PROTOCOL FOR 18srRNA REAL TIME QPCR

1.0 Purpose

This protocol provides a method for performing Real-time PCR assay targeting *P. falciparum* 18s ribosomal RNA (18srRNA) gene using genomic DNA extracted from dried blood spots on Whatman 3MM filter paper.

2.0 References

2.1 Cornelus C. Hermsen, Denise S.C. Telgt, Ellen H.P. Linders, Louis A.T.F. van de Locht, Wijnand M.C. Eling, Ewald J.B.M. Mensink, Robert W. Sauerwein. Detection of Plasmodium falciparum malaria parasites in vivo by real-time quantitative PCR. Molecular & Biochemical Parasitology 118 (2001) 247–251.

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 Manual DNA Extraction Protocol for Dried Blood Spot Filter papers DNA Extraction (Modified for use with QIAGEN QIAamp DNA 96 Blood Kit, Cat #s: 51161 OR 51162) (This protocol can be found at: http://medschool.umaryland.edu/malaria/)

2.4 Applied Biosystems 7300 Real Time PCR System User Manual (Applied Biosystems, Foster City, CA, USA)

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 TagMan® Universal PCR Master Mix (Applied Biosystems cat# 4304437)

3.3 MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems; cat# N801-0560)

3.4 MicroAmp™ Optical Adhesive Film (Applied Biosystems; cat# 4311971)

3.5 RNAse and DNAse Free Water (Gibco cat# 15230-147 or comparable)

3.6 Control DNA (3D7) (MR4 cat# MRA-102G)
Note: Prepare a standard curve by making dilutions of 3D7 or NF54 control DNA in water, for example: 3.0 ng/mL, 1.5 ng/mL, 0.75 ng/mL, 0.375 ng/mL, 0.1875 ng/mL, 0.09375 ng/mL, 0.046875 and 0 ng/mL (no DNA).

3.7 1.5 mL polypropylene centrifuge or 15mL conical tubes for master mix (depending on number of samples)

3.8 Scissors (or razors) (for cutting strips of filter paper with dried blood spots)

3.9 70% alcohol (Ethanol or Isopropanol)

3.10 PCR primers and probe **(see Table 1)**

4.0 Equipment:

4.1 Water baths

- 4.1.1 85 °C, 56 °C and 70 °C if using with QIAamp DNA mini Kit procedure for DNA extraction
- 4.1.2 OR 56°C incubator if performing manual extractions using QIAamp DNA 96 Blood Kit (Modified version)

4.2 Micro centrifuge (Beckman Coulter microfuge 16) or table top centrifuge (Eppendorf 5810R with Eppendorf A-2-DWP Centrifuge head), as appropriate

4.3 Applied Biosystems 7300 or 7500 Real Time PCR System (and Software)

4.4 BioRobot® Universal automated system (Qiagen) (optional; for processing very large number of specimens)

4.5 Calibrated 8 or 12 well multichannel pipets (2-20 µL and 20-200 µL) (Matrix or comparable) and aerosol resistant pipette tips

4.6 Calibrated Micropipets (10 µL, 20 µL & 1000 µL) (Matrix EDP Plus or comparable) and aerosol resistant pipette tips
4.7 Refrigerator (2-8 °C) and Freezer (-20°C and/or -80 °C, for long tern storage of DNA)
4.8 Ice bath (for setting up master mix & PCR reactions)

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 Take utmost care during pipetting steps to prevent cross contamination of samples or PCR products.
5.5 Use an ice bath to hold the PCR master mix and/or PCR reagents during PCR master mix preparation.
5.6 Do not vortex the PCR mix. Mix gently. Use light centrifugation to collect all liquid to the bottom of the PCR plate or tube.
5.7 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 in conjunction with the Biorobot® Universal System (for larger numbers of specimens). Follow instructions in the respective manuals when using these kits.
6.1.3 Alternately, a modified protocol has also been development for manual extraction of large numbers of specimens using the Qiagen QIAamp 96 Blood Kit (step 2.3 under References section).

6.2 Setting up PCR Reactions:

6.2.1 Use information on the Excel spreadsheet template **(Figure 2)** for setting up PCR reactions and the thermocycler cycling conditions.
6.2.2 Determine the number of samples (n) you need to amplify.
6.2.3 Use a MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems; cat# N801-0560). A representative plate is shown in **(Figure 1).**
6.2.4 Calculate the volume of master mix 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 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 PCR master mix.

