Copy number estimation of *P. falciparum* pfmdr1 v1.1

Procedure

Molecular Module

WorldWide Antimalarial Resistance Network (WWARN)

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Version History

Version number	Revision(s) & reason for amendment	Release date
1.1	Change from 50 ng/uL template DNA to 50 pg/uL	17/03/2011
1.0	Creation of procedure	18/11/2010

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Contents

1. Purpose

This procedure is designed to estimate the copy number of the *Plasmodium falciparum* multi-drug resistance 1 locus (pfmdr1) in DNA extracted from samples of infected blood.

2. Scope

This procedure is intended for use in clinical trials and molecular studies where whole blood or dried blood spot samples are collected for genotyping of *P. falciparum* infections. It describes the relative quantification of the pfmdr1 gene associated with resistance to multiple antimalarials. This procedure is applicable for well-equipped laboratories familiar with real-time PCR.

3. Abbreviations

DNA	Deoxyribonucleic Acid
pfmdr1	*Plasodium falciparum* multi-drug resistance 1 locus
PCR	Polymerase Chain Reaction
µL	microlitre
mL	millilitre
pg	picogram
nM	nanomolar
ΔΔ C_T	Delta delta Cycle threshold
C_T	Cycle threshold

4. Duties and Responsibilities

N/A

5. Materials and Equipment

5.1 Materials

- 2X TaqMan Master Mix (ABI, part # 4304437) or comparable reagent
- MicroAMP Optical 96-well plates (ABI, part # N801-0560) or comparable optical PCR tubes/plates

- MicroAMP Optical Adhesive Film Covers (ABI, part # 4311971) or comparable optical PCR film/caps
- Micropipets and tips (10 µL, 200 µL and 1000 µL)
- 1.5 mL microcentrifuge tubes or 15 mL conical tubes for master mix (depending on number of samples)
- pfmdr1 primers and probe (target locus):
    - F: 5'-TGC ATC TAT AAA ACG ATC AGA CAA A-'3
    - R: 5'-TCG TGT GTT CCA TGT GAC TGT-'3
    - Probe: 5'-6FAM-TTT AAT AAC CCT GAT CGA AAT GGA ACC TTT G-TAMRA-'3
- β-tubulin primers and probe (endogenous locus):
    - F: 5'-AAA AAT ATG ATG TGC GCA AGT GA-'3
    - R: 5'-AAC TTC CTT TGT GGA CAT TCT TCC T-'3
    - Probe: 5'-VIC-TAG CAC ATG CCG TTA AAT ATC TTC CAT GTC T-TAMRA-'3
- DNA controls: at least one single-copy template and one multi-copy template (see Table 1) at 50 pg/µL.

Table 1. Commonly used pfmdr1 copy number control strains

Strain	Approx. number of copies
3D7	1
K1	1
D10	1
Fac8	2-3
Dd2	3-4

5.2 Equipment

- Real-Time PCR machine

6. Procedure

6.1 Real-time multiplex PCR

Real-time PCR can be performed using a number of real-time PCR machines and reagents. The procedure given here uses the ABI system and probes with 5'-6FAM or VIC fluorescent labels with a 3'-TAMRA quencher for detection. Other machines and reagents, such as Minor Groove Binding (MGB)-labeled primers[1], require different reaction mixes, sample volumes and/or cycling conditions.

Success of this assay may vary based on parasitaemia and quality of the sample, particularly when using dried blood spots. Greater success is generally achieved using DNA extracted from samples with > 10,000 parasites/µL[2].

I. Calculate the number of samples to be tested. Each control and sample is quantified in triplicate multiplex reactions for pfmdr1 and β-tubulin. A representative 96-well plate is given in Figure 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	3D7	3D7	3D7	5	5	5	13	13	13	21	21	21
B	K1	K1	K1	6	6	6	14	14	14	22	22	22
C	Dd2	Dd2	Dd2	7	7	7	15	15	15	23	23	23
D	water	water	water	8	8	8	16	16	16	24	24	24
E	1	1	1	9	9	9	17	17	17	25	25	25
F	2	2	2	10	10	10	18	18	18	26	26	26
G	3	3	3	11	11	11	19	19	19	27	27	27
H	4	4	4	12	12	12	20	20	20	28	28	28

pfmdr1 + β-tubulin

Figure 1. 96-well plate including template controls, a no-template control and 28 samples in triplicate multiplexed reactions. Image source: Meera Venkatesan, University of Maryland Baltimore.

II. Place a 1.5 mL or 15 mL tube on ice. Prepare PCR master mix by adding 2x TaqMan Master Mix reagent, forward and reverse primers and probes in the amounts given in Table 2. It is best to prepare the master mix for 2–4 extra samples so that it does not run out.

Table 2. Real-time multiplex PCR master mix

Reagent	Amount per reaction
2x TaqMan Master Mix reagent	12.5 µL
Pfmdr1 Forward primer	300 nM
Pfmdr1 Reverse primer	300 nM
Pfmdr1 probe	100 nM
β-tubulin Forward primer	300 nM
β-tubulin Reverse primer	300 nM
β-tubulin probe	100 nM
Water	to reach total of 20 µL

NOTE: Do not vortex the mix. Mix by hand-shaking and light centrifugation. Avoid prolonged exposure of mix to light.

III. Add 20 µL of master mix to each well. Add 5 µL of control DNA, water or sample DNA to each well as shown in Figure 1.

IV. Seal plate thoroughly and cycle under the PCR conditions listed in Table 3. Label the single-copy control (usually 3D7) as the calibrator.

Table 3. Real-time PCR thermal cycling conditions

Step	Temperature (°C)	Time
1	95	5:00
2	95	0:15
3*	**58**	**0:60**
4	Repeat steps 2–3 40 times	

*Data collection occurs in step 3 shown in **green**

6.2 Quality check and estimation of copy number

I. Check the quality of your reaction. Repeat sample or control if[2]:
   a. The spread of ΔΔ C_T values among replicates exceeds 1.5.
   b. C_T estimate > 35.
   c. Copy number estimate ranges between 1.3–1.6.

NOTE: Other quality cutoff rules are also commonly used, including repetition or exclusion of samples with a C_T estimate > 32, with an upper limit of the 95% confidence interval of the copy number estimate > 0.4 or a copy number estimate of < 0.5[3,4].

7. References

1. Nair S, Nash D, Sudimack D, Jaidee A, Barends M, Uhlemann AC, Krishna S, Nosten F, Anderson TJ. 2007. **Recurrent gene amplification and soft selective sweeps during evolution of multidrug resistance in malaria parasites.** Mol Biol Evol. 24:562-573.

2. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ, Nosten F, Krishna S. 2004. **Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number.** Lancet. 364:438-447.

3. Nair S, Miller B, Barends M, Jaidee A, Patel J, Mayxay M, Newton P, Nosten F, Ferdig MT, Anderson TJ. 2008. **Adaptive Copy Number Evolution in Malaria Parasites**. PLoS Genet. 4: e1000243. doi:10.1371/journal.pgen.1000243

4. Griffing S, Syphard L, Sridaran S, McCollum AM, Mixson-Hayden T, Vinayak S, Villegas L, Barnwell JW, Escalante AA, Udhayakumar V. 2010. **pfmdr1 amplification and fixation of pfcrt chloroquine resistance alleles in Plasmodium falciparum in Venezuela.** Antimicrob Agents Chemother. 54:1572-1579.