DETECTION OF PLASMODIUM INFECTION BY NESTED POLYMERASE CHAIN REACTION ASSAY

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Abstract

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Protocol

Step 1.

1. Scope and Applicability

This SOP applies to the MDEE platform and subsequently to members belonging to the platform who are involved in the management and conduct of laboratory work of malaria diagnosis within the MRC Unit. The SOP accordingly applies to Scientific Officers and laboratory technicians performing the work.

Step 2.

10. References

Georges Snounou & Suganya Viriyakosol (1993). High sensitivity of detection of malaria parasites by the use of nested polymerase chain reaction. Molecular and Biochemical Parasitology, 61, 315.

Mathieu Rougemont & Madeleine Van Saanen (2004). Detection of Four Plasmodium Species in Blood from Humans by 18SrRNA Gene Subunit-Based and Species-Specific Real-Time PCR Assays. Journal of clinical microbiology, 42, 5636.
Assay MDEE.. Detection of Plasmodium infection using Nested PCR Assay

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Malaria diagnosis using PCR is based on the amplification of the malaria parasite DNA sequences. This is usually conducted using a nested PCR which involves two rounds of DNA amplification reactions targeting the 18S rRNA gene. The 18S rRNA gene contains multiple copies and harbours both genus and species-specific variations utilised in the design of differentiation assays (Snounou et al. 1993, Mol Biochem Parasitol 61: 315-20; Rougemont et al., J. Microbiol 42: 5636-5643). The technique therefore aids in the diagnosis of malaria infection through the identification of Plasmodium species present in samples.

Step 3.

3. Prerequisites

3.1 Anti-contamination considerations

- DNA extraction, Pre PCR set-up (mastermix preparation), and post-PCR procedures should be performed in separate rooms as outlined by the Molecular Diagnostics Platform Himsworth block work guidelines.
- Benches, work stations, centrifuges, vortexes, and pipettes must be cleaned with 10% bleach and 70% ethanol before and after every PCR set-up as outlined in the Molecular Diagnostics Platform decontamination of work benches SOP (SOP-MMT-002, Version 1.0).
- Gloves should be changed regularly, particularly after preparation and before the dispensing of master mixes into PCR plates or strips to avoid cross contamination.
- It is recommended that aerosol resistant/ filtered tips and dedicated pipettes in the pre-PCR rooms (tagged blue) are used.

Step 4.

3.2 Preparation and storage of reagents

3.2.1 Stock reagents for PCR (e.g. Primers at 100mM, dNTP nucleotides for dNTPs) and enzymes should be stored at -20°C.

- Stock reagents should be diluted into working concentrations and aliquoted into separate tubes.
- Working dilutions of PCR reagents (10X buffer, dNTPs, MgCl2, primers at 10µM) should be stored in the -20°C freezer for long term storage (i.e. over one week) but can be stored in the fridge if in frequent use and retrieved for use when required.
- Working aliquots of sample and control DNA as well as primary PCR (Nest-1) products must be kept at -20°C for long term storage but if in daily use can be kept in the fridge.
- Working aliquots of PCR reagents must be discarded after opening several times (e.g. over 10 times).

Step 5.

3.3 Pipeline of work

3.3.1 The malaria PCR is performed on DNA samples. For sample reception, dried blood spot punching and DNA extraction, please refer to the relevant SOPs.
3.3.2 It is important that PCR assays are performed in specified order.
3.3.3 Genus specific identification should be carried out first on samples.
3.3.4 Plasmodium positive samples should be genotyped for P. falciparum species only or for all other species (P. vivax, P. ovale, and P. malariae), to determine mixed infections, as required by a project/ study.
3.3.5 Where required, all Plasmodium positive but P. falciparum negative samples, if only P. falciparum was initially genotyped, should be genotyped for other Plasmodium species (P. vivax, P. ovale, and P. malariae).
3.3.6 After all PCR runs as required by a study, a table scoring the results should be generated in an excel sheet.
PCR scoring should be done by two independent readers. Where there is a discrepancy in scoring between the first and second reader, a third reader (ideally the supervisor) is required to settle the discrepancy

Step 6.

4. Safety Precautions

4.1.0 The procedures outlined should be carried out in accordance with MRC Unit, The Gambia Health and Safety guidelines described in SOP-H&S-001.
4.1.1 In case of any need for advice on H&S, consult the laboratory H&S representative.
4.1.2 Gloves must be worn at all stages of the procedures outlined in the SOP to avoid infection hazards from blood samples and contaminating the samples with finger DNases/RNases.
4.1.3 Handle all reagents with care to avoid infections.
4.1.4 Protect face and eyes with a screen and visor when using UV light. Where possible, leave the room when UV light is in use.
Ethidium Bromide (EtBr) is a mutagen and should therefore be handled and disposed of with utmost care when used.

Step 7.

5. Equipment and Materials

1. Trough
2. Pipette
3. 10µl presterilized filter tips
4. 20µl presterilized filter tips
5. 200µl presterilized filter tips
6. 1000µl presterilized filter tips
7. 96 well plate
8. Plate seal

Step 8.

