

Evaluation of a novel melting curve-based qPCR for the detection, identification and quantification of *Plasmodium* species causing malaria in humans

Kim J. M. Bergen¹, Antoine R. Stuitje², Robert C. Akkers¹, Henricus J. Vermeer¹, Rob Castel¹, Theo G. Mank³

¹Result Laboratorium, Dordrecht, ²MRC Holland, Amsterdam,

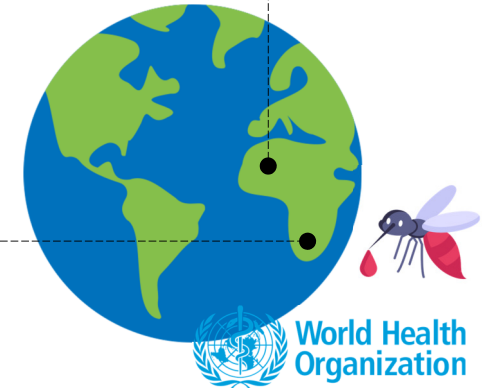
³Streeklaboratorium voor de Volksgezondheid Kennemerland, Haarlem



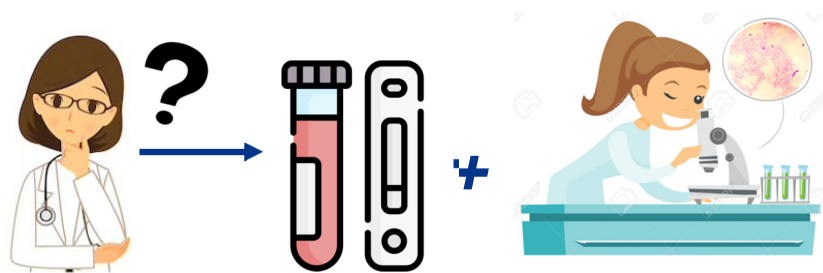
World Malaria Report 2022

247 million
New malaria cases

619.000
Malaria deaths



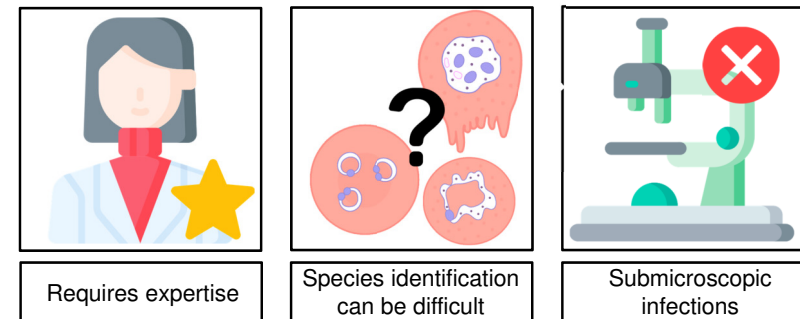
Malaria diagnostics



Plasmodium species

-*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*

Microscopy not always perfect

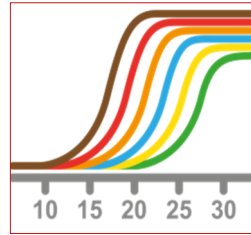


- Entry of PCR-based techniques into malaria diagnostics



MC004 assay

Single tube multiplex qPCR



- Discrimination between:
 - P. falciparum*
 - P. vivax*
 - P. malariae*
 - P. ovale wallikeri*
 - P. ovale curtisi*
 - P. knowlesi* (3 different strains)
 - P. cynomolgi* (3 different strains)

- Quantification of parasitaemia



Validation of the MC004 qPCR

- Reference samples (n=40)

P. falciparum
P. vivax
P. malariae
P. ovale
P. ovale wallikeri
P. ovale curtisi
P. knowlesi
P. cynomolgi
P. falciparum + *P. vivax*
 No parasites seen
Leishmania donovani infantum
Loa Loa
Trypanosoma brucei rhodesiensis
Babesia microti



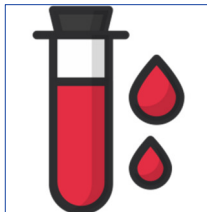
- Patient samples (n=304)

