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Diatheva COVID-19 PCR kit

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1. INTENDED USE

Diatheva COVID-19 PCR kit is a multiplex real-time RT-PCR one-step assay for the qualitative detection of RNA from Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in upper and lower respiratory samples during the acute phase of infection.
Positive results are indicative of the presence of RNA from SARS-CoV-2. Positive results do not rule out the possibility to have co-infections caused by the presence of other viruses or bacteria. To determine the patient's infection status, clinical correlation with the patient's history and other diagnostic information is required. Negative results do not exclude infections caused by SARS-CoV-2 and cannot be used as unique information for the clinical management of patients. The use of the product is intended for use by well trained personnel in the techniques of real-time PCR and *in vitro* diagnostic procedures. It is recommended to use the kit according to the instructions below

2. INTRODUCTION

SARS-CoV-2 virus, a single-stranded RNA virus, is the cause of coronavirus 2019 respiratory disease (COVID-19) [WHO, March 2, 2020]. SARS-CoV-2 is contagious to humans and the World Health Organization (WHO) has designated the current COVID-19 pandemic as a Public Health Emergency of International Concern. PCR testing of asymptomatic or mildly symptomatic patients can be considered in the clinical evaluation of individuals who have come into contact with a COVID-19 case. The test is based on the guidelines issued by the WHO.

3. PRINCIPLES OF THE ASSAY

The Diatheva COVID-19 PCR kit is a multiplex One-step Retro Transcriptional PCR (RT-PCR) assay based on the use of labeled probes to confirm the presence of SARS-CoV-2 RNA by simultaneous amplification of the **ORF1b** and **N** targets.
The assay also includes the **RNase P** target as an internal positive control (IC) to monitor that the sample is of acceptable quality, to monitor the RNA extraction process and the presence of PCR inhibitors.
The target regions and fluorophores are shown in Table 1:

Table 1: Target regions and fluorophores

Target Region	Fluorophore
ORF1b	FAM
N	Cal Fluor Red 610
RNase P	Quasar 670

4. KIT CONTENT

The kit provides all the reagents required for the analysis (Table 2). PCR negative and positive control are included.

Table 2: Kit content

Reagent	No vial x Volume	Cap colour
Mix 1	1 X 550 µL	Green
Mix 2	1 X 70 µL	Orange
Mix Primer/Probe	1 X 1040 µL	Blue
PCR Negative Control	1 X 100 µL	Clear
PCR Positive Control	1 X 50 µL	Red

5. REQUIRED MATERIAL NOT SUPPLIED

Disposable powder-free gloves
RNA isolation kit
Pipettes (adjustable)
Sterile pipette tips with aerosol-preventive filter
Vortex mixer
Bench microcentrifuge
Real-time PCR instrument
Consumables compatible with the real-time PCR instrument. White and clear plates, clear 8-well tubes strips, optical sealing foils and optical 8 caps tubes.
Sterile 1.5 mL tubes
Laboratory freezers

The kit is validated and can be used with the follow extraction systems and thermalcyclers:

Validated extraction methods

- QIAamp MinElute Virus spin kit (Qiagen)
- QIAamp Viral RNA Mini kit (Qiagen)
- High Pure Viral RNA/DNA Kit (Magen)
- Total RNA Purification kit (Norgen)
- MGISP-960 platform using the Virus DNA/RNA Extraction kit (MGI)
- KingFisher™ Flex Purification System using MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit (Thermofisher)
- Nextractor NX48-S platform using the NX48S VIRAL NA EXTRACTION KIT (Genolution)

For use with other extraction methods and/or Real-Time PCR instruments please contact Diatheva.

Thermalcyclers

- CFX96 Biorad
- ABI7500 Applied Biosystems
- ABI7500 Fast Applied Biosystems
- Rotor gene Q 5-plex Biorad
- Quant Studio 5 Applied Biosystems

6. ASSAY LIMITATIONS

- Samples must be collected, transported and stored using appropriate procedures and conditions. Improper sample collection, transfer, storage and processing may cause erroneous test results.
- The kit uses purified RNA as a sample for the analysis. The quality of the RNA recovered from biological samples is essential for the quality of results generated with this kit.

False negative results may arise from:

- Improper sample collection
- Degradation of viral RNA during shipping/storage
- Using poor extraction method
- The presence of RT-PCR inhibitors
- Possible polimorfisms in the target regions where the sequences hybridize (primers and probes) may affect the target detection
- Failure to follow the instruction for use

False positive results may arise from:

- Cross contamination during specimen collection, handling or preparation
- RNA contamination during product handling

7. STORAGE CONDITIONS AND STABILITY OF THE REAGENTS

Upon arrival, store at -20° C. If stored at the recommended temperature all reagents are stable until the expiration date.
The performance of kit components is not affected for up to 5 freezing and thawing. If the reagents are to be used only intermittently, they should be stored in aliquots.

