bio-protocol

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A Rapid FRET Real-Time PCR Protocol for Simultaneous Quantitative Detection and Discrimination of Human *Plasmodium* Parasites

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Abstract

Malaria is the most important parasitic disease worldwide, and accurate diagnosis and treatment without delay are essential for reducing morbidity and mortality, especially in *P. falciparum* malaria. Real-time PCR is highly sensitive and highly specific, therefore an excellent diagnostic tool for laboratory detection and species-specific diagnosis of malaria, especially in non-endemic regions where experience in microscopic malaria diagnostics is limited. In contrast to many other real-time PCR protocols, our new fluorescence resonance energy transfer-based real-time PCR (FRET-qPCR) allows the quantitative and species-specific detection of all human *Plasmodium* spp. in one run. Species identification is based on single nucleotide polymorphisms (SNPs) within the MalFL probe, detectable by melting curve analysis. A significant advantage of our FRET-qPCR is the short turn-around time of less than two hours, including DNA extraction, which qualifies it for routine diagnostics. Rapid and reliable species-specific malaria diagnosis is important, because treatment is species-dependent. Apart from first-line diagnosis, the quantitative results of our new FRET-qPCR can be helpful in therapy control, to detect early treatment failure.

Keywords: Malaria, *Plasmodium*, Diagnostics, PCR, Real-time PCR

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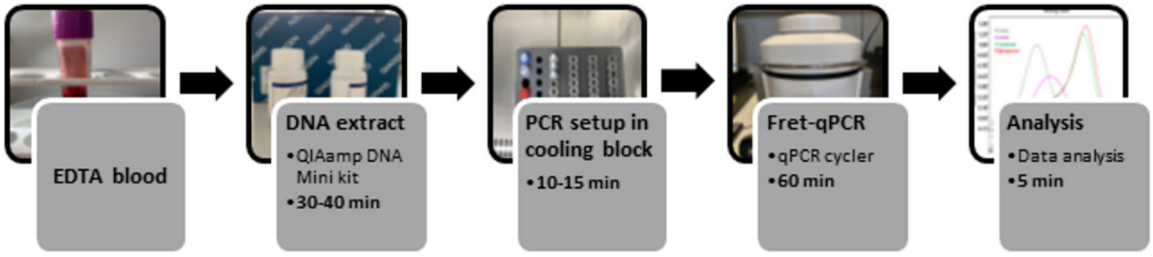
Graphic abstract



Background

Microscopy of Giemsa stained blood smears is still the gold standard for malaria diagnostics (Yin *et al*., 2018). However, microscopy requires considerable expertise, particularly at low-level parasitemia, which is often seen in imported malaria in non-endemic countries. Moreover, differentiating the *Plasmodium* species based on their morphological characteristics can be demanding, especially after chemoprophylaxis or auto-medication (Calderaro *et al*., 2018). Molecular detection and identification of *Plasmodium* spp. is a highly accurate and sensitive alternative method for the diagnosis of malaria. Particularly, real-time PCR has the advantage of fast and quantitative results and, compared to nested PCR, the risk of contamination is reduced. However, many published *Plasmodium* real-time PCR protocols have limitations, *e.g*., do not differentiate between species (Safeukui *et al*., 2008; Haanshuus *et al*., 2019; Farcas *et al*., 2004), do not detect all species (Perandin *et al*., 2004; Kim *et al*., 2014; Veron *et al*., 2009), or a positive genus-specific PCR has to be followed by two additional multiplex reactions, to obtain a species-specific result (Rougemont *et al*., 2004).