6.2.5 Add 24 µL of master mix to standard curve wells of the 96 well plate and 22.5 uL of master mix to sample wells.

6.2.6 Add 1 µL DNA standard curve dilutions in duplicate wells. Add 2.5 µL of test DNA sample to appropriate duplicate wells.

6.2.7 Seal the plate with MicroAmp™ Optical Adhesive Film (Applied Biosystems; cat# 4311971) and place it in the ABI Real Time PCR System.

6.2.8 Set up the PCR cycling conditions on the ABI System as shown in the spreadsheet in **(Figure 2)** and start the PCR reaction.

Note: (1) Refer to the ABI Real Time PCR System user manual for instructions on using the instrument and software.
(2) Ensure that the correct detectors (FAM/None) are selected in the ABI System software.

6.2.9 Obtain and record the Ct (cycle to thresh-hold) counts on the standard curve and unknown samples.

6.3 Interpretation:

6.3.1 Use the ABI Real Time PCR System software to calculate the threshold and ignore the background. The software will then calculate the quantity of parasite DNA present based on the standard curve run alongside the samples. If samples are run in replicate, the mean and standard deviation will also be calculated between replicates.

6.3.2 Cycling curves may also be reviewed to look for aberrant amplification. Curves should follow a sigmoid trajectory in parallel to the standard curve.

6.3.3 Samples with Ct values outside of the range of the standard curve or with abnormal amplification curves may be repeated with or without increasing the amount of DNA added to the reaction.

Table 1 (LDH PCR primers)

Primer name	Sequence (5'→3')	Purpose
18sTAQF	5'- GTA ATT GGA ATG ATA GGA ATT TAC AAG GT -3'	Forward primer
18sTAQR	5'- TCA ACT ACG AAC GTT TTA ACT GCA AC -3'	Reverse primer
18sProbe	5'- FAM GAA CGG GAG GTT AAC AA MGB-3'	FAM/MGB Probe

Figure 1 (Plate Template)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 3.0 ng/uL	Std 3.0 ng/uL	sample 1	sample 1								
B	Std 1.5 ng/uL	Std 1.5 ng/uL	sample 2	sample 2								
C	Std 0.75 ng/uL	Std 0.75 ng/uL										
D	Std 0.375 ng/uL	Std 0.375 ng/uL										
E	Std 0.1875 ng/uL	Std 0.1875 ng/uL										
F	Std 0.09375 ng/uL	Std 0.09375 ng/uL										
G	Std 0.046875 ng/uL	Std 0.046875 ng/uL										
H	Std 0 ng/uL	Std 0 ng/uL										

Figure 2 (PCR set-up and cycling conditions)

REAL TIME PCR SET-UP FOR 18srRNA:

PRIMERS FOR PCR:

18sTAQF	5'- GTA ATT GGA ATG ATA GGA ATT TAC AAG GT -3'
18sTAQR	5'- TCA ACT ACG AAC GTT TTA ACT GCA AC -3'
18sProbe	5'- FAM GAA CGG GAG GTT AAC AA MGB-3'

PCR Program

1. 95°C 15 Minutes
2. 95°C 15 seconds
3. 60°C 60 seconds (Data Collection)
4. Repeat to step 2, 45 times

Reaction Setup:

1 Plate:

Per Reaction		Rxn #	Master Mix	
0.75	uL 18sTAQF (10uM)	100	75.00	uL 18sTAQF (10uM)
0.75	uL 18sTAQR (10uM)		75.00	uL 18sTAQR (10uM)
0.50	uL 18sProbe (10uM)		50.00	uL 18sProbe (10uM)
12.50	uL 2x Mastermix		1250.00	uL 2x Mastermix
8.00	uL Water		800.00	uL Water
2.5	uL DNA			
25.00	uL Reaction			

3 Plates:

Per Reaction		Rxn #	Master Mix	
0.75	uL 18sTAQF (10uM)	300	225.00	uL 18sTAQF (10uM)
0.75	uL 18sTAQR (10uM)		225.00	uL 18sTAQR (10uM)
0.50	uL 18sProbe (10uM)		150.00	uL 18sProbe (10uM)
12.50	uL 2x Mastermix		3750.00	uL 2x Mastermix
8.00	uL Water		2400.00	uL Water
2.5	uL DNA			
25.00	uL Reaction			

7. Version History

Version	Type of Revision	Effective Date
1.0	Creation of SOP	30 January 2015