PCR PROTOCOL FOR *PfCRT* PYROSEQUENCING

(codons 72, 74, 75, 76, 220, 271, 326, 356, and 371)

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 *PfCRT* codons 72-76, 220, 271, 326, 356 and 371 by performing pyrosequencing.

2.0 References

2.1 Protocoldeveloped using DNA Engine Tetrad 2 Thermocycler for PCR(Bio-Rad Laboratories, Inc.)
2.2 Handbooks for Qiagen QIAamp DNA mini kit or DNA 96 Blood kit(optional, for processing large number of specimens) (or comparable DNA extraction kits)
2.3 Protocol for Pyrosequencing
2.4 PyroMark Q96 MD operating manual (Qiagen)
2.5 QIAxcel user manual (Qiagen)
2.6 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 (includes50 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 DNA (3D7 or HB3 and Dd2) (MR4 cat# MRA-102G or MRA-155G and MRA-150G 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 tern 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 the following (modified) version with the Qiagen QIAamp 96 Blood Kit:

Note: (a) Start incubator at 56 °C in advance for use in steps 3 and 5.
(b) Use Eppendorf A-2-DWP Centrifuge head with the Eppendorf 5810R table-top centrifuge (or equivalent) for steps 6.1.3.4 onward.

(c) All centrifugations are carried out at room temperature

6.1.3.1 Place 1 or 2 filter paper strips stained with dried blood into the wells of the round-well block (provided in kit)

6.1.3.2 Prepare ATL/Proteinase K solution:

For each extraction, take 180 µL of Buffer ATL. Add 20 µL reconstituted Proteinase K. Mix well. (Make extra volume to allow for loss during pipetting, use for negative control, etc).

6.1.3.3 Add 200 µL ATL/Proteinase K solution to each well of the block. Seal the wells using caps for the blocks provided. Incubate at 56 °C overnight with shaking.

6.1.3.4 Next day, briefly centrifuge to remove any solution from the caps.

6.1.3.5 Add 200 µL of Buffer AL to the sample, mix by thoroughly shaking for 15seconds (hold block and shake up and down). Centrifuge briefly at 1811 rcf to collect any solution from the caps and incubate at 56 °C for 15 min.

6.1.3.6 Add 200 µL ethanol (96 – 100%) to each well. Seal the wells with new caps & shake vigorously for 15seconds. Centrifuge briefly at 1811 rcf to collect any solution from the caps.

6.1.3.7 Place QIAamp 96 plate on top of an S-Block (both provided with kit).

6.1.3.8 Carefully apply the mixture from step 5 (600 µL per well) from the round well block to the QIAMP 96 plate. (Take care not to wet the rims of the wells to avoid aerosol formation)

6.1.3.9 Seal the QIAamp 96 plate with an AirPore tape sheet (provided). Centrifuge the QIAamp 96 plate + S-block at 2608 rcf for 8 min.

6.1.3.10 Remove the tape. Carefully add 500uL of buffer AW1 to each well. Empty S-block and replace QIAamp 96 plate onto S-block. Seal the QIAamp 96 plate with a new AirPore Tape sheet.

6.1.3.11 Centrifuge at 2608 rcf for 5 min.

6.1.3.12 Remove the tape. Carefully add 500 µL of buffer AW2 to each well. Empty S-block and replace QIAamp 96 plate onto S-block. Do not Seal with AirPore Tape to allow for sufficient ethanol evaporation.

6.1.3.13 Centrifuge at 2608 rcf for 25 min.

6.1.3.14 Place the QIAamp 96 plate on top of a rack of elution microtubes (provided with kit).

6.1.3.15 To elute DNA, add 150 µL Buffer AE to each well using a multichannel pipette. Seal the QIAamp 96 plate with an AirPore tape sheet and incubate for 1 minute at room temperature.

6.1.3.16 Centrifuge at 2608 rcf for 8 min. Seal the elution plate with caps (provided with kit) and store extracted DNA (eluate) at -80 °C (2-8 °C storage is allowed for ≤ 24 hours; -20 °C storage is allowed for ≤ 2 weeks).

