A Real-Time PCR (qPCR) Assay for the Speciation of *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*

Product Insert

*ab*TES™ Malaria 5 qPCR II Kit
Kit Version: 1.0

CE IVD

REF 300229 (50 Reactions)
300230 (100 Reactions)

Store at -25°C to -15°C

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For use on Bio-Rad-CFX96 and *ab*CyderQ only.

1. Pathogen Information

Malaria is a vector-borne disease caused by protozoan parasite of the genus *Plasmodium*. It is widespread in tropical and subtropical regions such as Sub-Saharan Africa, Asia and America, resulting in annual 207 million clinical cases and 627 000 deaths worldwide (WHO, 2013). While *Anopheles* mosquito is the main vector of transmission, other transmission pathways such as blood transfusions, hypodermic needle sharing among intravenous drug users and transplantation remain eminent. Five species of *Plasmodium* were identified as human pathogens, namely *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. The disease results from the multiplication of malaria parasites within red blood cells, causing symptoms that typically include headaches, lassitude, nausea and fever, in severe cases progressing to coma and death.

2. Test Description

The *ab*TES™ Malaria 5 qPCR II Kit is a real-time polymerase chain reaction (qPCR) kit for the detection and speciation of *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi.* This kit contains all the necessary PCR reagents for rapid, sensitive and reproducible real-time detection and speciation of the five members of the Malaria complex using highly specific primer pairs and double-dye hydrolysis probes. The recommended sample type is EDTA blood (heparin blood is not recommended). It should only be used for samples that are already tested positive for malaria by other diagnostic kits.

3. Storage Conditions

The components of *ab*TES™ Malaria 5 qPCR II Kit should be stored in the dark, at -20°C in a **NON**-frost-free freezer. Frost-free freezers go through freeze-thaw cycles to remain frost-free and may cause accelerated degradation of enzymes and nucleic acids. Avoid repeated thawing and freezing (max 3 times) as this may lower the sensitivity. If reagents are used intermittently, it is suggested to keep the reagents frozen in aliquots.

4. Kit Components

Tubes	Items	300229 (50 rxns)	300230 (100 rxns)
1	Primer/Probe Mix	1x 100 µL	2 x 100 µL
2	Enzyme/Reactions Mix	1x 300 µL	2 x 300 µL
3	Malaria 5 Positive Control	1x 200 µL	1x 200 µL
4	Nuclease Free Water	1x 800 µL	2 x 800 µL

5. Additional Materials Required but not Provided

- Disposable powder-free gloves
- Nucleic acid extraction kit
- Vortex mixer
- Pipettes and pipette tips with filter
- Desktop centrifuge with rotor
- Real-time thermal cycler
- 0.2 mL PCR tubes/ 96-well PCR plates
- Ice box/cooling block

6. Limitations and General Precautions

- The use of this product and its data interpretation are intended for personnel trained in real-time PCR techniques and *in vitro* diagnostics procedures only.
- It is advisable to analyze the real time PCR graph at the end of the run to determine the validity of the Ct data.
- Appropriate specimen collection, transport, storage and nucleic acid extraction procedures are required for reliable results.
- Wear disposable gloves, laboratory coats and eye protection when handling samples and reagents. Wash hands thoroughly thereafter.
- Use sterile pipette tips with filters and replace the tip for every procedure.
- Store and extract positive materials (specimens, controls and amplicons) separately from all other reagents and add to the reaction mix in a separate facility, if possible.
- Thaw all the components thoroughly at room temperature before starting an assay.
- When the components are thawed, mix the components and centrifuge briefly.
- Do not use the kit after its expiration date.

7. Procedures

7.1. Nucleic Acids (NA) Extraction

Standard NA extraction kits are compatible with this assay. Please carry out NA extraction as per instructed in the manufacturer's extraction kit manual.

