Instruction for use

Kit for quantitative detection of *Plasmodium* species by Real-Time PCR

Plasmodium-test-Q

Version 1 from 14.03.2022

1) Manufacturer

TestGene LLC, www.testgen.ru

2) Assignment

The kit is assigned for quantitative detection of all clinically important species of the genus *Plasmodium*, including *P. falciparum, P. vivax, P. malariae*, *P. ovale curtisi*, *P. ovale wallikeri* and *P. knowlesi* without their differentiation, based on qPCR of DNA samples obtained from whole blood of patients.

The target is highly conserved fragment of 18S rRNA.

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

Analyzed targets are listed in table 1.

Table 1 – Analyzed targets

Fluor ıphore	
FAM / Green	**HEX / Yellow**
18S *Plasmodium* spp.	IC (internal control)

Total time of PCR is 55–75 minutes depending on type of thermocycler (without preanalytical phase).

3) Description of reagents

The kit is intended for 96 reactions that correspond to analyzing of 90 samples, PTC, NTC, Calibrator 1 and Calibrator 2 in the case of parallel analyses of all samples. In the case of analysis of 1 sample per time, there 13 samples could be analyzed (with PTC, NTC, Calibrator 1 and Calibrator 2 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
1.	PCR-buffer 5x	1 tube, 480 ul
2.	Oligos mix	1 tube, 480 ul
3.	PTC (Positive Template Control)	1 tube, 195 ul
4.	NTC (Negative Template Control)	1 tube, 1300 ul
5.	IC (Internal Control)	1 tube, 940 ul
6.	Calibrator 1	1 tube, 1300 ul
7.	Calibrator 2	1 tube, 1300 ul

Calibrator 1 contains DNA template equal to $1x10^6$ IU/ml DNA of *Plasmodium* spp.

Calibrator 2 contains DNA template equal to $5x10^3$ IU/ml DNA of *Plasmodium* spp.

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: DNA 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: TestGen LLC).

Important! Calibrator 1, calibrator 2 and NTC (Negative Template Control) are also going through DNA-extraction. Before extraction, 10 ul

of IC (Internal Control) should be added to 100 ul of whole blood, calibrator 1 and calibrator 2.

6) Preparing of the kit components

Before PCR thoroughly mix the mixtures of the tubes with the extracted DNA, 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 – 5 ul, oligos mix – 5 ul, sample (examined DNA samples, PTC, NTC, Calibrator-1, Calibrator-2) – 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] + 2 x Calibrator-1 + 2 x Calibrator-2 + 1 x PTC + 1 x NTC.

b) mark the tubes;

c) using separate 1.5 ml tube prepare PCR-mix using the formula and components: (n+8) x 5 ul PCR buffer 5x and (n+8) x 5 ul oligos mix, where n is a number of examined samples. Thoroughly mix the mixture using vortex for 3–5 s;

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

e) add 15 ul of analyzed DNA template to corresponding tubes

(not to tubes for PTC, NCT and Calibrators);

f) add 15 ul of PTC to corresponding tube;

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

h) add 15 ul of Calibrator-1 (after DNA extraction with added IC)

to 2 corresponding tubes;

i)add 15 ul of Calibrator-2 (after DNA extraction with added IC)

to 2 corresponding tubes;

[1] To improve accuracy, it is recommended to analyze each sample in duplicate.

j)place all tubes to thermocycler;

k) use the amplification program of table 3; type of the analyses:

quantitative with standards;

l) launch PCR, after finishing of it start interpretation.

Table 3 – PCR protocol

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	

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".

7) Interpretation of results

Before interpretation the threshold should be defined at the level of 5–10 % of a maximum fluorescence of PTC.

In a case of using Rotor-Gene 6000, Rotor-Gene 3000 and RotorGene 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 4.

If the results for NTC are different from those at the table 4, 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 4, it is required to repeat the analysis. When re-obtaining values for PTC differ from those indicated in table 4 again, it is necessary to use another reagents.

Table 4 – Correct results for PTC and NTC

Sample	Channels (fluorophores)	
	FAM / Green (*Plasmodium* spp.)	HEX / Yellow (IC)
NTC	> 35 or absent	> 35 or absent
PTC	Ct ≤ 30	Ct ≤ 32

a) Based on Ct values for Calibrator-1 (concentration: 10^6 IU/ml) and Calibrator-2 (concentration $5x10^3$ IU/ml), the calibration curve should be constructed. PCR efficiency must be equal to 90–110 %, otherwise all results will be not valid and analysis should be repeated again starting from DNA extraction.

Wrong value of PCR-efficiency could be a result of DNA extraction or degradation of Calibrator-1 or Calibrator-2 during storage or transportation (due to low concentration of Calibrator-2, its concentration could be changed during wrong conditions of storage and transportation). It is recommended to use another Calibrator-2 obtained by 200-fold dilution of Calibrator-1.

b) Calculate concentrations of for analyzed samples based on their Ct values and constructed calibration curve. Concentration measurement accuracy: ±0.5 lg concentration.

NOTE: The Ct values ≤ 35 are taken into account. If a Ct value > 35 is obtained for analyzed samples (with a Ct value of IC ≤ 32), the result is considered as doubtful.

NOTE: If volume of whole blood exceeded than 100 ul was used for DNA extraction with volume of calibrators 100 ul, it is necessary to make recalculation of the concentrations using coefficient 100/V, where V is used volume of whole blood.

c) see table 5;

NOTE: Invalid result 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 DNA 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 DNA extraction.

Table 5 – Interpretation for DNA samples

Channels (fluorophores)		Indicated Result
FAM / Green (*Plasmodium* spp.), IU/ml	HEX / Yellow (IC), Ct values	
$10^3 – 10^7$	n/a	specified concentration in IU/ml in whole blood
$< 10^3$	n/a	"less than 10^3 IU/ml in whole blood"
$> 10^7$	n/a	"more than 10^7 IU/ml in whole blood"
absent	≤ 32	negative result (concentration is not indicated)
absent	absent	invalid result

Note: "n/a" – the value is not taken into account.

8) Analytical efficiency

Analytical specificity: 18S of *Plasmodium* spp., including *P. falciparum, P. vivax, P. malariae*, *P. ovale* (including subspecies *curtisi* and *wallikeri*), and *P. knowlesi*.

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* with: *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.

Linear range of determined concentrations: $10^3 – 10^7$ IU/ml.