6.2 **Setting up PCR Reactions:**

6.2.1 Use the Excel spreadsheet template(s) **(figures 2A, 2B and/or 2C, depending on the codons selected for testing)**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 (extraction buffer only and PCR negative control).

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(s) in **figures 2A,2B and/or 2C**, 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 spreadsheets in **figure 2A, 2B and/or 2C** 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 pfCRT 72-76 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 For all codons analyzedin the AQ mode, allele frequencies 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
72 - 97	External Forward	pfcrt_72-97EF	GACCTTAACAGATGGCTCAC	20	347bp
	External Reverse	pfcrt_72-97ER	TTTTATATTGGTAGGTGGAATAG	23	
	Internal Forward	pfcrt_72-97 IF	B-GGTAAATGTGCTCATGTGTTTAAACTTATT	30	241bp
	Internal Reverse	pfcrt_72-97 IR	TTACTTTTGAATTTCCCTTTTTATTTCCA	29	
220	External Forward	pfcrt_220 EF	CGGAGCAGTTATTATTGTTG	20	307bp
	External Reverse	pfcrt_220 ER	CTATTTCCCTTGTCATGTTTG	21	
	Internal Forward	pfcrt_220 IF	TGAAATTATCTTTTGAAACACAAGAAG	27	249bp
	Internal Reverse	pfcrt_220 IR	B-TTTGAAAAGCATACAGGCTAAAAA	24	
271	External Forward	pfcrt_271 EF	GGCTATGGTATCCTTTTTCCAA	22	260bp
	External Reverse	pfcrt_271 ER	CGACTGTGTTTCTTCCCAAG	20	
	Internal Forward	pfcrt_271 IF	B -ATCCTTTTTCCAATTGTTCACTTC	24	221bp
	Internal Reverse	pfcrt_271 IR	CGAAACCATTTTTTATATTTGTCC	24	
326	External Forward	pfcrt_326 EF	GAATTGTGGTCTTGGTATGGC	21	303bp
	External Reverse	pfcrt_326 ER	TTCCCATATTTATTTCCTCTTGTAT	25	
	Internal Forward	pfcrt_326 IF	TGTGGTCTTGGTATGGCTAAGTTA	24	285bp
	Internal Reverse	pfcrt_326 IR	B- TCCTCTTGTATGTATCAACGTTTT	24	
356	External Forward	pfcrt_356 EF	AGATTATCGACAAATTTTCTACCATG	26	240bp
	External Reverse	pfcrt_356 ER	ATAAAATATGATACGTTGTACCATC	25	
	Internal Forward	pfcrt_356 IF	CGACAAATTTTCTACCATGACATA	24	225bp
	Internal Reverse	pfcrt_356 IR	B- TGATACGTTGTACCATCATAAACA	24	
371	External Forward	pfcrt_371 EF	TACATTTAAATGTTTATGATGGTAC	25	246bp
	External Reverse	pfcrt_371 ER	ATATTTAT ACGAACAAGCCATTTG	25	
	Internal Forward	pfcrt_371 IF	ATGTTTATGATGGTACAACGTATC	24	231bp
	Internal Reverse	pfcrt_371 IR	B- ATAACGAACAAGCCATTTGATATT	24	
72-76	Pyrosequencing Primers	pfcrt_72-76_R_pyro	AGTTCTTTTAGCAAAAATT	19	
220		pfcrt_220_F_pyro	CTATCATATTTAATCTTGTC	20	
271		pfcrt_271_R_pyro	TTTCCTAATTAATTCTTACG	20	
326		pfcrt_326_F_pyro	CGCATTGTTTTCCTTCT	17	
356		pfcrt_356_F_pyro	TAGTTGTATACAAGGTCCAG	20	
371		pfcrt_371_F_pyro	AATTTTATAGGGTGATGTTG	20	

