malaria in pregnancy remains of public health concern. As observed in this study and elsewhere (18–20), younger women in their first pregnancy are at greatest risk of infection and should be targeted with preventive and early treatment interventions. IPTp-SP is an important prophylactic therapy recommended for prevention of malaria in high endemic African regions to reduce morbidity/mortality among pregnant women and adverse pregnancy outcomes (2). The emerging high \textit{Pf} resistance to the SP is likely to render IPTp-SP prophylactic intervention ineffective.
Similar to our findings, other studies have reported high prevalence (78%–97%) of quintuple *PfDHFR*/*PfDHPS* haplotype mutations in western Kenya (10,21). Studies elsewhere have documented quadruple mutations (65%) in Equatorial Guinea (22) and 48% in DRC (23), and triple mutations (92%) in Gabon (24) and 61-71% in Burkina Faso (25) as the most prevalent. Similar to India, where double mutation was the most prevalent (26,27), a study in Brazil reported double mutation as most prevalent but did not find quintuple or quadruple mutations (28).

Findings from this study demonstrated very high prevalence of SNPs at the two important genes that confer SP resistance. The high prevalence of 89% observed at *PfDHFR51I* in this study was similarly to (85%-100%) documented in western Kenya and elsewhere (10,21–24,29), but contrary to the 21% reported in India (26). Although a study in DRC reported a conservative prevalence of 60% (23) at 59R, other studies found slightly higher prevalence (87%-98%) at this codon (10,21,22,24,26,29), compared to 78% demonstrated in this study. In agreement with our study, investigations elsewhere found a prevalence of ≥97% at 108N (10,21–24,26,27,29). In contrast to average prevalence (66%-68%) reported in India and Gabon (24,26), prevalence of >90% at *PfDHPS437G* was reported in this study, western Kenya and parts of central Africa. (10,21–23,29). *PfDHPS540E* mutation is highly prevalent in Kenya (10,21), but rare in several countries, which have reported a prevalence of only 0-5% (22-24,29).

*PfDHFR* polymorphism at 51I, 59R and 108N combined mutations of 89% observed in this study was similar to 89-97% documented in western Kenya among pregnant women(10,21). Proportion comparable to these have been reported elsewhere: 97% in Uganda (8); 87% in Equatorial Guinea (22); 98% in Cameroon (29), and 93% in Senegal (30). These frequencies were higher compared to 48% detected in DRC (23).
and 54 -74% in Burkina Faso (25,31). The high frequency (94%) of *PfDHPS* gene double mutation (437G and 540E) reported in this study concurs with findings from other studies in East Africa that reported 90-99% and 99% in western Kenya (10,21) and Uganda (8) respectively. However, double mutation in this gene are reportedly rare occurring with a prevalence of 4% in Equatorial Guinea (22), 1% in Burkina Faso (25), and 2% in DRC (23). An average mutation prevalence of 71.3% was seen at codon 540E in the coastal regions of Tanzania which was low when compared to the country prevalence of 92.4% (9).

Several studies have demonstrated that the differing degrees of antimalarial drug resistance are dependent upon the number and combination of mutations present (32). The *PfDHFR/PfDHPS* quintuple mutant, in either mixed or pure form, is the most clinically relevant molecular marker for SP resistance. In the East African region, the prevalence of molecular markers of SP resistance has been increasing since the emergence of the first resistance-conferring mutations in the 1950s (9). Therefore, continuous molecular surveillance will allow early detection of drug resistance susceptibility and high mutation prevalence within the *PfDHFR* and *PfDHPS* gene of the *Pf* parasite that reduce SP drug effectiveness as a prophylactic treatment for malaria in pregnancy (25). These finding show that there is high prevalence of *PfDHFR/PfDHPS* haplotype mutations believed to confer resistance to SP, the drug of choice for malaria prophylactic treatment in pregnant women. Our data aligned with findings in other parts of Kenya and other tropical regions in defining high prevalence of SP resistance markers within circulating *Pf* isolates.

**Conclusion**

Results from this study suggest that coastal Kenya has high prevalence of *PfDHFR*
triple mutation and PfDHPS double mutation that could potentially undermine the efficacy of SP drug for prophylactic treatment among pregnant women. Despite growing evidence of high prevalence of genetic mutation within the PfDHFR and PfDHPS genes associated with SP drug resistance, it is still the recommended drug for IPTp in Kenya and sub-Saharan Africa regions which carry a disproportionately high burden of malaria (2). There is therefore urgent need for development of safe and more effective malaria drugs for prophylactic treatment of malaria in pregnancy. Continuous monitoring and treatment of malaria infection among pregnant women is necessary to avert malaria-related adverse pregnancy outcomes.

