TestGene
New generation of genetics

TestGene LLC
9, 44th Inzhenerny proyezd, Ulyanovsk, 432072
TIN 7329006880 / KPP 732801001 PSRN 1127329000930
E-mail: info@testgen.ru / Phone: +7 499 705 03 75

Instruction for use

Kit for qualitative detection and differentiation of DNA/RNA of *Plasmodium* species: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* by Real-Time PCR

Plasmodium-species-test

incl. 2 configuration forms:
Plasmodium-species-test-DNA
Plasmodium-species-test-RNA

1) Manufacturer

TestGene LLC, www.testgen.ru

2) Assignment

The kit is assigned for qualitative detection and differentiation of clinically important species of the genus *Plasmodium*: *P. falciparum, P. vivax, P. malariae*, and *P. ovale* (including subspecies *curtisi* and *wallikeri* without differentiation), based on qPCR or RT-qPCR (depending on the configuration form of the kit) using nucleic acid samples obtained from whole blood of patients.

Type of analyzed sample: DNA or RNA samples extracted from whole blood of patients.

Analyzed targets are listed in table 1.

Table 1 – Analyzed targets

Fluorophores				
FAM / Green	**HEX / Yellow**	**ROX / Orange**	**Cy5 / Red**	**Cy5.5 / Crimson**
18S *Plasmodium falciparum*	IC (internal control)	18S *Plasmodium vivax*	18S *Plasmodium malariae*	18S *Plasmodium ovale*

Total time of PCR is 55–75 minutes depending on type of thermocycler (without preanalytical phase) for Configuration form 1 (*Plasmodium*-species-test-DNA) and 80–100 minutes for Configuration form 2 (*Plasmodium*-species-test-RNA).

3) Description of reagents

The kit is intended for 96 reactions that correspond to analyzing of 94 samples, PTC, and NTC in the case of parallel analyses of all samples. In the case of analysis of 1 sample per time, there 32 single samples could be analyzed (with PTC and NTC in each run).

The kit is validated on thermocyclers CFX96 (BioRad), QuantStudio 5 (Thermo Fisher Scientific), and Rotor-Gene Q (Qiagen).

Kit composition is described in table 2.

Table 2 – Kit composition

#	Reagent	Quantity and Volume
***Plasmodium*-species-test-DNA**		
1.	PCR-buffer 5x	1 tube, 480 ul
2.	Oligos mix	1 tube, 480 ul
3.	PTC (Positive Template Control)	1 tube, 480 ul
4.	NTC (Negative Template Control)	2 tubes, each 1600 ul
5.	IC (Internal Control)	1 tube, 940 ul
***Plasmodium*-species-test-RNA**		
1.	RT-PCR-buffer 5x	1 tube, 480 ul
2.	Oligos mix	1 tube, 480 ul
3.	PTC (Positive Template Control)	1 tube, 480 ul
4.	NTC (Negative Template Control)	2 tubes, each 1600 ul
5.	IC (Internal Control)	1 tube, 940 ul

4) Storage and transportation

Storage

At the temperatures from от -18 °C to -22 °C. Storage and transportation at temperatures from +2 °C to +6 °C are allowed during 30 days. It is allowed to freeze/thaw the kit no more than 10 times.

5) Clinical samples

Type of sample: nucleic acids extracted from whole blood.

Taking peripheral blood: in a test tube (vacuum tube) containing an EDTA solution, with a volume of 4- or 6-ml. Invert the tube 3-4 times immediately after taking blood to change the blood with EDTA. The use of heparin as an anticoagulant is strictly prohibited.

Transportation and storage whole blood: no more than 6 hours at a temperature from +2 °C to +8 °C; no more than 2 hours at room temperature.

DNA extraction: according to instruction of the kit for nucleic acid extraction; the recommended kit: kit for DNA/RNA Extraction from Clinical Material «NA-Extra» (manufacturer: TestGene LLC).

Important! NTC (Negative Template Control) is also going through NA-extraction. Before extraction, 10 ul of IC (Internal Control) should be added to 100 ul of whole blood prepared to NA-extraction.

6) Preparing of the kit components

Before PCR thoroughly mix the mixtures of the tubes with the extracted DNA and/or RNA, NTC (Negative Template Control), oligos mix, PCR buffer 5x, PTC (Positive Template Control), turning each tube 10 times or vortexing at low speed for 3–5 seconds, and then precipitate drops from the lids of the tubes by short centrifugation.

It could be noted that the volumes of reagents for 1 reaction: PCR buffer 5x or RT-PCR buffer 5x – 5 ul, oligos mix – 5 ul, sample (examined DNA samples, PTC, and NTC) – 15 ul. Total reaction volume is 25 ul.

7) PCR protocol

a) prepare the necessary number of PCR-tubes using the formula:

1 x examined DNA samples + 1 x PTC + 1 x NTC.

b) mark the PCR-tubes;

c) using separate 1.5 ml tube prepare master mix using the formula and components: (n+3) x 5 ul PCR buffer 5x or RT-PCR buffer 5x (depending on the configuration form of the kit) and (n+3) x 5 ul oligos mix, where n is a number of examined samples. Thoroughly mix the master mix using vortex for 3–5 s;

d) add 10 ul of master mix into all prepared PCR-tubes;

e) add 15 ul of analyzed DNA template to corresponding tubes (not to tubes for PTC and NCT);

f) add 15 ul of PTC to corresponding tube;

g) add 15 ul of NTC (after NA extraction without IC added) to corresponding tube;

h) place all tubes to thermocycler;

i) use the amplification program of table 3 for *Plasmodium*-species-test-DNA or table 4 for *Plasmodium*-species-test-RNA;

j) launch PCR/RT-PCR, start interpretation after finishing of it.