7.2. PCR Reaction Setup

Thoroughly thaw all components, mix and spin briefly. Keep all components and samples on ice. Prepare your PCR reaction based on the following pipetting scheme:

Reagent	Volume per reaction		
	Test sample reaction	Positive control reaction	Non template control reaction
Primer/Probe Mix	2 µL	2 µL	2 µL
Enzyme /Reaction Mix	6 µL	6.0 µL	6 µL
Nuclease-Free Water	12 µL	12 µL	17 µL
Positive Control	-	5 µL	-
Extracted Test Sample	5 µL	-	-
Total Volume	**25 µL**	**25 µL**	**25 µL**

7.3. PCR Cycling Conditions

The following cycling conditions were established and validated on Bio-Rad CFX96. You may need to adjust these conditions for other real-time platforms. **FAM** (*P. falciparum*), **ROX** (*P. vivax*), **HEX** (*P. malariae*), **Cy5** (*P. ovale*) and **Quasar 705/ Alexa Fluor 680** (*P. knowlesi*) should be chosen, and the fluorescence is measured at the end of amplification phase of each cycle.

Temperature Profile & Data Acquisition:

Phase	Description	No. of Cycles	Temperature	Duration
1	*Taq* activation	1	95 °C	2 min
2	Amplification	45	95 °C	5 sec
			*60 °C	20 sec

**Data acquisition at amplification phase*

8. Performances Characteristics

8.1. Analytical sensitivity

The Limit-of-detection (LoD) of the assay (not in consideration of DNA extraction) was estimated by analyzing a dilution series of synthetic positive controls *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* from 0.05 to 100000 copies/µl. The diluted samples were carried out in either 10-replicates (for concentrations ≥10000 copies/µl) or 14-replicates (for concentration <10000 copies/µl) using the *ab*TES™ Malaria 5 qPCR II Kit on the BioRad CFX96 Real-Time PCR and *ab*CyclerQ.

The LoD was then confirmed by running 20 replicates at its estimated limit-of-detection concentration.

Target	Detecting Channel	Analytical Sensitivity (95% confidence)
P. falciparum	FAM	10 copies/ µl
P. malariae	HEX	10 copies/ µl
P. vivax	ROX	12 copies/ µl
P. ovale	Cy5	30 copies/ µl
P. knowlesi	Quasar 705/ Alexa Fluor 680	15 copies/ µl

8.2. Analytical specificity

The assay was tested for potential cross-reactivity against the following panel of 49 organisms. All organisms were tested in 1000 copies/µl concentration except otherwise stated. No cross-reactivity was observed.

Organisms Tested for Analytical Specificity	
E.coli	Paravovirus
Streptococcus pyogenes	Enterovirus 71
Staphylococcus aureus	Epstein-barr virus
Streptococcus pneumoniae	West nile virus
Mobiluncus mulieris	Dengue 1 virus
Bacteriodes fragilis	Dengue 2 virus
Neisseria meningitidis	Dengue 3 virus
Gardnerella vaginalis	Dengue 4 virus
Trichomonas vaginalis	Human herpes virus 6
Enterococus faecalis	Rhinovirus
Klebsiella pneumoniae	Cytomegalovirus
Legionella pneumophila	Salmonella typhi
Candida albicans	Coxsackie A6
Pseudomonas aeruginosa	Coxsackie B1
Proteus mirabilis	Coxsackie B5
Ureaplasma urealyticum	Yellow fever
Mycoplasma genitalium	Influenza A (H1N1) pdm09 virus
Mycoplasma Hominis	Influenza A virus (H3N2)
C.trachomatis	Influenza B virus
N.gonorrhoeae	*Plasmodium falciparum*
Treponema pallidum	*Plasmodium malariae*
Haemophilus influenza	*Plasmodium vivax*
HSV-1	*Plasmodium ovale*
HSV-2	*Plasmodium knowlesi*
VZV	

8.3. Precision/Reproducibility

The inter-assay (variability between different runs) and intra-assay (variability within one run) precision of the assay was determined by performing the assay once per day in 7-replicates over a period of 2 days for 4 samples of different concentrations (total of 14 reactions per target). To also account for variability between technicians, the testing over the 2 days were tested by 2 different technicians.

For all the 5 targets, the qualitative results of all 70 reactions were 100% reproducible for both inter-assay and inter-technician.

