

Instructions for Use

RealStar[®]

Malaria Screen & Type PCR Kit 1.0

11/2021 EN

RealStar®

Malaria Screen & Type PCR Kit 1.0

For use with

Mx 3005P™ QPCR System (Stratagene)

VERSANT® kPCR Molecular System AD (Siemens Healthcare)

ABI Prism® 7500 SDS (Applied Biosystems)

ABI Prism® 7500 Fast SDS (Applied Biosystems)

Rotor-Gene® 6000 (Corbett Research)

Rotor-Gene® Q5/6 plex Platform (QIAGEN)

CFX96™ Real-Time PCR Detection System (Bio-Rad)

CFX96™ Deep Well Real-Time PCR Detection System (Bio-Rad)

LightCycler® 480 Instrument II (Roche)



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2 x 48



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Content

1.	Intended Use	6
2.	Kit Components	6
3.	Storage	7
4.	Material and Devices required but not provided	8
5.	Background Information	9
6.	Product Description	11
6.1	Real-Time PCR Instruments	13
7.	Warnings and Precautions	14
8.	Procedure	15
8.1	Sample Preparation	15
8.2	Master Mix Setup	16
8.3	Reaction Setup.....	18
9.	Programming the Real-Time PCR Instrument	19
9.1	Settings	19
9.2	Fluorescence Detectors (Dyes).....	19
9.3	Temperature Profile and Dye Acquisition	20
10.	Data Analysis	20
10.1	Validity of Diagnostic Test Runs	21
10.1.1	Valid Diagnostic Test Run (qualitative).....	21
10.1.2	Invalid Diagnostic Test Run (qualitative)	21
10.2	Interpretation of Results	22
10.2.1	Qualitative Analysis	22
11.	Performance Evaluation	23

11.1	Analytical Sensitivity.....	23
11.2	Analytical Specificity.....	27
11.3	Precision	27
11.4	Diagnostic Evaluation	30
12.	Limitations	33
13.	Quality Control	34
14.	Technical Assistance	34
15.	Literature.....	34
16.	Trademarks and Disclaimers	35
17.	Explanation of Symbols	36

1. Intended Use

The RealStar® Malaria Screen & Type PCR Kit 1.0 is an *in vitro* diagnostic test, based on real-time PCR technology, for the qualitative detection and differentiation of DNA of the human pathogenic *Plasmodium* species *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium knowlesi*, *Plasmodium vivax* and *Plasmodium falciparum*.

2. Kit Components

The kit contains two different PCR assays with 48 reactions each. It includes two different Positive Controls: one for the *Plasmodium (P.) knowlesi*, *P. malariae* and *P. ovale* specific amplification and detection system and another for the *P. falciparum* and *P. vivax* specific amplification and detection system.

Table 1: Kit Components

Lid color	Component	Number of vials	Volume [µl/Vial]
Blue	Master A Pk/Pm/Po ¹⁾	4	60
Light blue	Master A Pf/Pv ²⁾	4	60
Purple	Master B Pk/Pm/Po ¹⁾	4	180
Light purple	Master B Pf/Pv ²⁾	4	180
Green	Internal Control	1	1000
Red	Positive Control Pk/Pm/Po ¹⁾	1	250
Orange	Positive Control Pf/Pv ²⁾	1	250
White	Water (PCR grade)	1	500

¹⁾ Pk - *Plasmodium knowlesi*, Pm - *Plasmodium malariae*, Po - *Plasmodium ovale*

²⁾ Pf - *Plasmodium falciparum*, Pv - *Plasmodium vivax*

3. Storage

- The RealStar® Malaria Screen & Type PCR Kit 1.0 is shipped on dry ice. The components of the kit should arrive frozen. If one or more components are not frozen upon receipt, or if tubes have been compromised during shipment, contact Altona Diagnostics GmbH for assistance.
- All components should be stored at -25 °C to -15 °C upon arrival.
- Repeated thawing and freezing of Master reagents (more than twice) should be avoided, as this might affect the performance of the assay. The reagents should be frozen in aliquots, if they are to be used intermittently.
- Storage at +2 °C to +8 °C should not exceed a period of 2 hours.
- Protect Master A and Master B from light.

4. Material and Devices required but not provided

- Appropriate real-time PCR instrument (see chapter 6.1 Real-Time PCR Instruments)
- Appropriate nucleic acid extraction system or kit (see chapter 8.1 Sample Preparation)
- Desktop centrifuge with a rotor for 2 ml reaction tubes
- Centrifuge with a rotor for microtiter plates, if using 96 well reaction plates
- Vortex mixer
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material
- Pipettes (adjustable)
- Pipette tips with filters (disposable)
- Powder-free gloves (disposable)

NOTE



Please ensure that all instruments used have been installed, calibrated, checked and maintained according to the manufacturer's instructions and recommendations.

