Comparison of two real-time PCR assays for the detection of malaria from blood samples and feasibility study of an integral solution for malaria diagnostic in resource-limited settings.

Lopez-Quintana[1], B., Iglesias, N[1]., De Pablo, Fernandez-Romero, N[1]., Ta-Tang, TH[6]., Garcia-Aranda, P[6]., Martin-Ramirez, A[6,7]., A[1]. Rogers, J[2]. Reeves, P[2]., Madejon, A[3]., Toro, C[4]., Castan, P[2,5] and Rubio, J.M[6].

1 Fundacion Investigacion Biomedica Hospital Universitario La Paz (FIBHULP), 2 ARCIS Biotechnology Ltd., 3 CIBERehd isciii, 4 Hospital Universitario La Paz-Hospital Carlos III (Departamento de Microbiologia), 5 Hospital Universitario La Paz-Hospital Carlos III, 6 Instituto de Salud Carlos III (Unidad de Parasitosis Emergentes), 7 Hospital Universitario La Princesa

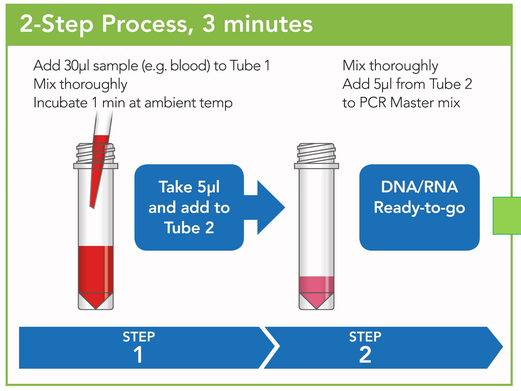
INTRODUCTION

Malaria remains one of the major health concerns on deployments in subtropical and tropical endemic settings, making rapid diagnosis in case of suspected malaria is mandatory. Traditional microscopy of Giemsa-stained thin and thick blood smears remains the diagnostic golden standard. However in resource-limited settings, alternative, less investigator-dependent diagnostic approaches are desirable. Rapid diagnostic tests (RDTs) are a frequently chosen option but their sensitivity of RDTs is still a matter of concern as much as the presence of false negatives due to the presence of heterophilic IgM antibodies in cases of exceedingly high parasitemia [**1**, **2**].

In this work we compare two molecular DNA-based assays as alternative approaches, with high sensitivity and specificity for malaria diagnostics, which can be standardized and require less experienced skills than microscopy.

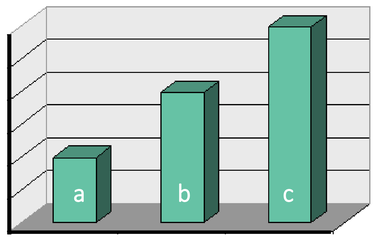
The reference polymerase chain reaction (PCR) is the generic reaction for real time detection of plasmodium described and validated in 2004 by Rougemont et al. in "Detection of four Plasmodium species in blood from humans by 18S rRNA gene subunit-based and species-specific real-time PCR assays" [**3**]. The challenging PCR belongs to an integral end-to-end solution developed by ARCIS-Biotechnology, whose commercial malaria retro-transcription-real-time-polymerase chain reaction (RTqPCR) assay aims for greater sensitivity by targeting the parasitic mRNA.

RESULTS



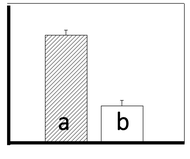
Protocol for ultra rapid sample prep process for extraction of plasmodium RNA and DNA from blood

SAMPLE	ICT	parasites/uL	Q/Rougemont (Gold Standard)				Q/Rougemont dilution factored in (7.5 CT)		A/Rougemont qPCR			ARCIS High sensitivity malaria RTqPCR Kit (MARK-2)			ARCIS High sensitivity malaria RTqPCR NZY boosted Kit (MARK-3)			Fraction of detection directly obtained from mRNA: using ARCIS RTqPCR Kit (MARK-2) (A/Rougemont-MK 2)	using ARCIS RTqPCR NZY boosted Kit (MARK-3) (A/Rougemont-MK 3)	Advantage in Ct		(vs Q/Rougemont)	
		Microscopy	Generic	Species					Generic			Generic			Generic			Generic	Generic	Q/Rougemont-MARK 2		Q/Rougemont-MARK 3	
1	+	0.3	23.97		23.38		30.88		38.19			18.24			14.95								
									38.16	38.10		18.05	18.11		12.89	13.84							
									37.94			18.03			13.68								
2	-	0	35.02	23/25	25.01	24.30	32.51	31.80	UND			37.88			UND								
									39	40.72	37.43	39.18	NA	22.93	UND	UND	16.98	14.50	20.45	1.37		7.32	
									42.43			UND			UND								
3	+	3	26.36		24.52		32.02		33.25			27.72			20.16								
									33.4	33.46		27.67	27.75		20.07	20.12							
									33.72			27.86			UND								
4	-	0	33.95		27.96		35.46		UND			29.41			22.86								
									41.99	42.72		28.66	29.25		22.56	22.54							
									43.45			29.68			22.21								
5	+	0.2	25.98	26/28	26.95	27.62	34.45	35.12	36.83			20.49			16.1								
									37.13	36.96	39.82	20.44	20.43	23.18	16.14	16.26	17.86	16.64	21.95	4.44		9.76	
									36.92			20.36			16.53								
6	+	<1	29.95		27.95		35.45		40.14			19.87			15.93								
									39.54	39.77		19.78	19.86		14.17	14.79							
									39.63			19.92			14.27								
7	+	0	40.95		32.92		40.42		43.11			28.66			20.94								
									UND	43.84		29.43	28.74		20.58	21.42							
									44.57			28.12			22.74								
8	-	0	32.03	29/32	31.57	32.48	39.07	39.98	38.46			23.92			17.96								
									39.26	38.56	41.67	23.73	23.86	29.12	17.7	17.82	21.60	12.55	20.07	3.36	4.20	10.88	11.04
									37.97			23.92			17.81								
9	+	<0.01	37.92		32.96		40.46		43.13			34.55			25.4								
									42.28	42.61		35.81	34.76		26.45	25.57							
									42.42			33.93			24.86								
10	+	<1	No PCR in StepOne		35.95		43.45		43.4			24.2			17.62								
									42.18	42.79		24.16	24.18		18.16	17.85							
									UND			24.18			17.76								
11	-	0	40.84	33/35	34.1	34.95	41.60	42.45	UND			35.43			26.81								
									44.28	44.64	43.55	35.94	36.25	31.48	26.98	26.21	22.73	12.07	20.82	3.47		12.22	
									44.99			37.39			24.84								
12	+	0	33.54		34.8		42.30		42			33.77			24.21								
									43.26	43.23		34.04	34.01		24.05	24.13							
									44.42			34.22			UND								
13	+	0	38.49		37.43		44.93		UND			26.01			20.62								
									43.58	43.58		25.65	25.77		20.84	20.22							
									UND			25.65			19.21								
									UND			33.34			25								
14	+	0	40.56	36/40	37.96	37.60	45.46	45.10	44.31	41.93	41.52	32.99	33.09	29.26	24.46	24.56	22.57	12.26	18.95	8.34		15.03	
									39.54			32.94			24.22								
									40.2			28.81			23.84								
15	+	0	38.6		37.4		44.90		UND	39.06		29.01	28.92		22.96	22.93							
									37.92			28.95			22								



