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IDEEL- UNC implementation of 18S species detection for Plasmodium

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We use this protocol and it's working

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Abstract

This protocol describes implementation of 18S qPCR for species identification for Plasmodium sp. Assays are run in single-plex to maintain the highest sensitivity. Assays are typically run for 40-45 cycles. Please see these references for information on running the assays for longer cycling parameters:

1. Gaither C, Morgan C, Kirby R, Karema C, Gashema P, Zuromski J, White SJ, Topazian HM, Giesbrecht D, Thwai K, Young NW, Goel V, Boyter K, Munyaneza T, Muvunyi CM, Butera JD, Bailey JA, Mazarati JB, Juliano JJ. Prevalence of asymptomatic non-falciparum and falciparum malaria in the 2014-15 Rwanda Demographic Health Survey. PLoS One. 2025 Sep 11;20(9):e0330480. doi: 10.1371/journal.pone.0330480. PMID: 40934175; PMCID: PMC12425214.
2. Sendor R, Mitchell CL, Chacky F, Mohamed A, Mhamilawa LE, Molteni F, Nyinondi S, Kabula B, Mkali H, Reaves EJ, Serbantez N, Kitojo C, Makene T, Kyaw T, Muller M, Mwanza A, Eckert EL, Parr JB, Lin JT, Juliano JJ, Ngasala B. Similar Prevalence of Plasmodium falciparum and Non-P. falciparum Malaria Infections among Schoolchildren, Tanzania[1]. Emerg Infect Dis. 2023 Jun;29(6):1143-1153. doi: 10.3201/eid2906.221016. PMID: 37209670; PMCID: PMC10202886.
3. Gumbo A, Topazian HM, Mwanza A, Mitchell CL, Puerto-Meredith S, Njiko R, Kayange M, Mwalilino D, Mvula B, Tegha G, Mvalo T, Hoffman I, Juliano JJ. Occurrence and Distribution of Nonfalciparum Malaria Parasite Species Among Adolescents and Adults in Malawi. J Infect Dis. 2022 Jan 18;225(2):257-268. doi: 10.1093/infdis/jiab353. PMID: 34244739; PMCID: PMC8763954.

These assays were derived from the following sources:
Falciaprum: Veron V et al. Exp Parasitol 2009. 121(4):346-51.
Vivax: Brazeau N, et. al. Nature Communications. 2021
Ovale sp.: Mitchell C, et. al. Journal of Infectious Diseases. 2021
Malariae: Koliopoulos, et al. Malar J. 2021 Feb 1; 20:66.

Guidelines

1. All reagents should be kept on ice or in cold blocks during set-up.
2. Real time PCR probes are light sensitive. Stocks should be kept in a black tube or covered.

ALL ASSAYS ARE DONE IN SINGLE-PLEX. EACH ASSAY IS PRESENTED AS A SEPARATE SECTION OF THE PROTOCOL.

Materials

Consumables:

1. P200 pipettor and tips (single or multi-channel)
2. P20 pipettor and tips (single or multi-channel)
3. 25µL reagent reservoir
4. 96-well optical PCR plates
5. 1.5ml microfuge tubes
6. optical PCR plate films
7. Film applicator (**for example**)

Reagents:

1. 80% Ethanol
2. 10% bleach
3. Roche FastStart Universal Probe Master Mix (Rox)
4. Molecular Grade Water (MGW)
5. Primers as probes as described for all assays below
6. *P. falciparum* plasmid control (https://www.beiresources.org/Catalog/BEIPlasmidVectors/MRA-177.aspx)
7. *P. malariae* plasmid control (https://www.beiresources.org/Catalog/BEIPlasmidVectors/MRA-179.aspx)
8. *P. ovale* plasmid control (https://www.beiresources.org/Catalog/BEIPlasmidVectors/MRA-180.aspx)
9. *P. vivax* plasmid control (https://www.beiresources.org/Catalog/BEIPlasmidVectors/MRA-178.aspx)

	A	B
	P. malariae-specific (18S)	
	ForwardPrimer(5'→3')	AGTTAAGGGAGTGAAGACGATCAGA
	ReversePrimer(5'→3')	CAACCCAAAGACTTTGATTTCTCATAA
	Probe(5'→3')	FAM/ATGAGTGTTTCTTTTAGATAGC/NFQ