NOTE



It is highly recommended to use the 72-well rotor with the appropriate 0.1 ml reaction tubes, if using the Rotor-Gene® 6000 (Corbett Research) or the Rotor-Gene® Q 5/6 plex (QIAGEN).

5. Background Information

Malaria is a vector-borne disease, caused by a protozoan infection. The parasites of the genus *Plasmodium* are transmitted to their vertebrate hosts during the blood meal of an infected, female mosquito of the genus *Anopheles*. The life cycle of the parasites encompasses a host change from the arthropod to the vertebrate host and is rather complex but can be divided into 3 core phases. These phases are based on the parasite's mosquito stage, the human liver stage and the human blood stage. There are 5 known human pathogenic *Plasmodium* species, namely *Plasmodium falciparum*, *Plasmodium knowlesi*, *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium malariae* [1].

The malaria disease presents itself in different manifestations, depending on the infecting *Plasmodium* species. In general, the earliest symptoms of malaria are very unspecific; fever, headache, general body weakness, myalgia, chills, dizziness, abdominal pain, diarrhoea, nausea and vomiting. *P. falciparum* and *P. knowlesi* are capable of causing severe malaria in humans [2,3]. *P. falciparum* is responsible for an annual case fatality rate of > 90 %, mostly in children [2].

P. knowlesi undergoes a short erythrocytic stage (24 hours) during which it rapidly reproduces [4]. The resulting hyperparasitaemia can lead to life-threatening complications such as multi-organ failure or to patient's death. *P. knowlesi* was often misdiagnosed as *P. malariae* due to the phenotypic similarities or as *P. vivax* based on the genetic similarities until the development of a PCR-based assay specific for *P. knowlesi* [5].

Although *P. vivax* is considered a benign parasite, it elicits incapacitating clinical manifestations and life threatening complications such as severe anaemia, thrombocytopenia, and dangerous paroxysms [6].

An infection with *P. ovale* is often mistaken for an infection with *P. vivax* due to its tertian fever. Infections with either parasite species show similar symptoms and are treated similarly, the only difference is the potential severity of an infection with *P. vivax*. Furthermore infections with *P. ovale* and *P. vivax* are characterized through repeated debilitating relapses from the dormant hypnozoites that persist in hepatocytes even after clearance of the parasites [1].

Infections with *P. malariae* are characterized through low parasitaemia and a mild course of the disease.

Malaria diagnosis by microscopy of Giemsa-stained thin or thick blood smears is the gold standard method [7]. Additionally rapid diagnostic tests are commonly used, and recommended by the World Health Organization (WHO). However the sensitivity and specificity of these methods are broadly limited and differentiation of the *Plasmodium* species is barely possible with either of the techniques [8]. There is an obvious need for more sensitive diagnostic tools that are fast, accurate and allow accurate typing of *Plasmodium* species, for an effective disease management and disease control. Molecular techniques, like real-time PCR, are getting more and more popular, as these are more sensitive, reliable [9,10] and easy to use alternatives to the gold standard. Correctly used sensitive and specific diagnostic tests can avoid an unnecessary use of antimalarial drugs and contribute to an appropriate and cost-effective disease management.

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- [9] Snounou, G., S. Viriyakosol, W. Jarra, S. Thaithong, and K. N. Brown (1993), Identification of the four human malarial species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol. Biochem. Parasitol.* 58:283–292.
- [10] Sethabutr, O., A. E. Brown, S. Panyim, K. C. Kain, K. Webster, and P. Echeverria (1992), Detection of *Plasmodium falciparum* by polymerase chain reaction in a field study. *J. Infect. Dis.* 166:145–148.

6. Product Description

The RealStar® Malaria Screen & Type PCR Kit 1.0 is an *in vitro* diagnostic test, based on real-time PCR technology, for the qualitative detection and differentiation of DNA of the human pathogenic *Plasmodium* species *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium knowlesi*, *Plasmodium vivax* and *Plasmodium falciparum*.

The RealStar® Malaria Screen & Type PCR Kit 1.0 consists of two independent assays, one targeting *P. ovale*, *P. malariae* and *P. knowlesi* specific DNA and another targeting *P. vivax* and *P. falciparum* specific DNA.

Both assays include a heterologous amplification system (Internal Control) to identify possible PCR inhibition and to confirm the integrity of the reagents.

Real-time PCR technology utilizes polymerase chain reaction (PCR) for the amplification of specific target sequences and target specific probes for the detection of the amplified DNA. The probes are labeled with fluorescent reporter and quencher dyes.