6. Reagents and Chemicals

1. Nuclease free H2O
2. Buffer (thermopol)
3. dNTPs
4. Taq polymerase
5. DNA
6. Forward and reverse primers

Step 9.

7. Procedure

7.1.0 Sample storage and punching of dried blood spots (DBS)
7.1.1 This should be done in accordance to the SOP document for DBS punching
7.1.2 DNA extraction
7.1.3 For DNA extraction, use the DNA extraction robot or manual extraction as specified by study and following the respective protocols
7.1.4 Store eluted DNA at -20°C until analysis is required.
7.1.5 It is recommended that a 20µl aliquot of DNA for each sample is transferred into a well in a sterile 96-well plate or eppendorf tube as appropriate for use in analysis (diagnostic PCR and speciation).
7.1.6 The remaining stock of the extracted DNA should be taken for long term storage immediately after extraction and before PCR begins

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Step 10.

7.2 **Summary of PCR**

7.2.1 Primary PCR/ Nest-1 PCR should be done using rPLU5 + rPLU6 primers.

7.2.2 For each plate include a negative control of PCR mix without a DNA template.

7.2.3 If required, include positive controls for falciparum, ovale, vivax and malariae species.

7.2.4 Nest-2 PCR to determine the presence of Plasmodium infection should be done using the genus-specific primers Plasmo1 + Plasmo 2.

7.2.5 Presence of Plasmodium falciparum species is determined by running a Nest-2 PCR for all plasmodium positive samples using species-specific primers rFAL 1 + rFAL 2.

7.2.6 If required to determine presence of other species from Plasmodium falciparum negative samples, run Nest-2 PCR assays using other species-specific primers

7.2.7 For the determination of the different Plasmodium species present in samples, use the species-specific primers indicated below in the PCR mastermix (mixes prepared separately):

Plasmodium falciparum use rFAL 1 + rFAL 2

Plasmodium ovale use rOVA 1 + rOVA 2

Plasmodium vivax use rVIV 1 + rVIV 2

Plasmodium malariae use rMAL 1 + rMAL 2

7.2.8 Note the PCR programme name and thermal cycler machine number (e.g. #B.....) and block number (in the case of the tetrad) used for the PCR reaction in your lab book.

7.2.9 Once the PCR run is complete, remove plate and briefly centrifuge to spin down products.

7.2.10 Store PCR plates in the fridge or freezer

7.2.11 Run the plate on the QIAxcel advanced system to determine if the PCR has worked and PCR fragment size. Alternatively samples can be run on Agarose gel electrophoresis using Ethidium Bromide.

7.2.12 Score results as presence or absence of bands comparing with control bands. Scoring should be done either by two independent readers, or by one reader and automated binary scoring using the QIAxcel screen gel software. Discrepancies are settled with a third read (ideally by the supervisor). Unresolved discrepancies should be repeated.

Step 11.

7.3 PCR Protocol

7.3.1 For each plate, eight controls (4 positive and 4 negative) should be included for punching in addition to the 80 samples. The controls should be randomised throughout the plate at the punching stage. Additional PCR controls (3D7 extracted DNA) should be included at the stage of PCR.

7.3.2 Prepare PCR master mix calculating the volume enough for number of samples +10 to account for pipetting loss and dead volume (left in the reaction container). E.g. for a whole plate of 88 samples (88DBS samples + 8 pcr controls), make a master mix for 105 samples.

7.3.3 For pre bar-coded samples scanned into item tracker in a plate plan format, print the plate plan and stick in the lab book with date, experiment, target gene for amplification, Nest-1 or Nest-2, PCR master mix recipe, thermal cycler machine number and block number as appropriate.

For samples without bar-codes prepare a plate plan with the above mentioned details in the lab book.

Step 12.

7.4 **Preparation of PCR Mastermix**

7.4.1 Prepare mastermix in a clean, DNA free area, preferably in a sterile hood with lamina flow or designated areas. Use only pre- PCR pipettes (tagged blue).

7.4.2 Disinfect pipettes and benches or hood before preparing the mastermix with 10% bleach followed by 70% ethanol.

7.4.3 Thaw, vortex briefly and spin down all reagents, except Taq polymerase enzyme. Leave Taq polymerase in freezer until all other reagents have been added.

7.4.4 Add PCR mastermix reagents together in a clean trough in the order listed in the mastermix in the tables below.

7.4.5 Mix mastermix by pipetting gently up and down several times with a P1000 pipette tip. Avoid foaming by tilting the trough slightly with the pipette tip fully immersed.

7.4.6 Label sterile PCR plates with study name initials (e.g. IM for Infant Malaria)/Plate number/primer name/PCR/ date and initials of the operator.

7.4.7 Dispense mastermix into sterile PCR plates according to plate plan. Add 11µl of master mix into each of the wells for primary/Nest-1 reactions and 14µl for Nest-2 reactions. Dispense 15µl into wells A12, B12, and C12 as PCR negative controls.