Validation of the MC004 qPCR

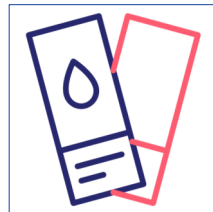
DNA samples



Blood samples



Blood smears



Total



Malaria diagnostics with the MC004 qPCR



1 PCR-reaction, 11 *Plasmodium* (sub)species.

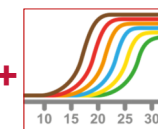
Pipetting



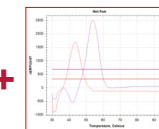
DNA-isolation



Amplification



Meltingcurve

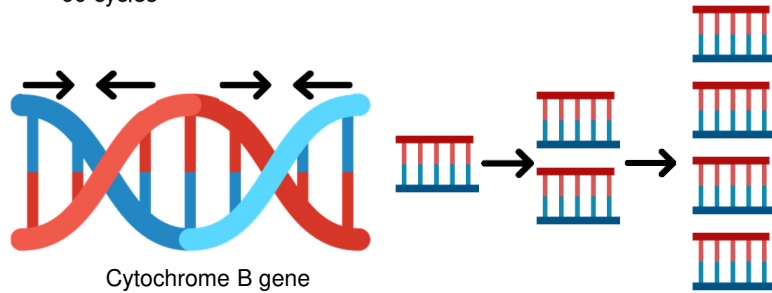


4 hours

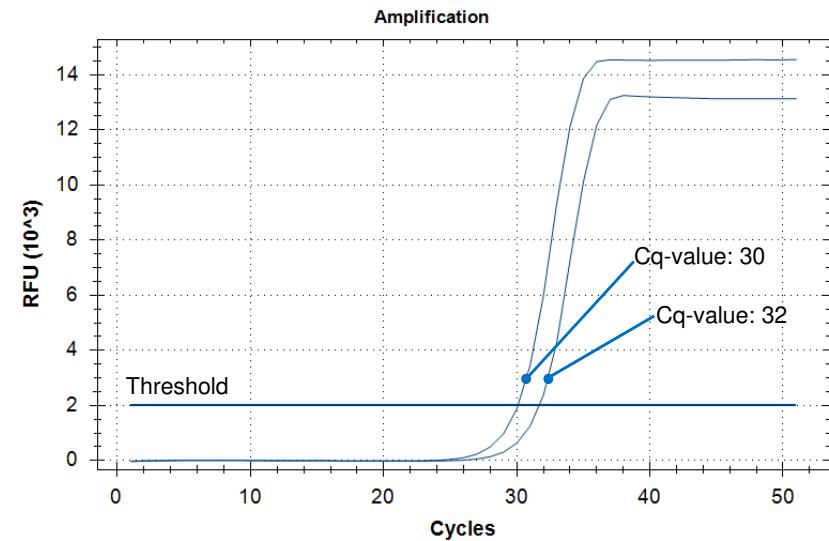


First step: amplification

- Target: Cytochrome B gene, mitochondrial DNA
- Multiplex PCR: 2 PCR products
- 50 cycles

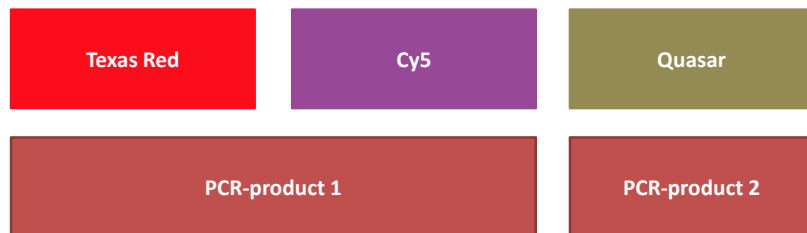


- Fluorescence can be followed “real-time”.

