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Protocol for Plasmodium Species Determination by PCR

P. Falciparum

P. Vivax

P. Malariae

P. Ovale

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Introduction

The purpose of the methodology described in this protocol is to detect and determine the species of Plasmodium present in dried blood spot samples. Genus-specific Polymerase Chain Reaction (PCR) is followed by a nested species-specific PCR of 18S small subunit ribosomal DNA. Species are determined on the basis of product size on 2.5% agarose gel. The steps for species detection and determination by PCR amplification are as follows:

Sample Preparation

Venous blood is sampled and dried on Whatman 3MM filter paper and an alcohol sterilized hole punch is used to cut circles for testing. DNA is isolated from dried blood spots (DBS) by using the Chelex extraction method described in Appendix 1. The minimum recommended DBS size is one 6mm circle. Along with the extraction of unknown sample DBS, prepare and extract a set of positive controls for all alleles of the genes being tested in this experiment. Due to the high sensitivity of this PCR amplification, please ensure there is no cross-contamination between samples.

PCR Amplification

PCR methods are adapted from Nsobya et al 2004 and Snounou et al 1993. Template DNA is amplified using nested PCR, with second round primers specific to the species *Falciparum*, *Vivax*, *Malariae*, and *Ovale*. Separate reactions are performed for each pair of nested primers. For example, one 96-well plate of primary PCR will become four 96-well plates of each species if all four are to be determined. A No Template Control (NTC) is used in all reactions and genomic DNA from laboratory strains or clinical isolates are used as a positive control for respective species.

Detection

PCR products are stained by ethidium bromide and resolved by gel electrophoresis on a 2.5% agarose gel. DNA size standards are separated alongside PCR products to allow sizing of species bands. Upon completion of the gel electrophoresis, gels are placed in a gel imaging cabinet and digitally photographed under UV light. Gel images are printed and corresponding sample lanes are scored visually for the presence of specific species. Since each nested PCR is specific to only one species if present, only the corresponding amplified products will appear in the gel lane.

Experimental Procedure

PCR Preparation

- All PCR reagents should be stored in the freezer (-20 °C) when not in use and necessary reagents allowed to thaw completely in time for preparation. The polymerase enzyme, however, should remain in the freezer until use as it is temperature sensitive and does not freeze.
- Begin the experiment in a lab notebook by designing a sample identification table in the standard 12 x 8 format. List all controls (no template control and four species positive controls) and samples to be tested. See the example below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	+C <i>P.f.</i>	+C <i>P.v.</i>	+C <i>P.m.</i>	+C <i>P.o.</i>	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
B	Sample 8	Sample 9										

- Primers for both rounds of PCR are listed below.

Primary Round:

Genus	Primer Name	Sequence
Plasmodium	rPLUf	5' - TTA AAA TTG TTG CAG TTA AAA CG
	rPLUr	5' - CCT GTT GTT GCC TTA AAC TTC

Nested Round:

Species	Primer Name	Sequence
<i>Falciparum</i>	rFALf	5' - TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT
	rFALr	5' - ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC
<i>Vivax</i>	rVIVf	5' - CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC
	rVIVr	5' - ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA
<i>Malariae</i>	rMALf	5' - ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC
	rMALr	5' - AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA
<i>Ovale</i>	rOVAf	5' - ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA
	rOVAR	5' - GGA AAA GGA CAC ATT AAT TGT ATC CTA GTG

- Work in a PCR hood to prepare the master mix as described accounting for 12.5% more than the number of samples/controls being tested. Use the table below to calculate the volumes of reagents in making the master mix. After calculating the volume of reagent needed, vortex each reagent and combine in a suitable tube or sterile trough to make a master mix. Prepare the primary PCR master mix and complete the amplification, then follow with nested PCR using primer pairs for all species in separate reactions. The PCR product of the primary PCR is used as the template in the nested PCR. The nested PCR is not multiplexed.