Relative performance (% of probability for positive detection of plasmodium calculated over the data in the table) for malaria detection :

a) Microscopy < 65%
b) Rougemont std. qPCR <84%
c) ARCIS MK2 RTqPCR <93%



% of probability to generate a false positive result for plasmodium loads over 39 Cts:

a) Rougemont std. qPCR >82%
b) ARCIS MK3 RTqPCR <35%

GENERAL CONCLUSION

The statistical analysis of the data obtained from the retrospective samples detailed in the table set the real gain in LOD obtained by application of RTqPCR instead of standard qPCR to be aligned with the transcription rates described in bibliography both for active plasmodium infection and latency periods (**4**, **5**).

The overall gains in performance of the extraction, ease of use and cost-effectiveness of ARCIS integral solution tested vs the reference PCR here suggest that a new viable solution for molecular detection under tropical deployment conditions may become available shortly.

MAPPENDIX I: METHODS

Rougemont standard qPCR for malaria identification was performed as described in J. Clin. Microbiol. 2004 Dec;42(12):5636-43. All samples tested with ARCIS kits for malaria detection were subjected to the following protocol:

-30 µl of blood + 3 µl of ARCIS solution + 167 µl of water **1**. Mix with vortex **2**. Incubate at RT 1 minute **3**. Wash the extracted sample with ARCIS buffer using the fixed ratio 2.5:10 (2.5 µl of extracted sample:10 µl of ARCIS buffer) **4**. Put 5 µl into the PCR

The following mastermixes were used containing 5 µL of template and 15 µL of mastermix in each case. **ARCIS Ultrasensitive Malaria ID Kit** (**MK3**), ie. mastermix 1 (RT mix boosted with PCR enzyme mix): reaction master mix containing AgPath-IDTM One-Step RT-PCR 25X Enzyme Mix + NZY qPCR 2X Master Mix (Nzytech), 0.1 µM each primer and 0.08 µM probe. **ARCIS Ultrasensitive Malaria ID Kit** (**MK2**), ie. mastermix 2 (standard RTqPCR): reaction master mix containing AgPath-IDTM One-Step RT-PCR 25X Enzyme Mix, 0.1 µM each primer and 0.08 µM probe.

MAPPENDIX IREFERENCES

1. Abba K, Kirkham AJ, Olliaro PL, Deeks JJ, Donegan S, Garner P, Takwoingi Y. Rapid diagnostic tests for diagnosing uncomplicated non-falciparum or Plasmodium vivax malaria in endemic countries. Cochrane Database Syst Rev 12, CD011431 (2014) **2.** Gillet P, Mori M, Van Esbroeck M, Van den Ende J, Jacobs J. Assessment of the prozone effect in malaria rapid diagnostic tests. Malar J 8, 271 (2009) **3.** Rougemont M1, Van Saanen M, Sahli R, Hinrikson HP, Bille J, Jaton K. Detection of four Plasmodium species in blood from humans by 18S rRNA gene subunit-based and species-specific real-time PCR assays. J Clin Microbiol. 2004 Dec;42(12):5636-43. **4.** Rubio JM, Post RJ, van Leeuwen WM, Henry MC, Lindergard G, Hommel M. Alternative polymerase chain reaction method to identify Plasmodium species in human blood samples: the semi-nested multiplex malaria PCR (SnM-PCR). Trans R Soc Trop Med Hyg. 2002 Apr;96 Suppl 1:S199-204. **5.** Jennifer S. Sims, Kevin T. Militello, Peter A. Sims, Vishal P. Patel, Jacob M. Kasper and Dyann F. Wirth. Patterns of Gene-Specific and Total Transcriptional Activity during the Plasmodium falciparum Intraerythrocytic Developmental Cycle. Eukaryotic Cell March 2009 vol. 8 no. 3 327-338