PCR PROTOCOL FOR *PfMDR1* PYROSEQUENCING
(codons 86, 184, 1034, 1042 and 1246)

1.0 Purpose

This protocol provides a method for performing PCR on genomic DNA extracted from dried blood spots on Whatman 3MM filter paper (Whatman cat# 3030-866; Fisher Scientific cat# 05-716-6B) for subsequent genotyping of *PfMDR1* codons 86, 184, 1034, 1042 and 1246 by performing pyrosequencing.

2.0 References

2.1 Protocol developed using DNA Engine Tetrad 2 Thermocycler for PCR (Bio-Rad Laboratories, Inc.)
2.2 Manual DNA Extraction Protocol for Dried Blood Spot Filter papers *(Modified for use with QIAGEN QIAamp® 96 DNA Blood Kit Cat #: 51161 OR 51162)*
2.3 Handbook for Qiagen QIAamp DNA mini kit or DNA 96 Blood kit (optional) (or comparable DNA extraction kits)
2.4 Protocol for Pyrosequencing
2.5 PyroMark Q96 MD operating manual (Qiagen)
2.6 QIAxcel user manual (Qiagen)
2.7 E-Gel user manual (Invitrogen)

3.0 Materials

3.1 QIAamp DNA mini Kit (Qiagen; cat# 51306) or QIAamp DNA 96 Blood Kit (Qiagen; cat# 51161) or comparable DNA extraction kit/ method for extraction of dried blood from filter paper
3.2 Hot Star™ TAQ Polymerase (includes 50 mM $MgCl_2$ and 10X buffer) (Qiagen; cat# 203205)
3.3 dNTP set; 100 mM (Invitrogen; cat# 10297-018)
Note: Prepare aliquots at 25 mM before use by combining equal volumes of each of the four dNTPs
3.4 TempPlate™ 0.2 mL polypropylene 96 well PCR Plates (USA Scientific; cat# 1402-9700) (or comparable)
3.5 Adhesive Film for PCR plates (VWR; cat# 60941-122) (or comparable)
3.6 Easy Peel™ peelable Heat Sealing Foil Sheets (Thermo Scientific; cat# AB-0745) (or comparable)
3.7 TempPlate® Sealing Foil Sheets (USA Scientific; cat# 2923-0100) (or comparable)
3.8 RNAse and DNAse Free Water (Gibco cat# 15230-147) (or comparable)
3.9 Control genomic DNA (3D7, Dd2 & 7G8) (MR4 cat# MRA-102G, MRA-150G & MRA-926G respectively, or equivalent)
3.10 1.5 mL centrifuge or 15mL conical tubes for master mix (depending on number of samples)
3.11 Scissors (or razors) (for cutting strips of filter paper with dried blood spots)
3.12 70% alcohol (Ethanol or Isopropanol)
3.13 QIAxcel DNA Screening Cartridge (Qiagen cat# 1050349)
3.14 E-Gel® 96 2% with SYBR® Safe (Invitrogen cat# G7208-02)
3.15 PCR and Pyrosequencing primers **(see Table 1)**

4.0 Equipment:

Note: Equipment listed in this protocol may be substituted with comparable equipment from other brands
4.1 Water baths (85 °C, 56 °C and 70 °C) for use with QIAamp DNA mini Kit procedure for DNA extraction

4.2 56 °C incubator (for use with manual extractions using QIAamp DNA 96 Blood Kit)
4.3 Micro centrifuge (Beckman Coulter microfuge 16) or table top centrifuge (Eppendorf 5810R with Eppendorf A-2-DWP Centrifuge head), as appropriate
4.4 DNA Engine Tetrad 2 Thermocycler (Bio-rad Laboratories)
4.5 PyroMark® Q96 MD Pyrosequencing machine (Qiagen)
4.6 Combi Thermosealer (ABGene; model# AB-0384/110)
4.7 BioRobot® Universal automated system (Qiagen) (optional; for processing very large number of specimens)
4.8 Calibrated 8 or 12 well multichannel pipets (2-20 µL and 20-200 µL) (Matrix or comparable) and aerosol resistant pipette tips
4.9 Calibrated Micropipets (10 µL, 20 µL & 1000 µL) (Matrix EDP Plus or comparable) and aerosol resistant pipette tips
4.10 Refrigerator (2-8 °C) and Freezer (-20°C and/or -80°C, for long term storage of DNA)
4.11 Ice bath (for setting up Master mixes & PCR reactions)
4.12 Equipment for running agarose gel electrophoresis
4.13 QIAxcel® Capillary Electrophoresis Instrument (Qiagen) (optional, in lieu of step 4.12)
4.14 E-Gel Agarose Electrophoresis apparatus (Invitrogen) (optional, in lieu of step 4.12)