The coefficient of variation (CV) of the cycle threshold (Ct) for the intra- and inter-assay precision are as follows:

Inter-assay precision data showing %CV (calculated from Ct values) at each concentration:

Target	1000 copies/ul	100 copies/ul	10 copies/ul	2 copies/ul
P. falciparum	1.06%	0.95%	0.99%	2.66%
P. malariae	0.96%	1.08%	1.44%	4.76%
P. vivax	0.71%	0.94%	2.23%	3.70%
P. ovale	1.17%	1.26%	3.71%	3.21%
P. knowlesi	0.96%	1.47%	2.06%	1.58%

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Intra-assay precision data showing %CV (calculated from Ct values) at each concentration:

Target	1000 copies/ul	100 copies/ul	10 copies/ul	2 copies/ul
P. falciparum	1.09%	0.93%	0.93%	2.66%
P. malariae	0.73%	1.05%	1.40%	3.54%
P. vivax	0.68%	0.98%	2.06%	3.24%
P. ovale	1.07%	1.30%	3.74%	3.75%
P. knowlesi	0.80%	1.42%	2.27%	1.22%

8.4. Diagnostic Evaluation

A total of 57 clinical samples which were previously tested positive, and their species were also confirmed by microscopy, were used to evaluate the clinical sensitivity and specificity of *ab*TES™ Malaria 5 qPCR kit. Because there was only a single P. Ovale identified from all clinical samples, 14 spike-in with P. Ovale control were included for validation purpose. The findings of the diagnostic sensitivity and specificity of each speciation are tabulated as below.

Sensitivity and specificity for *P. falciparum* (*P.f*) speciation

Reference Method (Microscopy)	*ab*TES (*n*=71)		Sensitivity/ Specificity
	P.f Positive	*P.f* Negative	
P.f Positive	12	0	100% sensitivity
P.f Negative	0	59	100% specificity
Total	12	59	

Sensitivity and specificity for *P. vivax* (*P.v*) speciation

Reference Method (Microscopy)	*ab*TES (*n*=71)		Sensitivity/ Specificity
	P.v Positive	*P.v* Negative	
P.v Positive	16	0	100% sensitivity
P.v Negative	0	55	100% specificity
Total	16	55	

Sensitivity and specificity for *P. malariae* (*P.m*) speciation

Reference Method (Microscopy)	*ab*TES (*n*=71)		Sensitivity/ Specificity
	P.m Positive	*P.m* Negative	
P.m Positive	13	0	100% sensitivity
P.m Negative	0	58	100% specificity
Total	13	58	

Sensitivity and specificity for *P. ovale (P.o)* speciation

Reference Method (Microscopy)	*ab*TES (*n*=71)		Sensitivity/ Specificity
	P.o Positive	*P.o* Negative	
P.o Positive	15	0	100% sensitivity
P.o Negative	0	56	100% specificity
Total	15	56	

Sensitivity and specificity for *P. knowlesi* (*P.k*) speciation

Reference Method (Microscopy)	*ab*TES (*n*=71)		Sensitivity/ Specificity
	P.k Positive	*P.k* Negative	
P.k Positive	15	0	100% sensitivity
P.k Negative	0	56	100% specificity
Total	15	56	

9. Interpretation of Data

A sample is considered as positive if the fluorescence level is higher than the threshold value.

Result	*P.f* (FAM)	*P.v* (ROX)	*P.m* (HEX)	*P.o* (Cy5)	*P.k* (Q705)
P. f positive	+	-	-	-	-
P. v positive	-	+	-	-	-
P. m positive	-	-	+	-	-
P. o positive	-	-	-	+	-
P. k positive	-	-	-	-	+
P.f, P.v, P.m, P.o and P.k positive	+	+	+	+	+
P.f, P.v, P.m, P.o and P.k negative	-	-	-	-	-

10. Troubleshooting

10.1 No signal observed with positive control

- Check programmed temperature settings against the protocol given.
- Affirm if proper storage was done and check the expiry date on the kit; Repeat the run with a new kit if needed.

10.2 Signal detected for negative control

- A contamination in the reagents or samples is highly possible.
- Repeat experiment protocol and take steps to locate source of contamination.

11. Explanation of Symbols

REF	Catalogue number
	Store at -25°C to -15°C
	Manufacturer
LOT	Lot number
	Use by
	Contains sufficient for <n> tests

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12. Reference

WHO (2013). *World Malaria Report 2013.* Switzerland: WHO Press, World Health Organization. Retrieved from http://www.who.int/malaria/publications/world_malaria_report_2013/en/

Electronic copy of the product insert can be downloaded at http://aitbiotech.com/malaria/

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