Note: B = Biotinylated

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

	wild		mutant				Pyrosequencing	
pfCRT	codon	Amino acid	SNP	Amino acid	wild	mutant	Sequence to Analyze	Dispensation order
76	AAA	Lys	ACA	Thr	HB3	DD2/7G8	G/TTA/TTT/CA/CATTACACA/TTACACTTAAATA	CGTATCATAGCACATGAC
220	GCC	Ala	TCC	Ser	HB3	DD2	TTAATTAGTG/TCCTTAATTGTAAGAAAACAAAATAT	GTATAGTGTCT
271	CAA	Gln	GAA	Glu	HB3	DD2	TTG/CTTTTAAAAATGGAAGGGTGTATACA	ATGCGTTATG
326	AAC	Asn	AGC	Ser	HB3	DD2	TTAG/ACATTTGTGATAATTTAATAACCAGC	GTAGTCATG
356	ATA	Ile	ACA	Thr	HB3	DD2	CAAT/CAGCAATTGCTTATTACTTTTAAATTC	GCATCTAGC
371	AGA	Arg	ATA	Ile	HB3	DD2	TAAG/TAGAACCAAGATTATTAGATTTCGTA	GTAGTCAGA

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	HB3 or 3D7 (DNA Ctrl) (Wild type)	Dd2 (DNA Ctrl) (Mutant)	Neg Ctrl (PCR)	Sample Extraction Buffer	Sample #1	Sample #2	Sample #3	Sample #4	Sample #5	Sample #6	Sample #7	Sample #8
B												
C												
D												
E												
F												
G												
H												

Figure 2A:

***pfcrt* gene codons 72, 74, 75, 76**												
Master Mix Preparation:												
External PCR (25ul):												
					Samples							
	Components		**Volume (µl)**	**final con.**	**1**		**External PCR Cycling Conditions:**					
							Hotstart	95°C	15 min			
	Water		17		17		Denaturation	94°C	30 sec			
	10x buffer	**10X**	2.5	1X	2.5		Annealing	45°C	45 sec	**40 cycles**		
	dNTP	**(25mM)**	0.2	(0.2mM)	0.2		Extension	72°C	1 min			
	$MgCl_2$	**(25mM)**	2	(2mM)	2		Final Extension	72°C	10 min			
	Primer pfcrt 76EF	**(5µM)**	**1**	(0.2µM)	1			4°C	HOLD			
	Primer pfcrt 76ER	**(5µM)**	**1**	(0.2µM)	1							
	Hotstar Taq Polymerase	**(5U/µl)**	0.3		0.3							
	DNA		1		**1**							
			25		**25**							
	Aliquot 24ul of Mastermix to positive controls and 25ul to negative control											
Internal PCR (25ul)												
					Samples							
	Components		**Volume (µl)**	**final con.**	**1**		**Internal PCR Cycling Conditions:**					
							Hotstart	95°C	15 min			
	Water		11		11		Denaturation	94°C	30 sec			
	10x buffer	**10X**	2.5	1X	2.5		Annealing	45°C	45 sec	**25 cycles**		
	dNTP	**(25mM)**	0.2	(0.2mM)	0.2		Extension	72°C	1 min			
	$MgCl_2$	**(25mM)**	2	(2mM)	2		Final Extension	72°C	10 min			
	Primer PCR F1	**(5µM)**	**4**	(0.8µM)	4			4°C	HOLD			
	Primer PCR R1	**(5µM)**	**4**	(0.8µM)	4							
	Hotstar Taq Polymerase	**(5U/µl)**	0.3	0.04 U/ul)	0.3							
	1º PCR product		1		**1**							
			25		**25**							
	Aliquot 24ul of Mastermix to positive controls and 25ul to negative control											

