

PROTOCOL FOR MSP-2 GENOTYPING

1.0 Purpose

This protocol provides a method for performing PCR on genomic DNA extracted from dried blood spots on Whatman 3MM filter paper for subsequent genotyping of *Plasmodium falciparum* polymorphic genes encoding merozoite surface protein 2 (MSP-2) by performing fragment analysis using multicolored capillary electrophoresis on an automated DNA sequencer or the QIAxcel capillary electrophoresis instrument.

2.0 References

- 2.1 Cattamanchi, A. et al. "Protocol for Genotyping: Merozoite Surface Protein 2 (MSP-2)", July 2005.
- 2.2 Eisen, D. et al. "Temporal Variation of the Merozoite Surface Protein-2 Gene of *Plasmodium falciparum*", Jan. 1998, p. 239-246.
- 2.3 Protocol developed using DNA Engine Tetrad 2 Thermocycler for PCR (Bio-Rad Laboratories, Inc.)
- 2.4 Handbooks for Qiagen QIAamp DNA mini kit or DNA 96 Blood kit (optional, for processing large number of specimens) (or comparable DNA extraction kits)
- 2.5 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 Taq DNA Polymerase [includes 50 mM MgCl₂ and 10X reaction buffer (-MgCl₂)] (Invitrogen cat# 10342-020)
- 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 and HB3) (MR4 cat#s MRA-102G and MRA-155G respectively, or equivalent)
Note: Control DNA must be diluted to 25 pg/μL in water prior to use.
- 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 Kit OR High Resolution Kit (Qiagen cat# 929004 OR 929002)
Note: High Resolution Kit is preferred.
- 3.14 E-Gel® 96 2% with SYBR® Safe (Invitrogen cat# G7208-02)
- 3.15 PCR Primers (see Table 1).

Note: If analysis is to be conducted using QIAxcel capillary electrophoresis only, the labeled primers (HEX and FAM, Table 1) used in the internal (secondary) PCR reaction may be substituted with the same primer without the label attached).

- 3.16 HiDi™ Formamide (for Genescan analysis on ABI DNA sequencer) (Applied Biosystems cat# 4311320)
- 3.17 GeneScan™ 600 LIZ® Size Standard (for Genescan) (Applied Biosystems cat# 4366589)
- 3.18 50 bp DNA ladder (Invitrogen cat# 10416-014)
- 3.19 QIAxcel QX Alignment marker 15 bp/1 Kb (Qiagen cat#929521)
- 3.20 QIAxcel QX size marker 50-800 bp (Qiagen cat# 929556)

4.0 Equipment:

- 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 Applied Biosystems 3730XL high-throughput 96-capillary DNA sequencer and ABI software and GeneMapper® software (for allele sizing) (Applied Biosystems).
- 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 proper lab etiquette 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.
- 5.9 Store extracted or amplified DNA at -80 °C (2-8 °C storage is allowed for ≤ 24 hours; -20 °C storage is allowed for ≤ 2 weeks).

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 QIAMP 96 plate + S-block at 2608rcf 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.

6.2 Setting up PCR Reactions:

Important: Secondary PCR (Internal primers) is set up in two separate plates or tubes – one for IC/3D7 allelic family and one for FC27 allelic family.

6.2.1 Use Excel spreadsheet template(**Figure 1**)for setting up PCR Master Mixes for the external (primary) and internal (secondary) PCR reactions and the thermocycler cycling 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. A representative plate is shown in(**figure 1**).

Note: Make sure to include 3D7 and HB3 control DNA for the IC/3D7 and FC27 allelic types, respectively, and negative control (no DNA control).

6.2.4 Calculate the volume of Master mixes to make according to the spreadsheet in (**Figure 2**).

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

(2) If you need to add more DNA, reduce an equal amount of water in the master mix.

(3) You can type the total number of samples that need to undergo PCR into the cell highlighted yellow in the spreadsheet(s) 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 Seal the plate with ThermoSeal plate sealer and place it in the PCR thermocycler.

6.2.7 Set up the PCR cycling conditions on the thermocycler as shown in the spreadsheets in(**Figure 2**) and start the PCR reaction.

6.2.8 Successful amplification can be confirmed by running the nested PCR product together with a 50 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 (optionally, a 2% agarose gel stained with ethidium bromide can also be used).