8. WARNING AND PRECAUTIONS

Before using the kit read the Product Information carefully and completely.
The operator should always pay attention to:

- Set up pre- and post-PCR areas. Do not share instruments or consumables (pipettes, tips, tubes, etc) between those areas;
- Store positive material separately from all other reagents and, if possible, add it to the reaction mix in a separated space;
- Do not use any reagent after the expiration date indicated on the label;
- Wear powder-free gloves during all procedures;
- Thaw all kit components and protect from light before starting the assay. After thawing, mix the components and centrifuge briefly;
- Do not substitute or mix reagents from different batches in order to maintain optimal performances;
- Include in each run at least 1 PCR Negative control and 1 PCR Positive Control;
- Minimize sample handling;
- Change gloves frequently;
- Wash the bench surfaces with 5% sodium hypochlorite;
- Use sterile disposable laboratory materials and do not re-use the tubes and tips;
- Store the reagents at the recommended temperature;
- Dispose waste in compliance with the local regulations;
- The quality of the sample preparation may influence the quality of the RT-PCR test.

9. INSTRUCTION FOR USE

9.1. SAMPLE PREPARATION

The samples should be extracted according to the corresponding requirements and procedures of the viral RNA extraction kits [https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance].
The kit has been tested on RNA samples extracted using the extraction methods listed on page 2. For additional extraction methods not listed in the table please contact Diatheva. The extracted RNA samples can be stored at below -70°C, avoiding repeated freeze-thaw cycles.

9.2. SETUP REAL TIME PCR INSTRUMENT

Before preparing the reaction mix, program the real-time PCR instrument with the following thermal profile:
Note: For use with ABI7500 fast Instrument select "ramp speed standard"

Table 3: Thermal profile setting

Phase	Temperature and time	Cycles
cDNA synthesis	48°C 20 min	1 X
Initial denaturation	95°C 2 min	1 X
Denaturation	95°C 10 sec	45 X*
Annealing-extension*	58°C 1 min	

*fluorescence of different fluorophores is detected during the **annealing-extension** step in the channels specified in the following table

Table 4: Fluorescence channels setting

Multiplex	Fluorescence reading channels		
	FAM (Green Channel)	**ROX/ Cal Fluor Red 610 (Orange Channel)**	**Cy5/ Quasar 670 (Red Channel)**
Target	ORF1b	N	RNaseP

- The final reaction volume is **20 µL**.
- **For each target, in the respective "Quencher" box, select "None" in the requesting instruments,** i.e.: ABI 7500, QS5
- **For requesting instruments, select "None" in the function "Select dye to use as passive reference"** i.e.: ABI 7500, QS5

9.3. REACTION SETUP

All the experiments must include a PCR negative control (NTC-No Template Control), containing all the reaction components except for the template. This enables the detection of potential contamination.

- Thaw the components protect from light.
- Gently vortex Mix 1 and Mix Primer/Probe for 6 sec and centrifuge briefly. Gently vortex Mix 2 for 2 sec and centrifuge briefly.
- In one sterile tube, prepare the amplification reaction mix (Master mix) needed for each sample to be tested plus one Negative control and one Positive control, following the scheme shown in table 5:

Table 5: Mastermix preparation

Reagent	Reagent volume for 1 reaction*
Mix 1	5 µL
Mix 2	0.625 µL
Mix Primer/Probe	9.375 µL
Total volume	15 µL

*For the analysis of more than one sample, simply multiply the volumes of Mix 1, Mix 2 and Mix Primer/Probe for the number of samples to be tested considering the NTC and Positive Control.

- Gently vortex the tube containing the Mastermix for 6" and centrifuge briefly
- Aliquot **15 µL** of Mastermix in the PCR tubes or in the wells of the plate prepared for the experiment for each sample to test, PCR Negative Control and PCR Positive Control.
- Add the PCR Negative Control in the corresponding tube/well
- In a separate area, add **5 µL** of RNA samples to be analyzed and the Positive Control in the corresponding wells in which the Mastermix has been previously aliquoted.
- Seal hermetically the PCR plate/tubes and load into the real-time PCR instrument, following the manufacturer's instructions

NOTE: It is recommended to centrifuge the tubes or the PCR plate before inserting them into the thermalcycler to eliminate air bubbles and collect all the reagents at the bottom of each well. Verify that the liquid is at the bottom of each well, otherwise centrifuge again at higher rpm for a longer time

9.4. ANALYSIS AND INTERPRETATION OF RESULTS

The analysis of the results must be performed using the PCR software of each specific instrument and referring to the manual for detailed information. Set the baseline and threshold values. Some software performs data analysis automatically, in this case it is advisable to check these settings. For manual data analysis, analyze the PCR file separately for the four fluorophores.