	A	B
	P. vivax-specific (18S)	
	ForwardPrimer(5'→3')	ACGCTTCT AGCTTAAT CCACATAA CT
	ReversePrimer(5'→3')	ATTTACTCA AAGTAACA AGGACTTC CAAGC
	Probe(5'→3')	FAM/TTCGT ATCG/ZEN/ ACTTTGTG CGCATTTT GC/3IABkF Q
	P. ovale-specific (18S)	
	ForwardPrimer(5'→3')	CCRACTAG GTTTTGGA TGAAAVRT TTTT
	ReversePrimer(5'→3')	AACCCAAA GACTTTGA TTTCTCATA A
	Probe(5'→3')	VIC/CRAAA GGAATTYT CTTATT/NF Q
	P. falciparum-specific (18s)	
	ForwardPrimer(5'→3')	ATTGCTTTT GAGAGGTT TTGTTACTT T
	ReversePrimer(5'→3')	GCTGTAGT ATTCAAAC ACAATGAA CTCAA
	Probe(5'→3')	FAM/ CATAACAG ACGGGTAG TCAT /NFQ

Before start

Clean work area/hood and pipettes per laboratory protocol. We recommend 10% bleach solution and UV irradiation.

Set Up

1 Dilute species specific primers to 20 micromolar (µM) with MGW per supplier's instructions.

2 Dilute species specific probes to 10 micromolar (µM) with Tris, ph 8.0 per supplier's instructions.

Controls

3 Negative controls: The negative controls for the assay can be MGW or human DNA used at a clinically relevant concentration (recommended). These should be done in duplicate.

4 Positive controls: Use the recommended plasmids for positive controls. The number and type of positive controls will depend on the goal for the assay. All positive controls should be done in duplicate.

4.1 Simple detection: A single concentration of plasmid is needed for this. Use on online calculator to determine the copies of plasmid per ul based on the concentration. Details of the plasmid size are available in the links to the reagent. As Plasmodium have multiple copies of 18S, we typically use 6 copies of the plasmid to equal a genomic equivalent (a parasite). Plasmid is diluted to a clinically representative concentration, for example 1,000 genomic equivalents per ul (6,000 copies per ul).

4.2 Quantification: In order to quantify relative parasitemia, a standard curve of plasmid is required. Typically we recommend at least 4 concentrations (tested in duplicate) across a range of clinically relevant concentrations. The average of the replicates at each concentration can be used to determine estimated parasitemia using the software on your real time PCR machine.

P. falciparum Detection

5 In a 1.5ml microfuge tube combined the master mix as follows:

	A	B	C	D
		Final concentration	1 RXN	96 RXN*
	RocheFastStart	1X	6	633.6

	A	B	C	D
	UniversalProbe Master(Rox)			
	Fwd primer (20uM)	300nM	0.18	19.008
	Rev primer (20uM)	300nM	0.18	19.008
	Probe (10uM)	200nM	0.24	25.344
	MGW		3.4	359.04
	Total volume		10	1056

*: 96 reactions is made with 10% extra to ensure enough master mix for the plate.
Volumes are microliters.

6 Pipet 10 ul of mastermix into each well of the 96-well optical PCR plate.

6.1 Master mix can be added one well at a time. However, it can be placed in a reservoir to allow multichannel pipette use to more quickly set up the plate.

7 Pipet 2 ul of template DNA into each well, ensuring you have enough wells for non-template controls and positive controls as described above.

7.1 If template DNA is in strips or 96-well plates, this may be added using a multichannel pipette.

8 Add 2 ul of non-template controls or positive controls to each well as desired.

9 Seal PCR plate with optical film being sure to apply pressure across the entire plate to gain a good seal. Typically, this is best achieved using a hard plastic tool film applicator to smoothly work across the plate

10 Place PCR plate in your real time PCR thermocycler and program the following cycling conditions:

	A	B	C
	Temp	Time	Cycles
	50C	2 min	1
	95C	10 min	1
	95C	15 sec	40-45
	60C	1 min	

Be sure to set your machine to the approrpriate detecter dye, quencher, and ROX background. Enter controls into the machine to allow for relative quantification if desired.

11 After the run is complete, export the data for analysis per your laboratory protocol.

P. vivax Detection

12 In a 1.5ml microfuge tube combined the master mix as follows:

	A	B	C	D
		Final concentration	1 RXN	96 RXN*
	RocheFastStartUniversalProbeMaster(Rox)	1X	6	633.6
	Fwd primer (20uM)	400nM	0.24	25.344
	Rev primer (20uM)	400nM	0.24	25.344
	Probe (10uM)	200nM	0.24	25.344
	MGW		3.28	346.368
	Total volume		10	1056

*: 96 reactions is made with 10% extra to ensure enough master mix for the plate.
Volumes are microliters.