Master Mix Pk/Pm/Po: The probe specific for *P. knowlesi* DNA is labeled with the fluorophore ROX™, the probe specific for *P. malariae* DNA is labeled with the fluorophore FAM™, and the probe specific for *P. ovale* DNA is labeled with the fluorophore Cy5.

Master Mix Pf/Pv: The probe specific for *P. falciparum* DNA is labeled with the fluorophore FAM™, and the probe specific for *P. vivax* DNA is labeled with the fluorophore Cy5.

The probe specific for the Internal Control (IC) is labeled with the fluorophore JOE™.

Using probes linked to distinguishable dyes enables the parallel detection of *P. knowlesi*, *P. malariae*, *P. ovale*, *P. falciparum* and *P. vivax* specific DNA as well as the detection of the Internal Control in corresponding detector channels of the real-time PCR instrument.

The test consists of two processes in a single tube assay:

- PCR amplification of target DNA and Internal Control
- Simultaneous detection of PCR amplicons by fluorescent dye labeled probes

The RealStar® Malaria Screen & Type PCR Kit 1.0 consists of:

- Master A Pk/Pm/Po¹⁾
- Master A Pf/Pv²⁾
- Master B Pk/Pm/Po¹⁾
- Master B Pf/Pv²⁾
- Internal Control
- Positive Control Pk/Pm/Po¹⁾
- Positive Control Pf/Pv²⁾
- Water (PCR grade)

¹⁾ Pk - *Plasmodium knowlesi*, Pm - *Plasmodium malariae*, Po - *Plasmodium ovale*

²⁾ Pf - *Plasmodium falciparum*, Pv - *Plasmodium vivax*

The Master A and Master B Pk/Pm/Po set contains all components (PCR buffer, DNA polymerase, magnesium salt, primers and probes) to allow PCR mediated amplification and detection of *P. knowlesi*, *P. malariae* and *P. ovale* specific DNA as well as Internal Control in one reaction setup.

The Master A and Master B Pf/Pv set contains all components (PCR buffer, DNA polymerase, magnesium salt, primers and probes) to allow PCR mediated amplification and detection of *P. falciparum* and *P. vivax* specific DNA as well as Internal Control in one reaction setup.

6.1 Real-Time PCR Instruments

The RealStar® Malaria Screen & Type PCR Kit 1.0 was developed and validated to be used with the following real-time PCR instruments:

- Mx 3005P™ QPCR System (Stratagene)
- VERSANT® kPCR Molecular System AD (Siemens Healthcare)
- ABI Prism® 7500 SDS (Applied Biosystems)
- ABI Prism® 7500 Fast SDS (Applied Biosystems)
- Rotor-Gene® 6000 (Corbett Research)
- Rotor-Gene® Q5/6 plex Platform (QIAGEN)
- CFX96™ Real-Time PCR Detection System (Bio-Rad)
- CFX96™ Deep Well Real-Time PCR Detection System (Bio-Rad)
- LightCycler® 480 Instrument II (Roche)

7. Warnings and Precautions

Read the Instructions for Use carefully before using the product.

- Before first use check the product and its components for:
 - Integrity
 - Completeness with respect to number, type and filling (see chapter 2. Kit Components)
 - Correct labelling
 - Frozenness upon arrival
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.
- Specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures.
- Wear protective disposable powder-free gloves, a laboratory coat and eye protection when handling specimens.
- Avoid microbial and nuclease (DNase/RNase) contamination of the specimens and the components of the kit.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (i) sample preparation, (ii) reaction setup and (iii) amplification/detection activities. The workflow in the laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area and change them before entering a different area.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.

- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.
- Do not autoclave reaction tubes after the PCR, since this will not degrade the amplified nucleic acid and will bear the risk to contaminate the laboratory area.
- Do not use components of the kit that have passed their expiration date.
- Discard sample and assay waste according to your local safety regulations.

8. Procedure

8.1 Sample Preparation

Extracted DNA is the starting material for the RealStar® Malaria Screen & Type PCR Kit 1.0.

The quality of the extracted DNA has a profound impact on the performance of the entire test system. It is recommended to ensure that the system used for nucleic acid extraction is compatible with real-time PCR technology. The following kits and systems are suitable for nucleic acid extraction:

- QIAamp® DNA Mini Kit (QIAGEN)
- QIAamp® DNA Blood Mini Kit (QIAGEN)
- QIAsymphony® (QIAGEN)
- NucliSENS® easyMAG® (bioMérieux)
- MagNA Pure 96 System (Roche)
- m2000sp (Abbott)
- Maxwell® 16 IVD Instrument (Promega)
- VERSANT® kPCR Molecular System SP (Siemens Healthcare)

Alternative nucleic acid extraction systems and kits might also be appropriate. The suitability of the nucleic acid extraction procedure for use with RealStar® Malaria Screen & Type PCR Kit 1.0 has to be validated by the user.