NOTE: for a correct result interpretation, pay attention to the amplification curves. In particular, verify that the threshold is set to intersect the exponential phase of the fluorescence curve and above any background signal. The threshold value of different instruments may vary due to different signal intensities.

Using the instruments CFX96, Rotorgene Q 5-plex, ABI 7500, QuantStudio5 refer respectively to **appendices A, B, C, D** for the set-up of the analysis parameters.

Before interpreting the sample results it is necessary to verify the validity of the PCR analysis.
Proceed with checking the controls based on the following table:

Table 6: Reaction controls check

Control	Fluorescence Channel		
	FAM (Green Channel)	**ROX/Cal Fluor red 610 (Orange Channel)**	**Cy5/Quasar 670 (Red Channel)**
PCR Positive Control	Positive	Positive	Positive
PCR Negative Control (NTC)	No amplification signal	No amplification signal	No amplification signal

If the run is valid, continue with the interpretation of the sample results according to the table below.
Alternatively, if the run is not valid, please repeat the test.

The following table lists an example of expected results for the kit with valid positive and negative control. The Ct cut-off values shown below were derived from product verification and validation studies. The user should determine the specific Ct cut-off values to achieve optimal performance.

Table 7: Analysis of results

Amplification			Interpretation of results
Target: ORF1b	**Target: N**	**Target: RNaseP**	
FAM (Green Channel)	**ROX/ Cal Fluor Red 610 (Orange Channel)**	**Cy5/ Quasar 670 (Red Channel)**	
≤40	≤40	≤35*	Positive to SARS-CoV-2 RNA
N/A	≤40	≤35*	Positive to SARS-CoV-2 RNA
≤40	N/A	≤35*	Positive to SARS-CoV-2 RNA
N/A	N/A	≤35	Negative to SARS-CoV-2 RNA
N/A	N/A	>35 o N/A	Invalid result (repeat the test starting from the extraction step). If the same result is obtained again, a new sampling must be required.

* The amplification of the RNaseP internal control may be absent when in presence of positive samples in which the viral target genes are preferentially amplified.

Note: In the case of samples with very low SARS-CoV-2 titer, near or below the detection limit, it is possible that one of the two targets of the pathogen, the ORF1b gene or the N gene, may be randomly negative due to low number of copies. Since the two target genes, ORF1b and N, are both specific for SARS-CoV-2, a positive result for only one of the two targets is sufficient to attribute SARS-CoV-2 positivity to the tested sample.