Hence, we developed and evaluated a new fluorescence resonance energy transfer-based real-time PCR (FRET-qPCR) that allows a quantitative, rapid, sensitive, and species-specific diagnosis of malaria (Schneider *et al*., 2021). The sequences of the hybridization probes MalFL and MalLC640 match *P. falciparum*. Fluorescence at 640 nm was generated by FRET, following the annealing of both probes to their adjacent complementary sequences. Species discrimination is based on the presence of single nucleotide polymorphisms (SNPs), reducing the affinity of the MalFLprobe for *P. vivax/knowlesi* (2 mismatches), *P. ovale* (1 mismatch), and *P. malariae* (1 mismatch), and thus lowering the melting temperature (Tm) in the melting curve analysis (Figure 1). The current paper is a step-by-step protocol for the performance of this new FRET-qPCR, focusing on the proper handling and storage of all components. The details described in this protocol are crucial comments to achieve excellent amplification and melting curves, a prerequisite for correct species identification based on single nucleotide polymorphisms (SNPs).

The advantage of requiring only one reaction per patient, and the short turn-around time of less than two hours (including DNA extraction), makes it a valuable diagnostic tool, especially in the absence of experienced microscopists. Moreover, results are objective and reproducible, DNA extraction can be automated, and the use of only one set of primers and probes is a significant advantage in routine clinical applications. One technician, well-trained in molecular methods, will be able to test a large number of patient samples in a relatively short turn-around time.

This protocol can be helpful for the rapid and reliable diagnosis of malaria in individual patients, particularly travelers returning from endemic areas, migrants, and refugees. Another field of application is treatment monitoring, by evaluating the quantitative results, and the crossing point (Cp) values. These allow the determination of the reduction in parasite number, important to detect early treatment failure (Rougemont *et al*., 2004). The potential of the FRET-qPCRs to detect asymptomatic infections can be useful for screening travelers returning to non-endemic regions. Moreover, it can also be performed in well-equipped malaria reference laboratories in endemic countries.

Materials and Reagents

1. 1.5 mL microcentrifuge tubes, sterile (Sarstedt, catalog number: 72.690.01)
2. 0.5–10 µL extra-long, 100–1,250 µL extra-long, 10–100 µL filtered pipette tips (Biozym, catalog numbers: VT0200, 770600; Eppendorf, catalog number: 30.077.547)
3. QIAamp® DNA Mini Kit (Qiagen, catalog number: 51304)
4. Ethanol absolute (Merck, catalog number: 1.00983.2500), do not use denatured alcohol
5. DNase- and RNase free water (Promega, catalog number: P1193)
6. LightCycler® Capillaries 20 µL (Roche, catalog number: 4929292001)
7. LightCycler® Fast Start DNA Master HybProbe (Roche, catalog number: 12239272001)
8. Primers and hybridization probes (TIB Molbiol GmbH; Table 1)
9. Primer reconstitution and storage (working stock preparation instructions).
10. EDTA blood samples

Table 1. Primers and FRET-probes targeting a 157–165 bp fragment of the small subunit 18S rRNA gene, for the detection and simultaneous differentiation of the respective *Plasmodium* species.

Oligonucleotide	Sequence
Plasmo 1[a]	5′-GTTAAGGGAGTGAAGACGATCAGA-3′
Plasmo 2[a]	5′-AACCCAAAGACTTTGATTTCTCATAA-3′
MalFL[b]	5′-CTTTCATCCAACACCTAGTCGGC; 3′label fluorescein
MalLC640[b]	5′-TAGTTTATGGTTAAGATTACGACGGT; 5′label, LC red 640, 3′phosphorylated

[a]Primers Plasmo 1 and Plasmo 2 were published by Rougemont *et al*. (2004)
[b]FRET-probes MalFL and MalLC640 were published by Schneider *et al*. (2021)

Equipment

1. 0.5–10 µL, 10–100 µL, and 100–1,000 µL micropipettes (Eppendorf, catalog numbers: 3123000020, 3123000047, 3123000063)
2. ThermoMixer (Eppendorf ThermoMixer C + Smart Block for 1.5 mL tubes; Eppendorf, catalog numbers: 5382000015, EPS360000036)
3. Microcentrifuge (Thermo Scientific™, Pico™ 21 Microcentrifuge, catalog number: 75002415)
4. LightCycler® Instrument 2.0 (Roche, catalog number: 03531414001)
5. LightCycler® Adapters (Roche, catalog number: 11909312001)
6. Vortex (Vortex-Genie 2, Sigma-Aldrich, catalog number: Z258423)

Procedure

The preparation of primer stock solutions and the FRET-qPCR setup were carried out separately in different biosafety cabinets.