If using a spin column-based sample preparation procedure including washing buffers containing ethanol, it is highly recommended to perform an additional centrifugation step for 1 min at approximately 17,000 x g (~ 13,000 rpm), using a new collection tube, prior to the elution of the nucleic acid.

CAUTION



If your sample preparation system is using washing buffers containing ethanol, make sure to eliminate any traces of ethanol prior to elution of the nucleic acid. Ethanol is a strong inhibitor of real-time PCR.

CAUTION



The use of carrier RNA is crucial for extraction efficiency and stability of the extracted nucleic acid.

For additional information and technical support regarding pre-treatment and sample preparation please contact our Technical Support (see chapter 14. Technical Assistance).

8.2 Master Mix Setup

All reagents and samples should be thawed completely, mixed (by pipetting or gentle vortexing) and centrifuged briefly before use.

The RealStar® Malaria Screen & Type PCR Kit 1.0 contains a heterologous Internal Control (IC), which can either be used as a PCR inhibition control or as a control of the sample preparation procedure (nucleic acid extraction) and as a PCR inhibition control.

- ▶ If the IC is used as a PCR inhibition control, but not as a control for the sample preparation procedure, set up each Master Mix (Master Mix Pk/Pm/Po and Master Mix Pf/PV) according to the following pipetting scheme:

Number of Reactions (rxns)	1	12
Master A	5 µl	60 µl
Master B	15 µl	180 µl
Internal Control	1 µl	12 µl
Volume Master Mix	21 µl	252 µl

- ▶ If the IC is used as a control for the sample preparation procedure and as a PCR inhibition control, add the IC during the nucleic acid extraction procedure.
- ▶ No matter which method/system is used for nucleic acid extraction, the IC **must not** be added directly to the specimen. The IC should always be added to the specimen/lysis buffer mixture. The volume of the IC which has to be added, always and only depends on the elution volume. It represents 10 % of the elution volume. For instance, if the nucleic acid is going to be eluted in 60 µl of elution buffer or water, 6 µl of IC per sample must be added into the specimen/lysis buffer mixture.
- ▶ If the IC was added during the sample preparation procedure, set up each Master Mix (Master Mix Pk/Pm/Po and Master Mix Pf/PV) according to the following pipetting scheme:

Number of Reactions (rxns)	1	12
Master A	5 µl	60 µl
Master B	15 µl	180 µl
Volume Master Mix	20 µl	240 µl

CAUTION



If the IC (Internal Control) was added during the sample preparation procedure, at least the negative control must include the IC.

CAUTION

No matter which method/system is used for nucleic acid extraction, never add the IC directly to the specimen.

8.3 Reaction Setup

- ▶ Pipette 20 µl of the Master Mix Pk/Pm/Po or the Master Mix Pf/Pv into each required well of an appropriate optical 96-well reaction plate or an appropriate optical reaction tube.
- ▶ Add 10 µl of the sample (eluate from the nucleic acid extraction) or 10 µl of the controls (Positive or Negative Control).

Reaction Setup	
Master Mix	20 µl
Sample or Control	10 µl
Total Volume	30 µl

- ▶ Make sure that at least one Positive Control (Positive Control Pk/Pm/Po for Master Mix Pk/Pm/Po and Positive Control Pf/Pv for Master Mix Pf/Pv) and at least one Negative Control is used per Master Mix and run.
- ▶ Thoroughly mix the samples and controls with the Master Mix by pipetting up and down.
- ▶ Close the 96-well reaction plate with appropriate lids or optical adhesive film and the reaction tubes with appropriate lids.
- ▶ Centrifuge the 96-well reaction plate in a centrifuge with a microtiter plate rotor for 30 seconds at approximately 1,000 x g (~ 3,000 rpm).

9. Programming the Real-Time PCR Instrument

For basic information regarding the setup and programming of the different real-time PCR instruments, please refer to the user manual of the respective instrument.

For detailed programming instructions regarding the use of the RealStar® Malaria Screen & Type PCR Kit 1.0 on specific real-time PCR instruments please contact our Technical Support (see chapter 14. Technical Assistance).