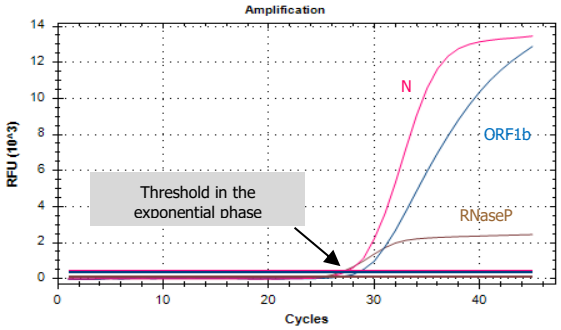
1. TROUBLESHOOTING

OBSERVATION	PROBABLE CAUSES	SOLUTION
No amplification signal for positive control and samples	Instrument not correctly set up	Verify the thermal protocol settings and repeat the test with the right settings
	Wrong reagent conservation or use of the reagents beyond the expiration date.	Verify the storage conditions and use a new kit if necessary
No amplification signal or very low signal of endogenous internal control (RNase P)	Pathogen's nucleic acid in high concentration	If in presence of a pathogen amplification signal but not the internal control signal probably the Internal Control is inhibited by the high viral titer.
	Incorrect sample collection	Repeat the sample collection and repeat the test from the RNA extraction step.
	Problem in the extraction process	Repeat the test starting from the extraction step
Presumed false positive or target signal in the PCR Negative Control	Contamination	Decontaminate all the surfaces and the work areas with sodium hypochlorite. Use filter tips. Repeat the entire procedure starting from the extraction step.
Presumed false negative or signal absence for the positive control	Wrong sample collection	Verify the sample collection method.
	Wrong RNA sample conservation	Collect the sample again and repeat the entire procedure starting from the extraction step following the recommendations.
	Incorrect nucleic acid extraction	Verify the extraction protocol and repeat the entire procedure
	Presence of PCR inhibitors	Dilute the sample
	Wrong nucleic acid addition in the amplification tube/well	Verify the correct sample addition to the corresponding amplification tube/well basing on the extraction method used.
	Incorrect reaction mix preparation	Confirm the addition of all the components to the reaction mix. All the reagents should be well mixed and centrifuged before the use.
Presence of abnormal fluorescence signals	Amplification reaction not centrifuged	Repeat the amplification reaction after spinning the plate and/or the tubes before starting.
	Sample evaporation in the thermal cycler due to erroneous reaction tubes' sealing	Repeat the amplification reaction making sure to properly seal the plate or the tubes before starting.
	Not suitable thermal cycler plastics	Contact Diatheva and repeat the test with the suggested plastics
	Presence of bubbles in the reaction tube/well	Repeat the amplification reaction after spinning the plate and /or the tubes, verify the absence of bubbles before starting the reaction.

APPENDIX A - CFX96 Analysis parameters

In the Setting topic select "baseline setting" and set up "baseline substracted curve fit". To set the threshold, manually move the line in the exponential phase of the fluorescence curves (Fig. 1). If in some samples the fluorescence signal has an anomalous trend or artifacts appear, it is possible to improve the trend of the curves by selecting "settings", "Baseline threshold": select the samples with anomalous trend and set the value 20 in the respective "baseline end " box

Fig 1: Parameter setting and data analysis on CFX96

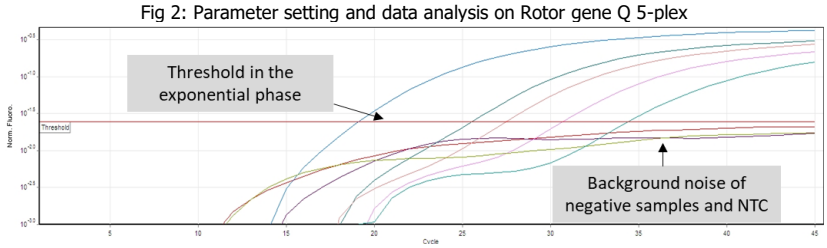


APPENDIX B - Rotor gene Q 5-plex Analysis parameters

For each fluorophore proceed with the analysis as follows:

Select the "Dinamic Tube" function. Select the "Ignore First" function by entering the value 10 in the "Cycles" box. To set the threshold, manually move the line in the exponential phase of the fluorescence curves (Fig. 2).

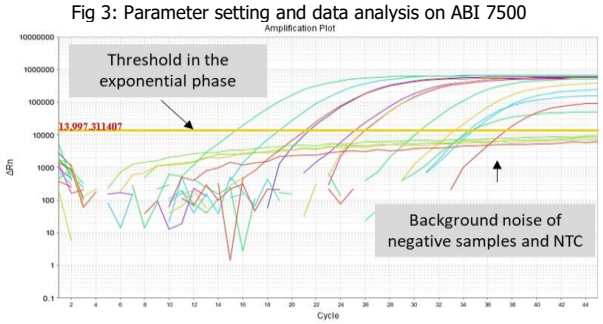
Fig 2: Parameter setting and data analysis on Rotor gene Q 5-plex



APPENDIX C - ABI 7500 Analysis parameters

For analysis, in the screen "Amplification plot" and function "Options", deselect "Auto" respectively at the threshold and select "Auto Baseline". To set the threshold, manually move the line in the exponential phase of the fluorescence curves (Fig. 3).

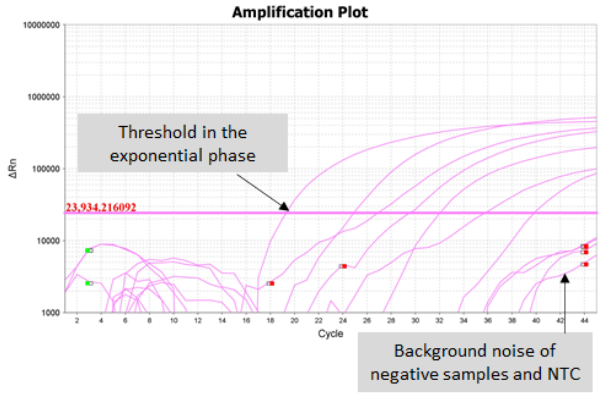
Fig 3: Parameter setting and data analysis on ABI 7500



APPENDIX D - Quant Studio 5 Analysis parameters

For the analysis, on the "Results" screen, select "Amplification Plot" from the drop-down menu. Maintain the setting "Auto Baseline" and manually move the threshold above the background noise (Fig. 4).