5.0 Safety and Precautions

5.1 Use universal BSL2 precautions when working in the laboratory. Wear gloves, labcoat and safety glasses when handling specimens.
5.2 Use appropriate safety precautions when using razor blade or scissors to cut filter strips with dried blood. Wipe the cutting tool with 70% alcohol between samples.
5.3 Ensure that all equipment (pipettes, centrifuges, water baths, etc.) is properly calibrated prior to use.
5.4 The Thermosealer has hot surfaces. Use with caution.
5.5 Take care when applying or removing plate sealant, to prevent cross-contamination of adjacent wells.
5.6 Take utmost care during pipetting steps to prevent cross contamination of samples or PCR products.
5.7 Use an ice bath to hold the PCR master mix and/or PCR reagents during PCR master mix preparation.
5.8 Do not vortex the PCR mix . Mix gently. Use light centrifugation to collect all liquid to the bottom of the PCR plate or tube.

6.0 Procedure

6.1 DNA extraction from blood spotted filter papers:

6.1.1 Using clean scissors (or razor blade) cut an approximately 3mm by 5 mm strip of filter paper stained on both sides with dried blood
Note: Use two strips if only one side of the filter has the blood spot, or if lower quantity/quality of DNA is anticipated.
6.1.2 For extraction of genomic DNA from blood spots on filter paper our laboratory has successfully used the Qiagen QIAamp DNA mini kits (for fewer than 24 specimens) and Qiagen DNA 96 Blood kit (for very large number of specimens, used in conjunction with the Biorobot® Universal System, for processing large number of specimens). Follow instructions in the respective manuals when using these kits.
6.1.3 For manual extraction of large number of specimens, we have successfully used a modified version with the Qiagen QIAamp 96 Blood Kit (see 2.2 under References section).

6.2 **Setting up PCR Reactions:**

6.2.1 Use the Excel spreadsheet template **(figure 2)** for setting up PCR Master mixes for the external and internal (nested) PCR reactions and the thermocycler conditions.

6.2.2 Determine the number of samples (n) you need to amplify.

6.2.3 You can use a 96 well PCR plate if you have more than 24 samples for PCR amplification. **A representative plate is shown in figure 1** (make sure to include controls for drug-susceptible and drug-resistant DNA and negative controls (and external and nested PCR negative controls).

6.2.4 Calculate the volume of Master mixes to make according to the spreadsheet in figure 2.

Note: (1) Prepare master mix for 2-5 extra samples (eg: n+5) to allow sufficient volume for pipetting.

(2) If you need to add more DNA, reduce the amount of water in the master mix. Final reaction volume is 25 µL.

(3) You can type the total number of samples that need to undergo PCR into the cell highlighted yellow in the spreadsheet in **figure 2** by double clicking on the embedded spreadsheet, and it will calculate the volume of reagents you need to make up the external and internal PCR Master mixes.

6.2.5 Add 24 µL of Master mix to the PCR tube or 96 well plate (add 25 µL to Negative PCR Control).

6.2.6 Add 1 µL of genomic control DNA or DNA extracted from the filter paper.

Note: More DNA can be used, if required, and the volume of water in the Master Mix must be adjusted accordingly.

6.2.7 Seal the plate with Thermoseal plate sealer and place it in the PCR thermocycler.

6.2.8 Set up the PCR cycling conditions on the thermocycler as shown in the spreadsheet in **figure 2** and start the PCR reaction.