Table 3 – PCR protocol (for *Plasmodium*-species-test-DNA)

Stage	Temperature, °C	Time, minutes:seconds	Detection	Number of cycles
1	95	02:00	–	–
2	95	00:15	–	5
	64	00:20		
3	95	00:15	–	45
	64	00:20	FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson	

NOTE: if it is necessary to use several kits in a single run of thermocycler that required reverse transcription stage, it is possible to add the starting stage “52°C – 25 minutes”.

Table 4 – RT-PCR protocol (for *Plasmodium*-species-test-RNA)

Stage	Temperature, °C	Time, minutes:seconds	Detection	Number of cycles
1	52	25:00	–	–
2	95	02:00	–	–
3	95	00:15	–	5
	64	00:20		
4	95	00:15	–	45
	64	00:20	FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson	

7) Interpretation of results

Before interpretation the threshold should be defined at the level of 10–20 % of a maximum fluorescence of PTC.

In a case of using Rotor-Gene 6000, Rotor-Gene 3000 and Rotor-Gene Q (Qiagen) thermocyclers, use the functions Dynamic Tube, Noise Slope correction, set 10% Outlier Removal.

In a case of using CFX96 (BioRad) thermocycler, for correct baseline correction choose from 5 to 45 or 10 to 45 Cycles to Analyze.

At the beginning the results in PTC and NTC are analyzed, where Ct values should be as in table 5.

If the results for NTC are different from those at the table 5, all results are regarded as doubtful and possible contamination could be found. In this case it is necessary to carry out measures to eliminate possible contamination and repeat analysis.

If the results for PTC are different from those at the table 5, it is required to repeat the analysis. When re-obtaining values for PTC differ from those indicated in table 5 again, it is necessary to use another reagents.

Table 5 – Correct results for PTC and NTC

Sample	Channels (fluorophores)				
	FAM / Green (*P. falciparum*)	HEX / Yellow (BKO)	ROX / Orange (*P. vivax*)	Cy5 / Red (*P. malariae*)	Cy5.5 / Crimson (*P. ovale*)
NTC	> 35 or absent	> 35 or absent	> 35 or absent	> 35 or absent	> 35 or absent
PTC	Ct ≤ 30	Ct ≤ 32	Ct ≤ 30	Ct ≤ 30	Ct ≤ 30

Use table 6 for interpretation of examined samples.

NOTE: Invalid test results could be obtained because of inhibitors presented in sample or incorrectly conducted analysis. Doubtful or negative results could be obtained because of low concentration of NA in a sample. In the cases of doubtful or invalid results, it is necessary to repeat analysis starting from material sampling. It is recommended for doubtful results to use large volume of blood for NA extraction.

Table 6 – Interpretation for NA samples

Ct value					Results
FAM / Green	ROX / Orange	Cy5 / Red	Cy5.5 / Crimson	HEX / Yellow	
P. falciparum	*P. vivax*	*P. malariae*	*P. ovale*	IC	
absent				Ct ≤ 32	NA of *P. falciparum, P. vivax, P. malariae*, and *P. ovale* not detected
Ct ≤ 35				n/a	NA of corresponding species with Ct ≤ 35 is detected
Ct > 35 or absent				Ct > 32 or absent	Invalid result
Ct > 35				Ct ≤ 32	Doubtful result

Note: "n/a" – the value is not taken into account; "NA" – nucleic acid (DNA for *Plasmodium*-species-test-DNA and RNA for *Plasmodium*-species-test-RNA).

If NA of *P. vivax* (ROX/Orange) is detected, it could be possible to get not specific positive results for Cy5/Red and Cy5.5/Crimson. Values on Cy5/Red and Cy5.5/Crimson are taken into account, if $Ct_{Cy5} – Ct_{ROX} > 1,5$ and $Ct_{Cy5.5} – Ct_{ROX} > 1,5$.

8) Analytical efficiency

Analytical specificity: 18S of *Plasmodium* spp., including *P. falciparum, P. vivax, P. malariae*, and *P. ovale* (without differentiation of subspecies *curtisi* and *wallikeri*).

Shown absence of cross-reactivity *in vitro* with: Chikungunya virus, Dengue 1 virus, Dengue 2 virus, Dengue 3 virus, Dengue 4 virus, Zika virus, human immunodeficiency virus 1 (HIV-1), human immunodeficiency virus 2 (HIV-2), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV); *in silico*: *Bartonella quintana*, *B. henselae*, *Borrelia bisetti*, *B. garinii*, *B. japonica*, *B. spielmanii*, *Coxiella burnetii*, *Dobrava-Belgrade orthohantavirus*, Japanese Encephalitis virus, *Leptospira interrogans*, *L. kirshneri, L. borgpetersenii*, Puumala orthohantavirus, *Rickettsia conorii*, *R. hejlonjiangensis*, Tick Borne Encephalitis Virus (TBEV), *Treponema pallidum*, *Trypanosoma cruzi*, West Nile virus, and Yellow Fever virus.

Analytical sensitivity: 500 IU/ml of each species.