9.1 Settings

- Define the following settings:

Settings	
Reaction Volume	30 µl
Ramp Rate	Default
Passive Reference	None

9.2 Fluorescence Detectors (Dyes)

- Define the fluorescence detectors (dyes):

Target	Master Mix	Detector Name	Reporter	Quencher
<i>P. knowlesi</i> specific DNA	Pk/Pm/Po	<i>P. knowlesi</i>	ROX™	(None)
<i>P. malariae</i> specific DNA		<i>P. malariae</i>	FAM™	(None)
<i>P. ovale</i> specific DNA		<i>P. ovale</i>	Cy5	(None)
<i>P. falciparum</i> specific DNA	Pf/Pv	<i>P. falciparum</i>	FAM™	(None)
<i>P. vivax</i> specific DNA		<i>P. vivax</i>	Cy5	(None)
Internal Control	Pk/Pm/Po and Pf/Pv	IC	JOE™	(None)

9.3 Temperature Profile and Dye Acquisition

- Define the temperature profile and dye acquisition:

	Stage	Cycle Repeats	Acquisition	Temperature [°C]	Time [min:sec]
Denaturation	Hold	1	-	95	02:00
Amplification	Cycling	45	-	95	00:15
			yes	58	00:45
			-	72	00:15

10. Data Analysis

For basic information regarding data analysis on specific real-time PCR instruments, please refer to the user manual of the respective instrument.

For detailed instructions regarding the analysis of the data generated with the RealStar® Malaria Screen & Type PCR Kit 1.0 on different real-time PCR instruments please contact our Technical Support (see chapter 14. Technical Assistance).

10.1 Validity of Diagnostic Test Runs

10.1.1 Valid Diagnostic Test Run (qualitative)

A **qualitative** diagnostic test run is **valid**, if the following control conditions are met:

Control ID	Detection Channel			
	ROX™	FAM™	Cy5	JOE™
Positive Control <i>P. knowlesi</i> , <i>P. malariae</i> , <i>P. ovale</i>	+	+	+	+/-*
Positive Control <i>P. falciparum</i> , <i>P. vivax</i>	-	+	+	+/-*
Negative Control	-	-	-	+

* The presence or absence of a signal in the JOE™ channel is not relevant for the validity of the test run.

10.1.2 Invalid Diagnostic Test Run (qualitative)

A **qualitative** diagnostic test run is **invalid**, (i) if the run has not been completed or (ii) if any of the control conditions for a **valid** diagnostic test run are not met.

In case of an **invalid** diagnostic test run, repeat testing by using the remaining purified nucleic acids or start from the original samples again.

10.2 Interpretation of Results

10.2.1 Qualitative Analysis

Table 2: Qualitative Analysis using Master Mix Pk/Pm/Po

Detection Channel				Master Mix	Result Interpretation
ROX™	FAM™	Cy5	JOE™		
+	+	+	+	Pk/Pm/ Po	<i>P. knowlesi</i> , <i>P. malariae</i> and <i>P. ovale</i> specific DNA detected.
+	-	-	+		<i>P. knowlesi</i> specific DNA detected.
-	+	-	+		<i>P. malariae</i> specific DNA detected.
-	-	+	+		<i>P. ovale</i> specific DNA detected.
+	+	-	+		<i>P. knowlesi</i> and <i>P. malariae</i> specific DNA detected.
+	-	+	+		<i>P. knowlesi</i> and <i>P. ovale</i> specific DNA detected.
-	+	+	+		<i>P. malariae</i> and <i>P. ovale</i> specific DNA detected.
-	-	-	+		Neither <i>P. knowlesi</i> nor <i>P. malariae</i> nor <i>P. ovale</i> specific DNA detected.
-	-	-	-		PCR inhibition or reagent failure. Repeat testing from original sample or collect and test a new sample.

Table 3: Qualitative Analysis using Master Mix Pf/Pv

Detection Channel				Master Mix	Result Interpretation
ROX™	FAM™	Cy5	JOE™		
N/A	+	+	+	Pf/Pv	<i>P. falciparum</i> and <i>P. vivax</i> specific DNA detected.
	+	-	+		<i>P. falciparum</i> specific DNA detected.
	-	+	+		<i>P. vivax</i> specific DNA detected.
	-	-	+		Neither <i>P. falciparum</i> nor <i>P. vivax</i> specific DNA detected.
	-	-	-		PCR inhibition or reagent failure. Repeat testing from original sample or collect and test a new sample.

* Detection of the Internal Control in the JOE™ detection channel is not required for positive results neither in the Cy5, FAM™ nor in the ROX™ detection channel. A high *Plasmodium* spp. DNA load in the sample can lead to a reduced or absent Internal Control signal.

11. Performance Evaluation

Performance evaluation of the RealStar® Malaria Screen & Type PCR Kit 1.0 was done using quantified PCR products and genomic DNA from *Plasmodium* species.