13 Pipet 10 ul of mastermix into each well of the 96-well optical PCR plate.

13.1 Master mix can be added one well at a time. However, it can be placed in a reservoir to allow multichannel pipette use to more quickly set up the plate.

14 Pipet 2 ul of template DNA into each well, ensuring you have enough wells for non-template controls and positive controls as described above.

14.1 If template DNA is in strips or 96-well plates, this may be added using a multichannel pipette.

15 Add 2 ul of non-template controls or positive controls to each well as desired.

16 Seal PCR plate with optical film being sure to apply pressure across the entire plate to gain a good seal. Typically, this is best achieved using a hard plastic tool film applicator to smoothly work across the plate

17 Place PCR plate in your real time PCR thermocycler and program the following cycling conditions:

	A	B	C
	Temp	Time	Cycles
	50C	2 min	1
	95C	10 min	1
	95C	15 sec	40-45
	60C	1 min	

Be sure to set your machine to the approrpriate detecter dye, quencher, and ROX background. Enter controls into the machine to allow for relative quantification if desired.

18 After the run is complete, export the data for analysis per your laboratory protocol.

P. ovale sp. Detection

19 In a 1.5ml microfuge tube combined the master mix as follows:

	A	B	C	D
		Final concentration	1 RXN	96 RXN*
	RocheFastStartUniversalProbeMaster(Rox)	1X	6	633.6
	Fwd primer (20uM)	400nM	0.24	25.344
	Rev primer (20uM)	400nM	0.24	25.344
	Probe (10uM)	200nM	0.24	25.344
	MGW		3.28	346.368
	Total volume		10	1056

*: 96 reactions is made with 10% extra to ensure enough master mix for the plate.
Volumes are microliters.

20 Pipet 10 ul of mastermix into each well of the 96-well optical PCR plate.

20.1 Master mix can be added one well at a time. However, it can be placed in a reservoir to allow multichannel pipette use to more quickly set up the plate.

21 Pipet 2 ul of template DNA into each well, ensuring you have enough wells for non-template controls and positive controls as described above.

21.1 If template DNA is in strips or 96-well plates, this may be added using a multichannel pipette.

22 Add 2 ul of non-template controls or positive controls to each well as desired.

23 Seal PCR plate with optical film being sure to apply pressure across the entire plate to gain a good seal. Typically, this is best achieved using a hard plastic tool film applicator to smoothly work across the plate

24 Place PCR plate in your real time PCR thermocycler and program the following cycling conditions:

	A	B	C
	Temp	Time	Cycles
	50C	2 min	1
	95C	10 min	1
	95C	15 sec	40-45
	60C	1 min	

Be sure to set your machine to the approrpriate detecter dye, quencher, and ROX background. Enter controls into the machine to allow for relative quantification if desired.

25 After the run is complete, export the data for analysis per your laboratory protocol.

P. malariae Detection

26 In a 1.5ml microfuge tube combined the master mix as follows:

	A	B	C	D
		Final concentration	1 RXN	96 RXN*
	RocheFastStartUniversalProbeMaster(Rox)	1X	6	633.6
	Fwd primer (20uM)	300nM	0.18	19.008
	Rev primer (20uM)	300nM	0.18	19.008
	Probe (10uM)	200nM	0.24	25.344
	MGW		3.4	359.04
	Total volume		10	1056

*: 96 reactions is made with 10% extra to ensure enough master mix for the plate.
Volumes are microliters.

27 Pipet 10 ul of mastermix into each well of the 96-well optical PCR plate.

27.1 Master mix can be added one well at a time. However, it can be placed in a reservoir to allow multichannel pipette use to more quickly set up the plate.

28 Pipet 2 ul of template DNA into each well, ensuring you have enough wells for non-template controls and positive controls as described above.

28.1 If template DNA is in strips or 96-well plates, this may be added using a multichannel pipette.

29 Add 2 ul of non-template controls or positive controls to each well as desired.

30 Seal PCR plate with optical film being sure to apply pressure across the entire plate to gain a good seal. Typically, this is best achieved using a hard plastic tool film applicator to smoothly work across the plate

31 Place PCR plate in your real time PCR thermocycler and program the following cycling conditions:

	A	B	C
	Temp	Time	Cycles
	50C	2 min	1
	95C	10 min	1
	95C	15 sec	40-45
	60C	1 min	

Be sure to set your machine to the approrpriate detecter dye, quencher, and ROX background. Enter controls into the machine to allow for relative quantification if desired.

32 After the run is complete, export the data for analysis per your laboratory protocol.