6.2.9 If amplification is successful, prepare secondary PCR products for multiplexed fragment analysis on the ABI multicolored capillary sequencer or the QIAxcel capillary electrophoresis instrument as follows:

6.2.9.1 ABI multicolored capillary sequencer analysis:

6.2.9.1.1 Prepare a 1:10 dilution of the secondary PCR product in water by adding 1 µL of DNA from EACH plate (IC/3D7 and FC27) to 8 µL water.

6.2.9.1.2 For each sample to be analyzed, take 1 µL of the diluted DNA (from step 6.2.9.1) and mix with 0.5 µL of Genescan™ 600_LIZ(R) Size Standard and 8.5 µL of HiDi® Formamide and submit this sample for capillary electrophoresis on the Applied Biosystems 3730XL Capillary DNA Sequencer.

6.2.9.1.3 Use the ABI Genemapper® Software version 4.0 for scoring the peaks for the IC/3D7 alleles (Green peaks) and the FC27 alleles (Blue peaks).

6.2.9.1.4 The 3D7 control should give a single green peak with an expected size of 262 bp. The HB3 control should give a single blue peak with an expected size of 370 bp.

Note: To reduce fluorescent artifacts (such as stutter peaks and non-specific low background peaks often seen with samples containing high parasite densities e.g. with the 3D7 and HB3 control DNA), set a cut-off value for the rfu's (relative fluorescent units) such that it allows for the true peak while excluding other minor artifact peaks. **Apply the same cut-off to all the samples in the run.**

6.2.9.2 QIAxcel analysis:

6.2.9.2.1 Use the QIAxcel DNA High Resolution kit (recommended for higher band resolution) or the Screening kit.

6.2.9.2.2 Use the QX Alignment marker 15 bp/1 Kb (Qiagen cat#929521) and the QX size marker 50-800 bp (Qiagen cat# 929556).

6.2.9.2.3 Run the secondary PCR products from step 6.2.7.

6.2.9.2.4 The 3D7 control should give a single band of 262 bp. The HB3 control should give a single band of 370 bp.

Note: (1) Electropherograms of the resultant peaks can be examined and analyzed using the Biocalculator software that comes with the QIAxcel instrument. Peaks that look like artifacts (seen with some samples containing high parasite densities or non-specific background DNA amplification) can be reduced by increasing the positive threshold value in the parameter setup dialog box (refer to the QIAxcel User Manual). **Apply the same threshold value to all the samples in the run.**

(2) Resulting band sizes from QIAxcel analysis can be copied into an Excel file that allows for objective analysis and comparison of bands between any two episodes under investigation for both IC/3D7 and FC27 families (Refer to the QIAxcel User Manual).

6.2.9.3 Interpretation:

6.2.9.3.1 IC/3D7 alleles range in size from 170 to 510 bp and FC27 alleles range in size from 280 to 590 bp (Cattamanchi et al; Am J of Trop Med; 2003)

6.2.9.3.2 Alleles belonging to IC/3D7 family show a central repeat region made up of variable numbers of 12 to 24 bp repeats separated by 6 bp sequences. The FC27 family members have variants of a central 96 bp sequence that may be present in one to four copies followed by a 21 bp partial repeat and a variably represented 36 bp sequence that may be present in one to five copies (Eisen et al; Infection and Immunity; Jan. 1998, p. 239-246).

6.2.9.3.3 For each PCR product, bands (i.e. alleles) that are within 10bp of each other are considered the same.

6.2.9.3.4 Presence of at least one allele in the PCR product from the failure day that is within 10 bp of the allele in the PCR product from the initial day of infection is classified as a recrudescence infection.

6.2.9.3.5 Absence of alleles from the failure day that are within 10 bp of the alleles from the initial infection constitutes a new infection.