The DNA extraction should be performed on a clean working desk, that is only used for DNA extraction, with a separate set of pipettes, used only for this purpose. Additionally, false positive results are prevented by the use of aerosol-resistant pipette tips. During DNA extraction and FRET-qPCR setup, the gloves should be changed several times, to avoid cross contamination between different (sometimes high positive) samples. When removing the capillaries from the cycler after the PCR reaction is finished, take care that no capillary breaks! A broken capillary containing a positive sample bears the risk of a carry-over contamination, and has to be carefully cleaned, and gloves changed! Additionally, in the FastStart DNA Master HybProbe mix, dTTP is replaced by dUTP, allowing the use of uracil-N-glycosylase (UNG) as an additional carryover prevention measure.

A. Human blood samples

We recommend tubes containing ethylenediaminetetraacetic acid (EDTA) to prevent the coagulation of blood. In contrast to heparin (also used as anticoagulant), EDTA does not inhibit PCR and is frequently used for hematological, as well as molecular diagnostics. Gloves have to be worn all the time, because human blood samples might be infectious. Additionally, eye protection should be worn to minimise the chance of infection in case of aerosol generation, splashes, or other accidental spillages. Postal transport at room temperature should not exceed 48 h. Then, the blood samples should be stored at 4°C and DNA extraction should be performed the next day. Alternatively, store of the blood samples at -20°C until DNA extraction is possible.

B. DNA extraction (QIAamp DNA Mini kit)

Kit contents: QIAamp Mini Spin Columns, Collection tubes 2mL, Buffer AL, Buffer AW1 (concentrate), Buffer AW2 (concentrate), Buffer AE, Proteinase K. All components are stable for up to one year after delivery at room temperature (15–25°C).

All centrifugation steps are performed at room temperature (15–25°C). Brief centrifugation means 5–10 s at maximum speed; this is sufficient to remove liquid from the lid, and collect the content at the bottom of the tube.

1. Add absolute ethanol to Buffer AW1 and AW2, as indicated on the bottles.
2. Pipet 20 µL of Proteinase K into the bottom of a 1.5-mL microcentrifuge tube.
3. Add 200 µL of EDTA blood (invert the tube several times before pipetting the 200 µL!)
4. Add 200 µL of Buffer AL, and mix by vortexing for 15 s.

> *Note: Do not add Proteinase K directly to Buffer AL.*

5. Incubate in the ThermoMixer with shaking at 56°C for 10 min.
6. Briefly centrifuge the tubes, to remove drops from the lid.
7. Add 200 µL of absolute ethanol, vortex for 15 s, and briefly centrifuge the tubes.
8. Carefully apply the mixture (620 µL) to the QIAamp Mini spin column (in a 2-mL collection tube). Close the cap and centrifuge at ≥6,000 × *g* for 1 min. Place the spin column in a fresh 2-mL collection tube, and discard the tube containing the filtrate.
9. Add 500 µL of Buffer AW1, and centrifuge at ≥6,000 × *g* for 1 min.
10. Discard the filtrate and the collection tube.
11. Place the spin column in a fresh 2-mL collection tube, add 500 µL of Buffer AW2, and centrifuge at 14,000 × *g* for 3 min.
12. Discard the filtrate and the collection tube.
13. Place the spin column in a fresh 2-mL collection tube (not provided), and centrifuge at 14,000 × *g* for 1 min, to eliminate possible Buffer AW2 carryover.
14. Place the spin column in a clean 1.5-mL microcentrifuge tube, add 100 µL of AE buffer, and incubate at room temperature for **5 min.** Centrifuge at ≥6,000 × *g* for 1 min, to elute the purified DNA from the spin column.
15. Store the DNA at 4°C until qPCR-setup (stable for several weeks at 4°C, or stable for 5 years when frozen at -20°C).