Master Mix for BOTH Primary and Nested PCR Rounds

	Stock Concentration	Final Concentration	Volume (μL)	Multiple (# wells + 12.5%)	Vol. in Master Mix (μL)
H ₂ O	--	--	16.05	X _____	
Primer 1	10 μM	0.2 μM	0.5		
Primer 2	10 μM	0.2 μM	0.5		
Buffer	10x	1x	2.5		
dNTP	2mM each	200 μM each	2.5		
MgCl ₂	50 mM	1.5mM	0.75		
Taq Polymerase	5 U/ μL (INV)	1 Unit	0.2		
Template	unknown	--	2.0		
Final volume			25.0 μL total		

5. Add 23 μL of master mix to each well of a standard PCR plate that corresponds to the arrangement of controls and samples using a pipettor with aerosol barrier tips.
6. Add 2.0 μL of samples and controls to appropriate reaction wells using a pipettor with a new aerosol barrier tip for each sample. It is optional to add 2.0 μL of PCR water to the NTC well. Be careful to keep track of sample locations and avoid cross contamination. Cap the tubes and apply pressure for a tight seal.

PCR Amplification

1. Place the sealed PCR plate in the thermal cycler block.
2. Program the thermal cycler by creating the programs as follows:

<p>Species, Primary Round PCR Program: 5 hrs</p> <p><u>Initial denaturation</u> 94°C x 1m</p> <p><u>PCR</u> 35 cycles of 94°C x 1m, 58°C x 2m, 72°C x 5 m</p> <p><u>Final Elongation</u> 72°C x 5 m</p> <p><u>Hold @ 4 °C</u></p>	<p>Species, Nested Round PCR Program: 4.5 hrs</p> <p><u>Initial denaturation</u> 94°C x 1m</p> <p><u>PCR</u> 30 cycles:94°C x 1m, 58°C x 2m, 72°C x 5 m</p> <p><u>Final Elongation</u> 72°C x 5 m</p> <p><u>Hold @ 4 °C</u></p>
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Detection by gel electrophoresis

1. Prepare enough 2.5% agarose gels to be used for band detection. See Appendix 2 for formulas to prepare gels and other necessary reagents. Use 30-well combs when casting gels to run 29 samples plus 1 ladder on each gel. Ready gels are placed in the gel tank filled with enough 1X TBE buffer to cover the gel by 5-10mm.
2. Add 3 μL of loading dye to each PCR well using a pipettor (multichannel recommended) with a new aerosol barrier tip for each sample.
3. Mix each sample/dye by pipetting up and down and carefully load 15 μL into the appropriate well on a gel. Reserve the first and last well for the DNA size standard.
4. Once all 29 wells are loaded, vortex the vial containing prepared 50bp DNA ladder and load 15 μL into the first well of the gel. Work efficiently so the sample DNA does not diffuse excessively into the gel before applying electrical current.

5. Once all wells are loaded, align the gel horizontally in the gel box, cover, and apply electrical current. Be sure to connect the gel tank so that the DNA electrophoreses towards the red/positive side of the gel.
6. Set the electrical supply to 80-85 Volts and confirm that electrophoresis has begun by observing bubbles rising from the electrodes in the TBE buffer.
7. The gel has run sufficiently and should be photographed before the bromophenol blue dye has run off the bottom of the gel.
8. Gently remove the gel from the tank and place into the gel imaging cabinet. Using UV light, digitally photograph the gel and print the gel image for placement in the lab notebook. Refer to separate instructions for the gel imaging cabinet.
9. Immediately after the images are taken, check on the validity of the results. If any bands are present in the No Template Control, the entire experiment is invalid. For each positive control a single band of the correct size should be present for each species as described below.