Figure 2B:

pfcrt gene codons 220, 271																	
Master Mix Preparation:																	
External PCR (25ul):																	
					Samples												
Components		Volume (µl)		final con.	1		External PCR Cycling Conditions:										
							Hotstart	95°C	15 min								
	Water		18		18		Denaturation	94°C	30 sec								
	10x buffer	10X	2.5	1X	2.5		Annealing	67°C	45 sec	(-0.5C per cycle)		10 cycles (Touch-down)					
	dNTP	(25mM)	0.2	(0.2mM)	0.2		Extension	72°C	1 min								
	$MgCl_2$	(25mM)	2	(2mM)	2		Denaturation	94°C	30 sec								
Primer 220EF		(10µM)	0.5	(0.2µM)	0.5		Annealing	62°C	45 sec	20 cycles							
Primer 220ER		(10µM)	0.5	(0.2µM)	0.5		Extension	72°C	1 min								
Hotstar Taq Polymerase		(5U/µl)	0.3		0.3		Final Extension	72°C	10 min								
	DNA		1		1			4°C	HOLD								
			25		25												
	Aliquot 24ul of Mastermix to positive controls and 25ul to negative control																
Internal PCR (25ul)																	
					Samples												
Components		Volume (µl)		final con.	1		Internal PCR Cycling Conditions:										
							Hotstart	95°C	15 min								
	Water		18		18		Denaturation	94°C	30 sec								
	10x buffer	10X	2.5	1X	2.5		Annealing	62°C	45 sec	20 cycles							
	dNTP	(25mM)	0.2	(0.2mM)	0.2		Extension	72°C	1 min								
	$MgCl_2$	(25mM)	2	(2mM)	2		Final Extension	72°C	10 min								
Primer 220IF		(10µM)	0.5	(0.2µM)	0.5			4°C	HOLD								
Primer 220IR		(10µM)	0.5	(0.2µM)	0.5												
Hotstar Taq Polymerase		(5U/µl)	0.3	0.04 U/ul)	0.3												
1° PCR product			1		1												
			25		25												
	Aliquot 24ul of Mastermix to positive controls and 25ul to negative control																

Figure 2C:

***pfcrt* gene codons 326, 356, 371**															
Master Mix Preparation:															
External PCR (25ul):															
					Samples										
	Components		**Volume (µl)**	**final con.**	**1**		**External PCR Cycling Conditions:**								
							Hotstart	95°C	15 min						
	Water		18		18		Denaturation	94°C	30 sec						
	10x buffer	**10X**	2.5	1X	2.5		Annealing	62°C	45 sec	(-0.5C per cycle)			**10 cycles (Touch-down)**		
	dNTP	**(25mM)**	0.2	(0.2mM)	0.2		Extension	72°C	1 min						
	$MgCl_2$	**(25mM)**	2	(2mM)	2		Denaturation	94°C	30 sec						
	Primer 220EF	**(10µM)**	**0.5**	(0.2µM)	0.5		Annealing	57°C	45 sec	**20 cycles**					
	Primer 220ER	**(10µM)**	**0.5**	(0.2µM)	0.5		Extension	72°C	1 min						
	Hotstar Taq Polymerase	**(5U/µl)**	0.3		0.3		Final Extension	72°C	10 min						
	DNA		1		1			4°C	**HOLD**						
			25		**25**										
	Aliquot 24ul of Mastermix to positive controls and 25ul to negative control														
Internal PCR (25ul)															
					Samples										
	Components		**Volume (µl)**	**final con.**	**1**		**Internal PCR Cycling Conditions:**								
							Hotstart	95°C	15 min						
	Water		18		18		Denaturation	94°C	30 sec						
	10x buffer	**10X**	2.5	1X	2.5		Annelaing	57°C	45 sec	**20 cycles**					
	dNTP	**(25mM)**	0.2	(0.2mM)	0.2		Extension	72°C	1 min						
	$MgCl_2$	**(25mM)**	2	(2mM)	2		Final Extension	72°C	10 min						
	Primer 220IF	**(10µM)**	**0.5**	(0.2µM)	0.5			4°C	HOLD						
	Primer 220IR	**(10µM)**	**0.5**	(0.2µM)	0.5										
	Hotstar Taq Polymerase	**(5U/µl)**	0.3	0.04 U/ul)	0.3										
	1º PCR product		1		1										
			25		**25**										
	Aliquot 24ul of Mastermix to positive controls and 25ul to negative control														