Fig 4: Parameter setting and data analysis on Quant Studio 5



APPENDIX E – KIT PERFORMANCES

ANALYTICAL SPECIFICITY

The analytical specificity of the *Diatheva COVID-19 PCR kit* is guaranteed by the careful and specific choice of primers and probes published in the international guidelines issued by WHO and CDC.

ANALYTICAL SENSITIVITY

The analytical sensitivity of the Diatheva COVID-19 PCR kit was determined using the TWIST SYNTHETIC SARS-CoV-2 RNA CONTROL 2 genomic RNA (Cat. No. 102024, TWIST BIOSCIENCE). The RNA was subjected to serial dilutions in a pool of RNA previously diagnosed as negative for SARS-CoV-2. Results were analyzed by Probit analysis (p = 0.005, 95% CI) on the CFX96 instrument. The LOD determined for the N gene corresponds to 5.37 copies / PCR while for the ORF1b gene it corresponds to 6.47 copies / PCR.

REPEATABILITY

The repeatability of the test (intra-assay variability) was assessed by analysing 3 replicates of 3 different clinical samples in one run on CFX96 instrument (Biorad). Tested samples include a high SARS-CoV-2 positive sample, a medium SARS-CoV-2 positive sample, a low SARS-CoV-2 positive sample. The percentage coefficient of variation (CV%) of the ORF1b, N and RNase P is shown in Table A and it is always <2.5:

Table A: mean Ct and CV% of SARS-CoV-2 positive samples

	Mean Ct value (CV%)		
	High positive SARS-CoV-2 sample	**Medium positive SARS-CoV-2 sample**	**Low positive SARS-CoV-2 sample**
ORF1b	18.68 (2.46)	25.53 (0.29)	32.32 (0.41)
N	15.42 (2.16)	22.96 (0.40)	29.70 (0.40)
RNase P	24.28 (1.08)	24.48 (0.45)	23.78 (0.14)

REPRODUCIBILITY

The reproducibility of the assay (inter-assay variability) was evaluated by analyzing 3 clinical samples in two different runs on the CFX96 instrument. Tested samples include a high SARS-CoV-2 positive sample, a medium SARS-CoV-2 positive sample and a low SARS-CoV-2 positive sample. The percentage coefficient of variation (CV%) of the ORF1b, N and RNase P is shown in Table B and it is always <3.0:

Table B: mean Ct and CV% of SARS-CoV-2 positive samples

	Mean Ct value (CV%)		
	High positive SARS-CoV-2 sample	**Medium positive SARS-CoV-2 sample**	**Low positive SARS-CoV-2 sample**
ORF1b	19.14 (1.03)	26.63 (1.22)	33.16 (0.45)
N	15.44 (1.83)	23.16 (1.53)	29.92 (1.44)
RNase P	25.43 (2.14)	25.77 (2.96)	24.82 (2.39)

ROBUSTNESS

The robustness of the method was evaluated by analyzing the impact of different operators and different thermal cyclers (Table C).

Table C: Positive samples diagnosed in different thermal cyclers by different operators

	Diagnosed positive samples/Total positive samples	**% Diagnosed samples**
CFX96 (Operator 1)	85/85	100
ABI 7500 (Operator 2)	40/40	100
QuantStudio 5 (Operator 3)	36/36	100
RGQ 5-plex (Operator 2)	52/52	100

For all instruments and all operators, 100% agreement was obtained with respect to the diagnosis of the samples.

CLINICAL EVALUATION

Clinical specificity was evaluated by testing 67 SARS-CoV-2 negative samples and clinical sensitivity was assessed by testing 186 SARS-CoV-2 positive samples. All samples analyzed were diagnosed by CE-IVD marked RT-PCR methods. RNAs were extracted using the validated extraction kits. Results are summarized in Table D.

Table D: Specificity, sensitivity and Kappa of Cohen value for SARS-CoV-2

SARS-CoV-2		**CE-IVD Competitor**		
		Positive	**Negative**	**Total**
Diatheva COVID-19 PCR kit	**Positive**	186	0	186
	Negative	0	67	67
	Total	186	67	253

- Clinical sensitivity: 100%
- Clinical specificity: 100%
- Kappa of Cohen value: 1.00 (perfect concordance)