Declaration

ETHICAL APPROVAL

Written informed consent was obtained from each study participant before study participation. The ethical approvals were granted by the Kenyatta National Hospital Ethical Review committee; #P85/03/2013, the Institutional Review Board for Human Studies at University of Cleveland Case Medical Center; #01-13-13 and the KEMRI Scientific Ethics and Review Committee (SERU); #SSC3134.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The dataset analyzed for this study is available from the corresponding author on reasonable request.

COMPETING INTERESTS

Authors declare no conflict of interest.

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**AUTHOR’S CONTRIBUTION**

SWG, KF, ALE, KJ, IM, ADL, CHK and MF designed the study; SWG and KT performed all laboratory analysis; ROO analyzed and presented the data; SWG and ROO drafted the paper, all authors reviewed and approved the final manuscript.

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### Tables

#### Table 1: Characteristics of pregnant women screened for malaria parasites between 2013 -2015 at Msambweni County Hospital in Mombasa, Kenya

| Characteristic                  | Negative (N=628) | Positive (N=135) | p value |
|--------------------------------|------------------|------------------|---------|
| Age (mean; SD) years           | 26.4 (6.31)      | 24.4 (6.34)      | <0.001  |
| Marital status (n, %)          |                  |                  |         |
| Single                         | 100 (16%)        | 32 (24%)         | 0.030   |
| Married                        | 468 (75%)        | 88 (65)          | 0.027   |
| Cohabiting                     | 51 (8%)          | 14 (10)          | 0.400   |
| Divorced/separated             | 9 (1%)           | 1 (1%)           | 0.521   |
| Education (n, %)               |                  |                  |         |
| None to Lower primary          | 149 (24%)        | 41 (30%)         | 0.105   |
| Upper Primary                  | 358 (57%)        | 72 (53%)         | 0.435   |
| Secondary                      | 121 (19%)        | 22 (16%)         | 0.422   |
| Occupation (n, %)              |                  |                  |         |
|                           | n    | %    | p-value |
|---------------------------|------|------|---------|
| Housewife/Not working     | 541  | 86%  | 0.862   |
| Employed (formal or self) | 87   | 14%  | 0.120   |
| Monthly household expenditure (n, %) | | | |
| <5000                     | 45   | 7%   | 0.490   |
| ≥5000                     | 583  | 93%  | 0.490   |
| Bed net (n, %)            |      |      |         |
| Yes                       | 596  | 95%  | 0.043   |
| No                        | 32   | 5%   | 0.043   |
| First Pregnancy (n, %)    |      |      |         |
| Yes                       | 144  | 23%  | 0.011   |
| No                        | 484  | 77%  | 0.011   |
| Gestation in weeks (mean, SD) | | | |
| Number of visits of Folic use (mean, SD) | 23.3 (5.17) | 22.1 (5.21) | 0.017 |
| Number of visits with SP use (mean, SD) | 3.4 (1.39) | 3.8 (1.36) | 0.007 |
| HB g/dl (mean, SD)        | 9.8  | 1.82 | 0.472   |
| Parasite load/µL of blood (median; IQR) | | | |
| Placenta (mean, SD)       |      |      |         |
| Weight (g)                | 550.3 (98.86) | 518.3 (96.74) | 0.002 |
| Length (cm)               | 19.3 | 1.77 | 0.162   |
| Width (cm)                | 17.3 | 1.91 | 0.178   |
| Height (cm)               | 2.2  | 0.38 | 0.826   |
Table 2: Characteristics of 84 pregnant women from whom *Plasmodium falciparum* isolates were recovered for genetic analysis

| Characteristic                                      | Mean, Median or Proportion |
|-----------------------------------------------------|-----------------------------|
| Age in years (mean; SD)                             | 23.7 (6.14)                 |
| Marital status:                                     |                             |
| Single                                              | 19 (23%)                    |
| Married                                             | 53 (63%)                    |
| Cohabiting                                          | 11 (13%)                    |
| Divorced                                            | 1 (1%)                      |
| Education:                                          |                             |
| None to Lower primary school                        | 24 (29%)                    |
| Upper Primary school                                | 48 (57%)                    |
| Secondary school                                    | 12 (14%)                    |
| Occupation:                                         |                             |
| Housewife/Not working                               | 79 (94%)                    |
| Employed                                            | 5 (6%)                      |
| Monthly household expenditure (KShs.):              |                             |
| < 5000                                              | 61 (73%)                    |
| ≥ 5000                                              | 23 (27%)                    |
| Bed net use:                                        |                             |
| Yes                                                 | 76 (90%)                    |
| No                                                  | 8 (10%)                     |
| First pregnancy?                                    |                             |
| Yes                                                 | 32 (38%)                    |
| No                                                  | 52 (62%)                    |
| Gestation in weeks (mean, SD)                       | 22.0 (5.19)                 |
| Number of Visits of Folic acid use (mean, SD)       | 3.7 (1.40)                  |
| Number of Visits of SP use (mean, SD)               | 3.5 (1.43)                  |
| Hb g/dL (mean, SD)                                  | 9.5 (2.31)                  |
| Parasite load/µL of blood (median; IQR)             | 2760 (1200-7133)            |
Table 3: Genetic analysis of *Pfdhfr* and *Pfdhps* SNPs in *Plasmodium falciparum* isolates from Mombasa, Kenya