6.2.9 Successful amplification can be confirmed by running the nested PCR product together with a 100 bp DNA ladder on the QIAxcel® capillary electrophoresis instrument (Qiagen) using the QIAxcel DNA Screening cartridge, or on the Invitrogen E-Gel apparatus using E-Gel® 96 2% with SYBR® Safe DNA gel stain (a 2% agarose gel stained with ethidium bromide can also be used optionally).

6.2.10 Use the protocol for pyrosequencing for genotyping the amplified DNA.

6.2.11 The Sequence to Analyze (STA) and the nucleotide dispensation order used entered into the pyrosequencing instrument are listed in **Table 2**. The sample genotype for **pfMDR1 codon 86** can be determined using the SNP mode of the PyroMark™ Q 96MD software **(see Protocol for Pyrosequencing)**. The sample genotypes for the remaining codons can be determined using the SNP and/or AQ (Allele Quantification) mode of the software.

6.2.12 Allele frequencies for all codons analyzed in the AQ mode should be corrected and adjusted using a laboratory-specific standard curve for the respective codon(s).

Table 1 (PCR and pyrosequencing primers)

Codon	Primer	Primer ID	Sequence (5' - 3')	Bases	Amplicon size
86	External Forward	pfMDR1_86/184 EF	TGAACAAAAAGAGTACCGCTGA	22	577 bp
	External Reverse	pfMDR1_86/184 ER	TTCTTATTACATATGACACCACAAACA	27	
	Internal Forward	pfMDR1_86 IF	CGTTTAAATGTTTACCTGCACAA	23	290 bp
	Internal Reverse	pfMDR1_86 IR	B- TTGTCCATCTTGATAAAAAACACT	24	
184	External Forward	pfMDR1_86/184 EF	TGAACAAAAAGAGTACCGCTGA	22	577 bp
	External Reverse	pfMDR1_86/184 ER	TTCTTATTACATATGACACCACAAACA	27	
	Internal Forward	pfMDR1_184 IF	AGTGAGTTCAGGAATTGGTACGA	23	127 bp
	Internal Reverse	pfMDR1_184 IR	B-CGGAAAAACGCAAGTAATACATAA	24	
1034 & 1042	External Forward	pfMDR1_1034/1042 EF	CAAGCGGAGTTTTTGCATTT	20	377 bp
	External Reverse	pfMDR1_1034/1042 ER	TTTTGCATTTTCTGAATCTCCTT	23	
	Internal Forward	pfMDR1_1034/1042 IF	TTATTGTAAATGCAGCTTTATGGG	24	178 bp
	Internal Reverse	pfMDR1_1034/1042 IR	B- AAGGACATTAATTTTCCAGCATAA	24	
1246	External Forward	pfMDR1_1246 EF	GCAATCGTTGGAGAAACAGG	20	562 bp
	External Reverse	pfMDR1_1246 ER	TGGAATCAAGTGATGATGTTGC	22	
	Internal Forward	pfMDR1_1246 IF	B-TTTTCAAACCAATCTGGATCTG	22	320 bp
	Internal Reverse	pfMDR1_1246 IR	CTCTGTTTTTGTCCACCTGATAAG	24	
86	Pyrosequencing Primers	pfMDR1_86_F_pyro	GTGTAATATTAAAGAACATG	20	
184		pfMDR1_184_F_pyro	CCAGTTCCTTTTTAGGTT	18	
1034 & 1042		pfMDR1_1034_1042_F_pyro	CAGCTTTATGGGGATT	16	
1246		pfMDR1_1246_R_pyro	ATTGAAAATAAGTTTCTAAG	20	

Note: B = Biotinylated; External Forward and Reverse primers are the same for codons 86 and 184.