11.1 Analytical Sensitivity

The analytical sensitivity of the RealStar® Malaria Screen & Type PCR Kit 1.0 is defined as the concentration (copies/μl of the eluate) of *Plasmodium* specific DNA molecules that can be detected with a positivity rate of 95 %. The analytical sensitivity was determined by analysis of dilution series of *Plasmodium* specific PCR products (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*).

Table 4: PCR results used for the calculation of the analytical sensitivity with respect to the detection of *P. falciparum* specific DNA

Input Conc. [copies/μl]	Number of Replicates	Number of Positives	Hit Rate [%]
316.228	24	24	100
100.000	24	24	100
31.622	24	24	100
10.000	24	24	100
3.161	24	24	100
1.000	24	24	100
0.316	24	18	75
0.100	24	14	58
0.032	23	7	30
0.000	23	0	0

Table 5: PCR results used for the calculation of the analytical sensitivity with respect to the detection of *P. vivax* specific DNA

Input Conc. [copies/μl]	Number of Replicates	Number of Positives	Hit Rate [%]
316.228	24	24	100
100.000	24	24	100
31.622	24	24	100
10.000	24	24	100
3.161	24	24	100
1.000	24	24	100
0.316	24	19	79
0.100	24	5	21
0.032	24	3	13
0.000	24	0	0

Table 6: PCR results used for the calculation of the analytical sensitivity with respect to the detection of *P. ovale* specific DNA

Input Conc. [copies/μl]	Number of Replicates	Number of Positives	Hit Rate [%]
316.228	24	24	100
100.000	24	24	100
31.622	24	24	100
10.000	24	24	100
3.161	24	24	100
1.000	24	21	88
0.316	24	15	63
0.100	24	4	17
0.032	24	1	4
0.000	24	0	0

Table 7: PCR results used for the calculation of the analytical sensitivity with respect to the detection of *P. malariae* specific DNA

Input Conc. [copies/μl]	Number of Replicates	Number of Positives	Hit Rate [%]
316.228	24	24	100
100.000	24	24	100
31.622	24	24	100
10.000	24	24	100
3.161	24	24	100
1.000	24	24	100
0.316	24	23	96
0.100	24	9	38
0.032	24	2	8
0.000	24	0	0

Table 8: PCR results used for the calculation of the analytical sensitivity with respect to the detection of *P. knowlesi* specific DNA

Input Conc. [copies/μl]	Number of Replicates	Number of Positives	Hit Rate [%]
316.228	24	24	100
100.000	24	24	100
31.622	24	24	100
10.000	24	24	100
3.161	24	24	100
1.000	24	21	88
0.316	24	8	33
0.100	24	5	21
0.032	24	2	8
0.000	24	0	0

The analytical sensitivity of the RealStar® Malaria Screen & Type PCR Kit 1.0 was determined by probit analysis:

- For the detection of *P. falciparum* specific DNA, the analytical sensitivity is 0.80 copies/μl eluate [95 % confidence interval (CI): 0.44 to 2.45 copies/μl]
- For the detection of *P. vivax* specific DNA, the analytical sensitivity is 0.73 copies/μl eluate [95 % confidence interval (CI): 0.46 to 1.62 copies/μl]
- For the detection of *P. ovale* specific DNA, the analytical sensitivity is 1.46 copies/μl eluate [95 % confidence interval (CI): 0.89 to 3.28 copies/μl]
- For the detection of *P. malariae* specific DNA, the analytical sensitivity is 0.36 copies/μl eluate [95 % confidence interval (CI): 0.24 to 0.74 copies/μl]
- For the detection of *P. knowlesi* specific DNA, the analytical sensitivity is 2.35 copies/μl eluate [95 % confidence interval (CI): 1.37 to 5.55 copies/μl]

11.2 Analytical Specificity

The analytical specificity of the RealStar® Malaria Screen & Type PCR Kit 1.0 was evaluated by testing a panel of genomic RNA/DNA extracted from pathogens related to *Plasmodium* and other pathogens causing similar symptoms as *Plasmodium*.

The RealStar® Malaria Screen & Type PCR Kit 1.0 did not cross-react with any of the following pathogens:

- Chikungunya virus
- Dengue virus
- Influenza A virus
- Influenza B virus
- West Nile virus
- *Babesia microti*
- *Leishmania donovani*
- *Leishmania infantum*
- *Leishmania major*
- *Toxoplasma gondii*
- *Trypanosoma brucei*
- *Trypanosoma cruzi*

To demonstrate that the RealStar® Malaria Screen & Type PCR Kit 1.0 is able to detect and correctly differentiate DNA from *P. falciparum*, *P. vivax*, *P. knowlesi*, *P. malariae* and *P. ovale* genomic DNA of the five *Plasmodium* species was tested using a CFX96™ Deep Well Real-Time PCR Detection System (Bio-Rad) for real-time PCR analysis. Each sample was tested positive for the respective *Plasmodium* species specific DNA but negative for the other four *Plasmodium* species.