Table 1 (PCR primers)

Primer name	Sequence (5'→3')	Purpose
MSP-2(1)	ATGAAGGTAATTTAAAACATTGTCTATTATA	External forward primer
MSP-2(4)	ATATGGCAAAGATAAAAACAAGTGTTGCTG	External reverse primer
MSP-2(A1)	HEX-CAGAAAGTAAGCCTTCTACTGG	Internal forward primer (IC3D7)
MSP-2(A2)	GATTTGTTTCGGCATTATTATGA	Internal reverse primer(IC3D7)
MSP-2(B1)	FAM-CAAATGAAGGTTCTAATACTA	External forward primer (FC27)
MSP-2(B2)	GCTTTGGGTCCTTCTTCAGTTGATTC	Internal reverse primer (FC27)

Figure 1 (Plate Template)

	1	2	3	4	5	6	7	8	9	10	11	12
A	3D7 Ctrl (IC/3D7)	HB3 Ctrl (FC27)	Neg Ctrl (PCR)	Sample Extraction Buffer	Sample #1 (Episode #1)	Sample #1 (Episode #2)	Sample #2 (Episode #1)	Sample #2 (Episode #2)				
B												
C												
D												
E												
F												
G												
H												

Figure 2 (PCR set-up and cycling conditions)

<u>MSP-2 PCR SET-UP FOR GENOTYPING IC/3D7 AND FC-27 ALLELIC FAMILIES:</u>							
Master Mix Preparations:							
External (primary) PCR (25 µL):							
MSP-2 (1): 5' - ATGAAGGTAATTAACATTGTCTATTATA -3'							
MSP-2 (4): 5' - ATATGGCAAAAGATAAAACAAGTGTGGCTG - 3'							
					External PCR Cycling Conditions:		
Components		Volume (µl)	final conc.	tubes 1			
					Initial Denaturation	94° C	5 min
					Denaturation	94° C	90 sec
					Annealing	55° C	45 sec
					Extension	72° C	90 sec
					Final Extension	72° C	10 min
						4° C	HOLD
Water		18.4		18.4	} x 40 cycles		
10x buffer		2.5		2.5			
dNTP (25mM)		0.2	(0.2mM)	0.2			
MgCl ₂ (50mM)		0.8	(1.5mM)	0.8			
Forward Primer MSP2-1	(10µM)	0.5	(0.2µM)	0.5			
Reverse Primer MSP2-4	(10µM)	0.5	(0.2µM)	0.5			
Taq Polymerase	(5U/µl)	0.2	(0.04U)	0.2			
DNA		2.0		2.0			
		25		25			
Internal (secondary) PCR (25 µL/ Plate):							
Plate 1 (IC3D7):							
MSP-2 (A1): 5' - 5HEX-CAGAAAGTAAGCCTTCTACTGG - 3'					} approx. 260 bp product		
MSP-2 (A2): 5' - GATTGTTCGGCATTATTATGA - 3'							
					Internal PCR Cycling Conditions (Plates 1 & 2):		
Components		Volume (µl)	final conc.	# tubes 1			
					Initial Denaturation	94° C	2 min
					Denaturation	94° C	90 sec
					Annealing	55° C	45 sec
					Extension	72° C	90 sec
					Final Extension	72° C	10 min
						4° C	HOLD
Water		18.4		18.4	} x 30 cycles		
10x buffer		2.5		2.5			
dNTP (25mM)		0.2	(0.2mM)	0.2			
MgCl ₂ (50mM)		0.8	(1.5mM)	0.8			
Forw. Primer MSP-2 (A1)	(10µM)	0.5	(0.2µM)	0.5			
Rev. Primer MSP-2 (A2)	(10µM)	0.5	(0.2µM)	0.5			
Taq Polymerase	(5U/µl)	0.2	(0.04 U)	0.2			
Primary PCR product		2.0		2.0			
		25		25			
Plate 2 (FC27):							
MSP-2 (B1): 5' - 6FAM-CAAATGAAGGTTCTAATACTA - 3'					} approx. 370 bp product		
MSP-2 (B2): 5' - GCTTTGGGTCCTTCCTCAGTTGATTC - 3'							
Components		Volume (µl)	final conc.	# tubes 1			
Water		18.4		18.4			
10x buffer		2.5		2.5			
dNTP (25mM)		0.2	(0.2mM)	0.2			
MgCl ₂ (50mM)		0.8	(1.5mM)	0.8			
Forw. Primer MSP-2 (B1)	(10µM)	0.5	(0.2µM)	0.5			
Rev. Primer MSP-2 (B2)	(10µM)	0.5	(0.2µM)	0.5			
Taq Polymerase	(5U/µl)	0.2	(0.04 U)	0.2			
Primary PCR product		2.0		2.0			
		25		25			