C. FRET-qPCR

1. Prepare primer and probe stock solutions (see working stock preparation instructions).
2. The LightCycler® Fast Start DNA Master HybProbe kit is stored at -25–-15°C: Kit Contents: vial 1a (LightCycler® Fast Start Enzym), vial 1b (LightCycler® Fast Start reaction mix), vial 2 ($MgCl_2$ stock solution, 25 mM), and vial 3 (H_2O, PCR-grade).
3. All centrifugation steps are at room temperature (15–25°C). Brief centrifugation means 5–10 s at maximum speed; this is sufficient to remove liquid from the lid, and collect the content at the bottom of the tube.
4. Prepare the LightCycler® Fast Start DNA Master HybProbe (10× concentrated): thaw one vial 1a, and one vial 1b.
5. Briefly centrifuge vial 1b and one vial 1a (enzyme).
6. Pipet 60 µL from vial 1b into vial 1a, and mix gently by pipetting 5 times up and down. DO NOT vortex!
7. Label vial 1a (red cap) with the new labels provided, it is now vial 1. Add the date; the reagent is stable at 4°C for 7 days.
8. Thaw and mix vial 2 and vial 3, until all frozen parts have disappeared. Briefly centrifuge all tubes listed in Table 2.

Table 2. FRET-qPCR mixture preparation

Reagent	Volume (µL) for one reaction
Water, PCR-grade (vial 3)	6.6
$MgCl_2$ stock solution, 25 mM (vial 2)	2.4
Plasmo 1 (20 µM)	1.0
Plasmo 2 (20 µM)	1.0
MalFL (4 µM)	1.0
MalLC640 (4 µM)	1.0
FastStart DNA Master HybProbe (vial 1)	2.0
Total volume	15.0

9. Prepare the FRET-qPCR master mix for the required number of reactions plus one additional reaction, as described in Table 2.
10. Mix all reagents carefully by pipetting up and down. DO NOT vortex the master mix!
11. Briefly centrifuge the FRET-qPCR master mix.
12. Take the precooled cooling block out of the fridge, and place the required number of capillaries into the precooled centrifuge adapters. Place the negative control at position 1 (PCR-grade water), and the positive control at position 2 (*Plasmodium falciparum* DNA), followed by the samples.
13. Pipet 15 µL of mix into each precooled LightCycler capillary.
14. Add 5 µL of DNA template, or respectively control.
15. Seal each capillary with a stopper, immediately after adding the DNA (avoids mistakes).
16. Place the adapters (containing the capillaries) into a standard benchtop microcentrifuge.
17. Centrifuge at 700 × *g* (3,000 rpm) for **exactly** 8 s.
18. Transfer the capillaries into the sample carousel of the LightCycler Instrument.
19. Cycle the samples as described in Table 3.

Table 3. LightCycler® 2.0 system FRET-qPCR Protocol
Set all protocol parameters not listed in the table to "0".

Analysis Mode	Cycles	Target Temperature [a]	Hold Time	Acquisition Mode [b]
None	1	Denaturation 95°C	 10 min	 none
None	10	Cycling (Touchdown) 95°C 69–58°C Slope =5°C/s 72°C	 5 s 10 s 15 s	 none single none
Quantification	35	Quantification 95°C 58°C Slope =5°C/s 72°C	 5 s 10 s 15 s	 none single none
Melting Curves	1	Melting Curves 95°C 50°C 70°C Slope =0.2°C/s	 20 s 20 s 0	 none none continuous
None	1	Cooling 40°C	 30 s	 none

[a]Temperature Transition Rate/Slope is 20°C/s, except where indicated.
[b]Default Channel 640 nm.