11.3 Precision

Precision of the RealStar® Malaria Screen & Type PCR Kit 1.0 was determined as intra-assay variability (variability within one experiment), inter-assay variability (variability between different experiments) and inter-lot variability (variability between different production lots). Total variability was calculated by combining the 3 analyses.

The variability data are expressed in terms of standard deviation and coefficient of variation. The data are based on threshold cycle (C_t) values. At least 6 replicates per sample were analyzed for intra-assay variability, inter-assay and inter-lot variability.

Table 9: Precision data for the detection of *P. ovale*, *P. malariae* and *P. knowlesi* specific DNA

<i>P. ovale</i> , <i>P. malariae</i> and <i>P. knowlesi</i>		Average Threshold Cycle (C _t)	Standard deviation	Coefficient of Variation (%)
Intra-Assay Variability	<i>P. malariae</i>	31.88	0.24	0.76
	<i>P. ovale</i>	30.29	0.12	0.40
	<i>P. knowlesi</i>	30.39	0.14	0.46
Inter-Assay Variability	<i>P. malariae</i>	31.89	0.18	0.58
	<i>P. ovale</i>	30.30	0.10	0.32
	<i>P. knowlesi</i>	30.53	0.14	0.45
Inter-Lot Variability	<i>P. malariae</i>	31.95	0.11	0.35
	<i>P. ovale</i>	30.26	0.11	0.35
	<i>P. knowlesi</i>	30.40	0.11	0.35
Total Variability	<i>P. malariae</i>	31.92	0.16	0.51
	<i>P. ovale</i>	30.27	0.10	0.32
	<i>P. knowlesi</i>	30.48	0.15	0.49

Table 10: Precision data for the detection of *P. falciparum* and *P. vivax* specific DNA

<i>P. falciparum</i> and <i>P. vivax</i>		Average Threshold Cycle (C _t)	Standard deviation	Coefficient of Variation (%)
Intra-Assay Variability	<i>P. falciparum</i>	31.72	0.11	0.35
	<i>P. vivax</i>	31.71	0.26	0.82
Inter-Assay Variability	<i>P. falciparum</i>	31.38	0.37	0.14
	<i>P. vivax</i>	31.57	0.24	0.77
Inter-Lot Variability	<i>P. falciparum</i>	31.42	0.33	1.04
	<i>P. vivax</i>	31.09	0.40	1.27
Total Variability	<i>P. falciparum</i>	31.29	0.33	1.04
	<i>P. vivax</i>	31.30	0.46	1.46

Table 11: Precision data for the detection of the Internal Control using Master Mix Pk/Pm/Po

Internal Control	Threshold Cycle (C _t)	Standard deviation	Coefficient of Variation (%)
Intra-Assay Variability	25.88	0.07	0.29
Inter-Assay Variability	25.64	0.27	1.05
Inter-Lot Variability	25.89	0.06	0.23
Total Variance	25.72	0.25	0.97

Table 12: Precision data for the detection of the Internal Control using Master Mix Pf/Pv

Internal Control	Threshold Cycle (C _t)	Standard deviation	Coefficient of Variation (%)
Intra-Assay Variability	26.73	0.13	0.47
Inter-Assay Variability	26.90	0.21	0.76
Inter-Lot Variability	26.96	0.13	0.49
Total Variance	26.89	0.17	0.63

11.4 Diagnostic Evaluation

The RealStar® Malaria Screen & Type PCR Kit 1.0 was evaluated in a comparative study with in-house conventional PCR [based on Rubio *et al.* (2002) and Ta *et al.* (2014)]. Retrospectively, 105 individual whole blood samples were tested:

- 75 whole blood samples from patients previously tested positive for human pathogenic *Plasmodium* spp.
- 15 whole blood samples from individual patients previously tested negative for human pathogenic *Plasmodium* spp.
- 15 whole blood samples from individual patients previously tested positive for other parasites than human pathogenic *Plasmodium* spp. causing diseases with similar symptoms like Malaria

The RealStar® Malaria Screen & Type PCR Kit 1.0 and the in-house conventional PCR [based on Rubio *et al.* (2002) and Ta *et al.* (2014)] were used in combination with the QIAamp® DNA Blood Mini QIAcube® Kit (QIAGEN) and the QIAcube® (QIAGEN).