Positive Control Sizes:

Species	Positive Control Band sizes (+/- 10bp)			
	<i>P.f.</i>	<i>P.v.</i>	<i>P.m.</i>	<i>P.o.</i>
<i>Falcip.</i>	205	Nothing	Nothing	Nothing
<i>Vivax</i>	Nothing	120	Nothing	Nothing
<i>Malariae</i>	Nothing	Nothing	140	Nothing
<i>Ovale</i>	Nothing	Nothing	Nothing	800

Interpretation of species-specific bands

After validating the experiment by visualizing a negative NTC and the correct band size for corresponding positive controls, enter all sample results into a standard database (*eg.* SPSS). It is best to indicate positive or negative for each species and each sample tested.

Appendix 1

Chelex DNA Extraction

Materials: Dried, blood-blotted filter papers; 10% saponin in water (stored at -20°C); 1XPBS (Calcium and Magnesium Free pH7.4); 20% Chelex (stored at room temperature); a heat block at 95°C

1. Cut the filter paper to appropriate size using a scissors or hole puncher. The same blade or hole puncher can be used after wiping with an alcohol swab.
2. Combine the dried, blood blotted filter papers with 1ml of PBS and 50ul of 10% saponin in a 1.5 ml Microfuge tube, invert several times, and store over night at 4°C.
3. Microfuge tubes for 5 seconds and aspirate the now reddish PBS/saponin from the tubes with a clean non-barrier tip attached to a Pasteur pipette at the end of a vacuum assembly using a new tip for each tube.
4. Add 1 ml of PBS/tube (no saponin), invert several times and incubate at 4°C for 15-30min. Turn on the heat block at this time to allow time for 95°C to be reached.
5. Microfuge and aspirate (as above) as much fluid as possible and afterwards use the tip to press the filter paper down into the lower third of the tube without packing it excessively.
6. Transfer 1ml of vortexed chelex stock solution to a 1.5 ml microfuge tube and using a tip with tapered end cut off. Dispense 50 ul to each sample, vortexing or inverting the tube every two or three transfers (to be sure you are not just transferring water with all the chelex settled to the bottom of the dispensing tube).
7. Add 100ul of sterile water to each tube.
8. Extract the parasite DNA by incubating the tubes for 10 min in a 95°C heat-block, vigorously vortexing each sample every 2 minutes or so throughout the incubation. It is best to briefly uncap each tube every two minutes or so in the block to release pressure, or else the tubes will "pop"
9. After incubation, microfuge the tubes for 5 minutes at high speed. Meanwhile, label two sets of 0.6mL microfuge tubes for transfer, the second set for final storage of the extracted DNA samples.
10. Transfer as much solution as possible from the spun tubes to the first set of microtubes with a 200uL barrier-tip, not worrying if chelex is carried over as well.
11. Spin tubes for 10 minutes at high speed and then transfer the final, white-to-yellowish supernatant (avoiding the pelleted chelex) to the final set of labeled tubes. Store at -20C.

Appendix 2

Reagent Formulas

2.5% Agarose Gels

Makes 2 gels

5.0g agarose

200 mL 1x TBE

15 uL ethidium bromide

Microwave 4 ½ minutes for 2 gels, 6 minutes for 4 gels, then cool to 60° C and add ethidium bromide. Mix gently to distribute ethidium bromide. Pour gel into casting tray with 20 well combs and allow to set (approximately 20 min). **Note: ethidium bromide is mutagenic and should be handled carefully. Gels containing ethidium bromide should be disposed of accordingly**

5X TBE Buffer Stock

Per liter of stock solution

54g tris base

27.5g boric acid

20 mL 0.5M EDTA pH 8

6X Loading Dye

Makes 10mL

25 mg (0.25%) bromophenol blue

3 mL (30%) glycerol

7 mL H₂O

DNA Size Marker With Dye

50 uL of 50bp ladder (Invitrogen)

170 uL 6X bromphenol blue loading dye

780 uL H₂O to make 1 mL

2mM dNTP

1 mL 100 mM dNTP from stock

11.5 mL H₂O

Aliquot into 1 mL volumes