Table 2 (*pfMDR1* gene SNPs and pyrosequencing STAs[1] and Dispensation Orders)

	wild		mutant		DNA (control strains)		Pyrosequencing	
pfMDR1	codon	Amino acid	SNP	Amino acid	wild	mutant	Sequence to Analyze	Dispensation order
86	AAT	Asn (N)	TAT	Tyr (Y)	3D7	Dd2	A/TATTTAGGTGATGATATTAATCCTAT	GTAGTAGTG
184	TAT	Tyr (Y)	TTT	Phe (F)	3D7	7G8	TATA/TTATTTGGTCATTAATAAAAAATGCA	GTATATATCGT
1034	AGT	Ser (S)	TGT	Cys (C)	3D7	7G8	CA/TGTCAAAGCGCTCAATTATTTATTA/GAT	GCATCGTCAGCGCTCATATATGA
1042	AAT	Asn (N)	GAT	Asp (D)	3D7	7G8	same as for codon 1034	same as for codon 1034
1246	GAT	Asp (D)	TAT	Tyr (Y)	3D7	7G8	ATA/CTCTTAAGTTATAATCACATATATTA	GATACGTCT

Note: Location of the SNP is shown in red font.

[1] STA = Sequence to Analyze

Figure 1:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41	Sample 49	Sample 57	Sample 65	Sample 73	Sample 81	Sample 89
B	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42	Sample 50	Sample 58	Sample 66	Sample 74	Sample 82	Sample 90
C	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43	Sample 51	Sample 59	Sample 67	Sample 75	Sample 83	Sample 91
D	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Sample 44	Sample 52	Sample 60	Sample 68	Sample 76	Sample 84	Ctrl 1 (wild type)
E	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37	Sample 45	Sample 53	Sample 61	Sample 69	Sample 77	Sample 85	Ctrl #2 (mutant)
F	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38	Sample 46	Sample 54	Sample 62	Sample 70	Sample 78	Sample 86	Ctrl #3 (wild/mutant) (optional)
G	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39	Sample 47	Sample 55	Sample 63	Sample 71	Sample 79	Sample 87	PCR 1 (neg Ctrl)
H	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40	Sample 48	Sample 56	Sample 64	Sample 72	Sample 80	Sample 88	PCR2 (neg Ctrl)

Figure 2:

pfmdr1 gene codons 86, 184, 1034, 1042 and 1246

Master Mix Preparation:

External PCR (25 µL):

Components		Volume (µL)	final conc.	Samples 1
Water		14		14
10x buffer	10X	2.5	1X	2.5
dNTP	(25 mM)	0.2	(0.2 mM)	0.2
$MgCl_2$	(25 mM)	2	(2 mM)	2
Primer EF	(5 µM)	**2.5**	(0.5 µM)	2.5
Primer ER	(5 µM)	**2.5**	(0.5 µM)	2.5
Hotstar Taq Polymerase	(5 U/µL)	0.3	(0.06 U/uL)	0.3
DNA		1		**1**
		25		**25**

Aliquot 24 µL of Mastermix to positive controls and 25 µL to negative control

External PCR Cycling Conditions:

Hotstart	95°C	15 min	
Denaturation	94°C	30 sec	**30 cycles**
Annealing	58°C	45 sec	
Extension	72°C	1 min	
Final Extension	72°C	10 min	
	4°C	HOLD	

Internal PCR (25 µL)

Components		Volume (µL)	final conc.	Samples 1
Water		14		14
10x buffer	10X	2.5	1X	2.5
dNTP	(25 mM)	0.2	(0.2 mM)	0.2
$MgCl_2$	(25 mM)	2	(2 mM)	2
Primer IF	(5 µM)	**2.5**	(0.5 µM)	2.5
Primer IR	(5 µM)	**2.5**	(0.5 µM)	2.5
Hotstar Taq Polymerase	(5 U/µL)	0.3	(0.06 U/µL)	0.3
1° PCR product		1		**1**
		25		**25**

Aliquot 24 µL of Mastermix to positive controls and 25 µL to negative control

Internal PCR Cycling Conditions:

Hotstart	95°C	15 min	
Denaturation	94°C	30 sec	**30 cycles**
Annealing	58°C	45 sec	
Extension	72°C	1 min	
Final Extension	72°C	10 min	
	4°C	HOLD	

7.0 Version History

Version	Type of Revision	Effective Date
1.0	Creation of SOP	27August 2013