For the qualitative analysis all samples with an invalid result for one or both assays were excluded.

Results for the remaining 105 samples are shown in Table 13.

Table 13: Results of the evaluation of the diagnostic sensitivity and specificity for *Plasmodium* spp. in whole blood samples

		In-house conventional PCR [based on Rubio <i>et al.</i> (2002) and Ta <i>et al.</i> (2014)]	
		POSITIVE	NEGATIVE
RealStar® Malaria Screen & Type PCR Kit 1.0	POSITIVE	74	0
	NEGATIVE	1	30

The diagnostic sensitivity and specificity of the RealStar® Malaria Screen & Type PCR Kit 1.0 compared to the in-house conventional PCR [based on Rubio *et al.* (2002) and Ta *et al.* (2014)] were 98.67 % (confidence interval: 92.79 % to 99.97 %) and 100.00 % (confidence interval: 88.43 % to 100.00 %), respectively.

Results for the evaluation of typing for *Plasmodium* spp. in whole blood samples are shown in Table 14.

Table 14: Results for the evaluation of typing for *Plasmodium* spp. in whole blood samples

		In-house conventional PCR [based on Rubio <i>et al.</i> (2002) and Ta <i>et al.</i> (2014)]						
		<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. malariae</i>	<i>P. ovale</i>	<i>P. knowlesi</i>	Negative	TOTAL
RealStar® Malaria Screen & Type PCR Kit 1.0	<i>P. falciparum</i>	15	0	0	0	0	0	15
	<i>P. vivax</i>	0	14	0	0	0	0	14
	<i>P. malariae</i>	0	0	15	0	0	0	15
	<i>P. ovale</i>	0	0	0	15	0	0	15
	<i>P. knowlesi</i>	0	0	0	0	15	0	15
	Negative	0	1	0	0	0	30	31
	TOTAL	15	15	15	15	15	30	105

The results in Table 14 show that with the exception of one sample the results of the RealStar® Malaria Screen & Type PCR Kit 1.0 and the reference assay were 100 % identical with respect to the identification of the *Plasmodium* spp. type.

12. Limitations

- Strict compliance with the Instructions for Use is required for optimal results.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.
- Good laboratory practice is essential for proper performance of this assay. Extreme care should be taken to preserve the purity of the components of the kit and reaction setups. All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded.
- Appropriate specimen collection, transport, storage and processing procedures are required for the optimal performance of this test.
- This assay must not be used on the specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The presence of PCR inhibitors (e.g. heparin) may cause false negative or invalid results.
- Potential mutations within the target regions of the *Plasmodium* spp. genome covered by the primers and/or probes used in the kit may result in failure to detect the presence of the pathogens.
- As with any diagnostic test, results of the RealStar® Malaria Screen & Type PCR Kit 1.0 need to be interpreted in consideration of all clinical and laboratory findings.

13. Quality Control

In accordance with the Altona Diagnostics GmbH EN ISO 13485-certified Quality Management System, each lot of RealStar® Malaria Screen & Type PCR Kit 1.0 is tested against predetermined specifications to ensure consistent product quality.

14. Technical Assistance

For customer support, please contact our Technical Support:

e-mail: **support@altona-diagnostics.com**

phone: **+49-(0)40-5480676-0**

15. Literature

Versalovic, James, Carroll, Karen C., Funke, Guido, Jorgensen, James H., Landry, Marie Louise and David W. Warnock (ed). Manual of Clinical Microbiology. 10th Edition. ASM Press, 2011.

Cohen, Jonathan, Powderly, William G, and Steven M Opal. Infectious Diseases, Third Edition. Mosby, 2010.

16. Trademarks and Disclaimers

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The RealStar® Malaria Screen & Type PCR Kit 1.0 is a CE-marked diagnostic kit according to the European *in vitro* diagnostic directive 98/79/EC.



Product not licensed with Health Canada and not FDA cleared or approved.

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17. Explanation of Symbols

Symbol	Explanation
	<i>In vitro</i> diagnostic medical device
	Batch code
	Cap color
	Catalogue number
	Content
	Number
	Component
	Global trade item number
	Consult instructions for use
	Contains sufficient for “n” tests/reactions (rxns)
	Temperature limit
	Use-by date
	Manufacturer
	Caution: Highlights operating instructions or procedures which, if not followed correctly, may result in personal injury or impact product performance. Contact Altona Diagnostics Technical Support for assistance.

Symbol	Explanation
	Note: Information is given to the user that is useful but not essential to the task at hand.
	Version

Notes:

always a drop ahead.

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