**a) Prevalence of *Pfdhfr* and *Pfdhps* SNPs in isolated *P. falciparum* malaria parasites**

| SNP (N=106) | Mutant Type n (%; 95% CI) | Mixed Type n (%; 95% CI) | Wild Type n (%; 95% CI) |
|-------------|----------------------------|--------------------------|-------------------------|
| 51          | 94 (88.68, 80.69 to 93.76) | 6 (5.66, 2.32 to 12.41)  | 5 (4.72, 1.75 to 7      |
| 59          | 83 (78.30, 69.03 to 85.48) | 21 (19.81, 12.95 to 28.91) | 2 (1.89, 0.33 to 7      |
| 108         | 99 (93.40, 86.40 to 97.08) | 6 (5.66, 2.32 to 12.41)  | 0 (0.00)                |
| 437         | 100 (94.34, 87.59 to 97.68) | 4 (3.77, 1.21 to 9.94)   | 2 (1.89, 0.33 to 7      |
| 540         | 97 (91.51, 84.07 to 95.80) | 1 (0.64, 0.05 to 5.90)   | 5 (4.72, 1.75 to 1      |

**b) Different combinations of *PFDHFR/PFDHPS* haplotype mutations**

| Genotype   | Mutants only n (%) | Mutants + Mixed n |
|------------|--------------------|-------------------|
| Double     | 7 (6.6)            | 0                 |
| Triple     | 7 (6.6)            | 4                 |
| Quadruple  | 22 (20.8)          | 11                |
| Quintuple  | 70 (66)            | 91                |

Table 4: Primer information

| No. | Primer Name | Sequence | Amount |
|-----|-------------|----------|--------|
| 1   | P.falc F    | 5’ GCTCTTTCTTGGAT TTCTTGGAT 3’ | 0.3uM |
| 2   | P.falc R    | 5’ AGCAGGTTAAGA TCTCGTTCG 3’ | 0.3uM |
| 3   | P.falc P    | 5’ CACGAACTAAAAA CGGCCAT 3’ | 0.2 uM |
| 4   | DHFR-51 F   | 5’ TGAGGTTTTTAAT AACTACACATTGA GAGGCTC 3’ | 0.3uM |
| 5   | DHFR-51 R   | 5’ TATCATTACATT ATCCACAGTTTCT TTGT 3’ | 0.3uM |
| 6   | DHFR-51 WTP | 5’ AATGTAATCCCT AGATATG 3’ | 0.2 uM |
| 7   | DHFR-51 MP  | 5’ AAATGTATTTCCC TAGATATG 3’ | 0.2 uM |
| 8   | DHFR-59 F   | 5’ Same primer sequence as 3’ | 0.3uM |
|   |   |   |   |
|---|---|---|---|
| 9 | DHFR-59 R | 5’ | Same primer sequence as DHFR - 51F |
| 10 | DHFR-59 WTP | 5’ | AATATTTTTGTGC AGTTACA |
| 11 | DHFR-59 MP | 5’ | TGAATATTTTCG TGCAGTTA |
| 12 | DHFR-108 F | 5’ | TGGATAATGTTAAA TGATATGCCTAAT TCTAA |
| 13 | DHFR-108 R | 5’ | AATCTTCTTTTTT AAGGTTCAGACA ATATAACA |
| 14 | DHFR-108 WTP | 5’ | AGAAACAAGCTGG GAAA |
| 15 | DHFR-108 MP | 5’ | AGAAACAACTGG GAAAG |
| 16 | DHPS-437 F | 5’ | TGAATATGATAATATG TGAAGGTGCTAGT GT |
| 17 | DHPS-437 R | 5’ | AATACAGGTACTA CTAAATCTCTTTTC SOOANAAAATTTTTT |
| 18 | DHPS-437 WTP | 5’ | AGATCCTCTGCT CCT |
| 19 | DHPS-437 MP | 5’ | AATCCTCTGGTCC TTT |
| 20 | DHPS-540 F | 5’ | AATGCATAAAAAGA GGAAATCCACAT |
| 21 | DHPS-540 R | 5’ | TCGGAATCTCCTAA TCCAATATCAAA |
| 22 | DHPS-540 WTP | 5’ | CAATGGATAAACT AACA |
| 23 | DHPS-540 MP | 5’ | AATGGATGAACATA ACAAA |

|   |   |   |
|---|---|---|
|  |  | 0.5 uM |
|  |  | 0.2 uM |
|  |  | 0.2 uM |
|  |  | 0.3 uM |
|  |  | 0.3 uM |
|  |  | 0.2 uM |
|  |  | 0.2 uM |
|  |  | 0.3 uM |
|  |  | 0.2 uM |