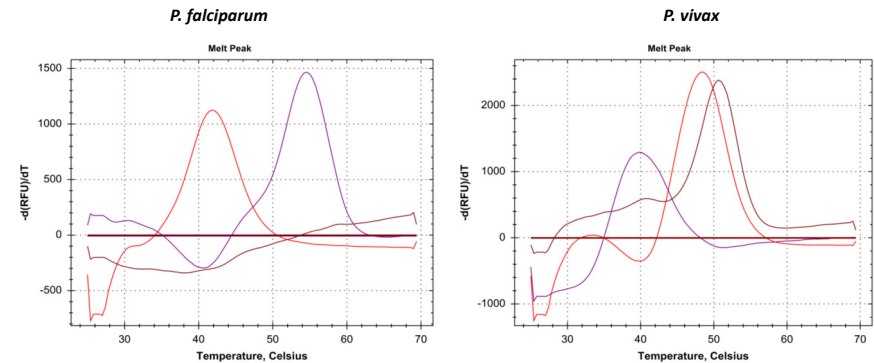


Second step: melting curve

- Three different Molecular beacon probes



Each *Plasmodium* species has a specific melting curve pattern



Representative melting curves of *P. falciparum* and *P. vivax*.

Discrepancies: MC004 correct

MC004 qPCR

37x	<i>P. falciparum</i>
9x	<i>P. vivax</i>
3x	<i>P. malariae</i>
5x	<i>P. ovale wallikeri</i>
2x	<i>P. ovale curtisi</i>
5x	<i>P. knowlesi</i>
1x	<i>P. cynomolgi</i>
1x	<i>P. falciparum</i> + <i>P. vivax</i>
281x	Negative
344	Total

Microscopy/RDT

✗	1x Misidentified as mixed (<i>Pf</i> + <i>Pv</i>).
✗	1x Missed <i>P. vivax</i> with microscopy.
✓	
✓	
✗	1x Misidentified as <i>P. vivax</i> with RDT.
✓	
✓	
✓	Included: <i>Babesia</i> , <i>Loa loa</i> , <i>Leishmania</i> and <i>Trypanosoma</i> .

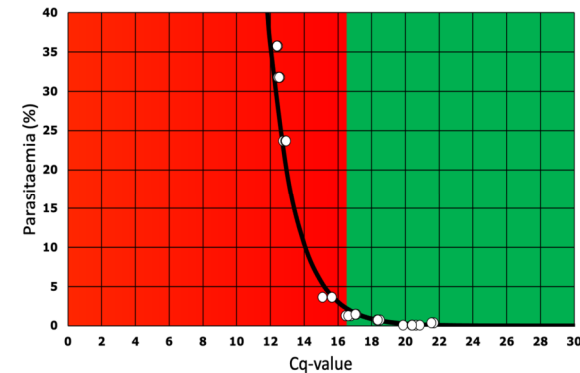
Follow-up patients:

9x	<i>P. falciparum</i>	✗	Positive with PCR,
2x	<i>P. vivax</i>	✗	Microscopy: no parasites seen.
1x	<i>P. malariae</i>	✗	

Quantification parasitaemia

Calibration line:

- 10 positive *P. falciparum* samples
- Microscopy estimated *P. falciparum* parasitaemia (y-axis)
- Cq-value (x-axis)



Parasitaemia microscopy versus MC004

Parasitaemia (%) [95% confidence interval] microscopy	Parasitaemia (%) MC004 assay
4.2 [3.0 – 5.4]	2.2
3.5 [2.4 – 4.6]	1.8
3.1 [2.0 – 4.2]	2.2
3.1 [2.0 – 4.2]	1.9
2.8 [1.8 – 3.8]	2.1
1.8 [1.0 – 2.6]	0.7
1.3 [0.6 – 2.0]	0.7
0.4 [0.0 – 0.8]	<0.1
0.3 [0.0 – 0.6]	0.8
0.3 [0.0 – 0.6]	0.2
0.2 [0.0 – 0.5]	0.4
0.2 [0.0 – 0.5]	0.2
0.1 [0.0 – 0.3]	<0.1
<0.1 [Not applicable]	0.1
<0.1 [Not applicable]	0.1
<0.1 [Not applicable]	<0.1
<0.1 [Not applicable]	<0.1
<0.1 [Not applicable]	<0.1
<0.1 [Not applicable]	<0.1

- 19 *P. falciparum* samples
- Tendency MC004 to underestimate
- Small differences Cq-value can result in large differences in calculated parasitaemia, especially in high parasitaemia

The MC004 PCR has improved the diagnosis of malaria