7.4.8 Briefly spin down DNA or Nest-1 products before opening (quick spin at maximum speed).

7.4.9 Add DNA (4µl) or Nest-1 product (1µl) to the relevant sample location according to plate plan. Seal plate carefully, spin down and place in a PCR machine to start the appropriate PCR run and cycling conditions (as indicated below).

7.4.10 After the PCR has finished, remove and spin plate briefly. Store in a fridge or at -20°C for further use for Nest-1 (to run a Nest-2 reaction) or proceed to QIAxcel for reading Nest-2 reaction product (SOP_MDX_QIAxcel operations guide/ QIAxcel DNA Hand book).

7.4.11 Sample IDs from the Item Tracker software can be exported and converted into XML format, using the dedicated software. This XML file of the plate plan can then be uploaded into the QIAxcel, therefore removing the need to transcribe IDs.

Step 13.

8. Calculations and Data Reporting (As applicable)

To Calculate concentrations and volumes, use the formula C1V1 = C2V2, transforming as appropriate e.g. V1 = C2V2/C1

Where
C1 = starting concentration
V1 = starting volume

C2 = final concentration
V2 = final volume

Always convert to the same units for concentrations (e.g. mM) and volumes (e.g. l, ml, µl etc.) on both sides of the equation.

Step 14.

Outer PCR (Primary/ Nest-1) 15µl

	Stock Conc.	Final Conc.	Initial Volume X1 (µl)	Final Volume 98X(µl)
Nuclease free H_2O			7.88	772.24
Buffer (Thermopol)		1X	1.5	147
rPLU5		0.4µM	0.6	58.8
rPLU6		0.4µM	0.6	58.8
dNTPs		10mM	0.3	29.4
Taq polymerase	5U/µl		0.12	11.76
DNA			4	

Inner PCR (Nest-2) 15µl

	Stock Conc.			
Nuclease free H_2O				
Buffer (Thermopol)		1X	1.5	147
Primer 1		0.3µM	0.45	44.1
Primer 2		0.3µM	0.45	44.1
dNTPs		10mM	0.3	29.4

Taq polymerase	5U/µl	0.12	11.76
DNA		1	

Please note, Primer 1 and Primer 2 can be the Plasmodium specific primers (Plasmo 1 and 2) or the species specific primers (falciparum, malariae, ovale and vivax) depending on the required assay.

Sequences of 18s rRNA primers for plasmodium PCR

Gene	Primers		Sequence	Product size
18S rRNA	Nest-1 forward rPLU5		5′-CCTGTTGTTGCCTTAAACTTC-3′	1,200 bp
	Nest-1 reverse rPLU6		5′-TTAAAATTGTTGCAGTTAAAACG-3′	
	Nest-2 forward Plasmo 1		5′-GTTAAGGGAGTGAAGACGATCAGA-3′	157–165 bp
	Nest-2 reverse Plasmo 2		5′-AACCCAAAGACTTTGATTTCTCATAA-3′	
	Nest-2 forward 1	rFAL	5′-TTAAACTGGTTTGGGAAAACCAAATATATT-3′	205 bp
	Nest-2 reverse 2	rFAL	5′-ACACAATGAACTCAATCATGACTACCCGTC-3′	
	Nest-2 forward Nest-2	rMAL	5′-ATAACATAGTTGTACGTTAAGAATAACCGC-3′	144 bp
	Nest-2 reverse 2	rMAL	5′-AAAATTCCCATGCATAAAAAATTATACAAA-3′	
	Nest-2 forward rOVA 1		5′-ATCTCTTTTGCTATTTTTTAGTATTGGAGA-3′	800 bp
	Nest-2 reverse 2	rOVA	5′-GGAAAAGGACACATTAATTGTATCCTAGTG-3′	

Nest-2 forward 1	rVIV	5’-CGCTTCTAGCTTAATCCACATAACTGATAC-3’	
Nest-2 reverse 2	rVIV	5’-ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3’	120 bp

Step 15.

PCR Cycling conditions

Nest-1:

rPLU5 + rPLU6

94°C for 3mins

94°C for 30secs

58°C for 30secs 24 Cycles

72°C for 45secs

72°C for 5mins

End

Nest-2:

Plasmo1 + Plasmo2

94°C for 3mins

94°C for 30secs

58°C for 30secs 29 Cycles

72°C for 45secs

72°C for 5mins

End

Nest-2:

rFAL1 + rFAL2; rMAL1 + rMAL2; rOVA1 + rOVA2; rVIV1 + rVIV2

94°C for 3mins

94°C for 30secs

60°C for 30secs 29 Cycles

72°C for 45secs

72°C for 5mins

End

Step 16.

References

Georges Snounou & Suganya Viriyakosol (1993). High sensitivity of detection of malaria parasites by the use of nested polymerase chain reaction. Molecular and Biochemical Parasitology, 61, 315.

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