Data analysis

LightCycler® Software version 4.05 or higher

1. Perform a Qualitative Detection Analysis, using controls.
2. Capillary 1 is defined as a negative control and contains no DNA.
3. Capillary 2 is defined as a positive control (*Plasmodium falciparum* DNA).
4. If one of the controls fails to behave as expected, the unknown sample reactions are considered unreliable by the software: "Invalid". If the controls perform as expected, the results for unknown samples are displayed as "Positive" or "Negative" in the combined column.
5. The Cp value is automatically generated by the software for each positive sample, including the positive control.
6. To perform a Melting Temperature analysis, select Tm calling from the Analysis toolbar. In the Melting Peaks display, the melting peaks and the calculated Tm values are displayed, and have to be controlled. Only when the software fails to calculate the Tm value properly, it shall be adjusted manually to peak maximum, using the manual Tm sliders in the Melting Peaks chart. (The difference in melting temperatures depends on the type of mismatch, the mismatch position within the probe sequence, and the base pairs immediately adjacent to the mismatch). According to the Tm value in the FRET-qPCR, the samples are assigned to *P. falciparum* (Tm 63.5–66°C), *P. malariae* (Tm 63.0–63.5°C), *P. ovale* (Tm 58–60°C), and *P. vivax/knowlesi* (Tm 56–57.5°C), respectively (Figure 1).
7. Figure 1 shows the malaria species differentiation (*P. vivax, P. ovale, P. malariae,* and *P. falciparum*) based on the Tm values (Schneider *et al*., 2021).

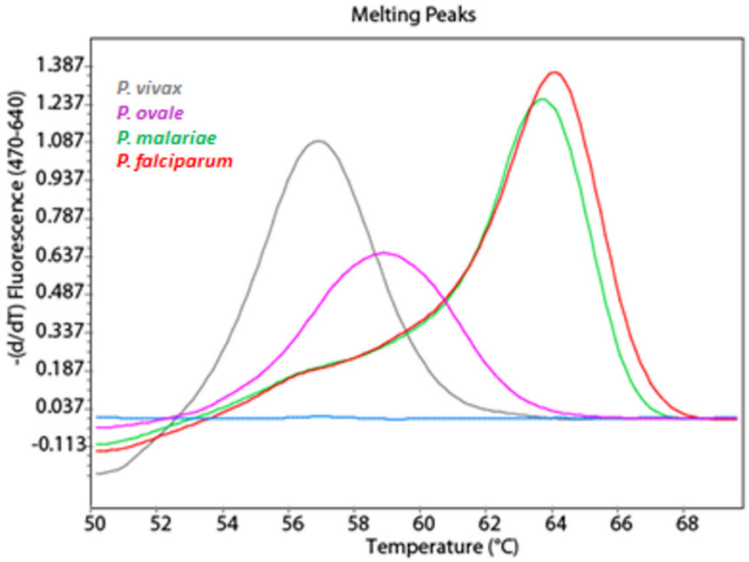


Figure 1. Species differentiation based on the Tm values.
Melting curves of amplicons post real-time PCR from *P. falciparum* (red), *P. malariae* (green), *P. ovale* (lilac), and *P. vivax* (grey) (Schneider *et al.*, 2021).

Working stock preparation instructions

Preparation of stock solution for primers and probes.

1. The lyophilized oligonucleotides should be stored in the dark at room temperature or at 4°C. Do not freeze them before dissolving! They are stable at room temperature for at least one year.
2. Briefly centrifuge the lyophilized primers Plasmo 1 and Plasmo 2 (exactly 5 nmol/tube), and probes MalFL and MalLC640 (exactly 1 nmol/tube), before opening in the safety cabinet.
3. Add 250 µL of molecular-grade water to each tube.
4. Vortex shortly and leave the tubes in the safety cabinet at room temperature for 30 min. Meanwhile, mix the tubes gently several times, and keep them away from direct sunlight.
5. Briefly centrifuge and prepare aliquots, store them at -20°C.
6. After thawing a set of aliquots, mix the four tubes gently, spin, and keep them away from excessive light. Store thawed primers and probes at 4°C, avoid multiple cycles of freezing and thawing. The shelf-life of the dissolved aliquots is at least 3 months at 4°C, or 1 year at -20°C.

Safety

When working with human blood samples, gloves and eye protection should be worn at all times to minimise the chance of infection.

Acknowledgments

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Competing interests

There are no conflicts of interest or competing interests.

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