



Via Sant'Anna 131-135
61030 Cartoceto PU (IT)
Telephone +39 (0)721 830605
FAX +39 (0)721837154
e-mail: info@diatheva.com
www.diatheva.com



COVID-19 Automatic PCR DIATHEVA Detection kit

Version: **November 2020**



INTENDED USE

COVID-19 Automatic PCR DIATHEVA Detection kit allows the qualitative detection of SARS-CoV-2-RNA in upper and lower respiratory samples during the acute phase of infection. Positive results are indicative of the presence of SARS-CoV-2 RNA; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Positive results do not rule out bacterial infection or co-infection with other viruses. Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological observations. Testing with the kit is intended for use by laboratory trained personnel in the technique of real-time PCR and in vitro diagnostic procedures.

INTRODUCTION

Coronaviruses are a large family of viruses that are common in people and many different species of animals including camels, cattle, cats, and bats. In December 2019, a cluster of patients with a novel coronavirus was identified in Wuhan, China. The virus named SARS-CoV-2 can cause the disease named coronavirus disease 2019 (COVID-2019) [WHO, 2 March 2020]. PCR testing of asymptomatic or mildly symptomatic contacts can be considered in the assessment of individuals who had contacted with a COVID-19 case. The test is based on the WHO guideline [Berlin protocol, 2nd revision Corman et al. 2020].

PRINCIPLE OF THE ASSAY

The **COVID-19 Automatic PCR DIATHEVA Detection kit** is a One-step real-time reverse transcription (RT-PCR) multiplex assay based on fluorescent-labelled probe used to confirm the presence of SARS-CoV-2-RNA by amplification of **RdRp** and **E gene**. The assay includes also **RNase P** target as internal positive control (IC) monitoring that the specimen is of acceptable quality, the RNA extraction process and also the presence of PCR inhibitors. The kit provides all the reagents required for the analysis (Table 1). PCR positive and PCR negative controls are also included.

KIT CONTENTS

Table 1: KIT content

Reagent	No vial x Volume
Mix 1	1 X 690 µL
Mix 2	1 X 86 µL
Mix Primer/Probe	2 X 880 µL
PCR Negative Control	1 X 100 µL
PCR Positive Control	1 X 50 µL

REQUIRED MATERIALS NOT SUPPLIED

This new revision of the IFU contains implementations on instruments, plastic and extraction methods compatible with the product. Composition, use and performance of the product remain completely unchanged.

- Disposable powder-free gloves
- RNA isolation kit
- Pipettes (adjustable)
- Sterile pipette tips with aerosol-preventive filters
- Vortex mixer
- Bench Microcentrifuge
- Real-time PCR instrument
- Consumables for real-time PCR instruments: Clear and white plastic; Optical Adhesive Film; Cap tubes

- 1.5mL tubes
- Laboratory freezers -30°C to -10°C/-70°C
- Cold block or ice

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.
- Diatheva can't respond of results obtained using instruments or accessorize other than recommended in the user manual
- 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
 - Mutation in the SARS-CoV-2 virus
 - Failure to follow instruction for use
- False positive results may arise from:
 - Cross contamination during specimen collection handling or preparation
 - RNA contamination during product handling

SHIPPING AND STORAGE CONDITIONS

Shipping in dry ice. Upon arrival, store at -20°C. If stored at the recommended temperature all reagents are stable until the expiration date.

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;
- Positive results are indicative of the presence of SARS-CoV-2 RNA
- The quality of the sample preparation may influence the quality of qPCR test.

INSTRUCTIONS FOR USE

1. SAMPLE PREPARATION

The samples should be extracted according to the corresponding requirements and procedures of viral RNA extraction kits [<https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance>]. The kit was tested on both RNA samples extracted in automatic and manual mode with the methods shown in the table 4, section 3. For extractions with methods other than those listed above, please contact Diatheva.

The extracted RNA sample can be stored at below -70°C, avoiding repeated freeze-thaw.

2. PROGRAM SETUP

Program the PCR instrument with the following thermal profile (Table 2) before preparing the reaction mix

Table 2: Thermal Profile setting

Step	Temperature and Times	Cycles
cDNA Synthesis	48°C 10 min	1 X
Initial denaturation	95°C 10 min	1 X
Denaturation	95°C 15 sec	50 X**
Annealing-extension*	58°C 30 sec	

*Fluorescence is detected during **annealing-extension** step on:

green channel (FAM /465-510) for the target **RdRp**;
 yellow channel (VIC/Cal Fluor orange 560 /533-580) for the **E gene**;
 red channel (quasar 670/Cy5/618-660) for **RNase P**.

** Use the setting with 45 cycles with the Rotor gene Q instrument

- Select **ROX** as passive reference dye for instruments that require it (es. Applied Biosystems).
- For the LC480 set the thermal profile maintaining the standard ramp rate
- The final reaction volume is **25 µL or 27 µL (to insert final reaction volume consider the RNA sample volume to be added according to the section 3, Table 4 based on the extraction system used)**.
- For the Rotor gene Q tool, place the NTC of the COVID-19 MIX in position 1 and set the "Auto-gain optimization setup" function, select the "Perform optimization before 1st acquisition" function. Finally select "Optimize acquiring" by selecting all the fluorescence reading channels related to each target.
- For the Light Cycler 480 II instrument, keep the default ramp rate settings: 4.4 °C/S for the 48 °C step for 10 minutes and 2.2 °C/S for the other steps.

3. PCR MIX PREPARATION

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

- Thaw the components protect from light. Vortex Mix 1, Mix Primer/Probe for 15 sec and centrifuge briefly. Vortex Mix 2 for 2 sec and centrifuge briefly.
- In one sterile 1.5 ml 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 pipette scheme reported in the Table 3:

Table 3: Master mix preparation

Reagent	Volume for 1 reaction*
Mix 1**	6.25 µL
Mix 2**	0.78 µL
Mix Primer/Probe	15.97 µL
Total volume	23 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.
 ** Reagents Mix 1 and Mix 2 contains component provided by Solis BioDyne

- Vortex for 15" the vial containing the prepared Master mix and centrifuge briefly
- Aliquot **23 µL of Master mix** in the PCR tubes or in the plate prepared for the experiment
- In a separate area, add PCR Negative control, RNA samples to be tested and PCR Positive control according to the table below into the corresponding PCR tubes or wells containing amplification mix:

Table 4: Samples and Controls loading

Extraction type	RNA sample volume
MGISP-960 platform using the Virus DNA/RNA Extraction kit	2 µL
- KingFisher™ Flex Purification System using MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit -Total RNA Purification kit (Norgen) -High Pure Viral RNA/DNA Kit (Magen) -Nextractor NX48-S platform using the NX48S VIRAL NA EXTRACTION KIT (Genolution) - OMNIA LH75 platform using the NucleoMag Pathogen (Macherey-Nagel) - RNeasy Mini kit (Qiagen) - QIAamp MinElute Virus spin kit (Qiagen)	4 µL
WARNING: In the case of using different extraction kits than those recommended, it is the user's responsibility to verify with standard samples (eg. VEQ) that this extraction does not lead to a reduction in the performance of the analysis system	

Seal hermetically the PCR plate or tubes and load into the real-time PCR instrument, following the manufacturer's instructions

Note: check that the reagents are at the bottom of each well, if not, centrifuge at 800 x g for 1 minute.

4. ANALYSIS OF RESULTS

The analysis of the results should be done with the PCR instrument program, please refer to the manual for detailed information. Set the baseline and threshold values. Some software performs the data analysis automatically in this case it is advisable to check these settings. For a manual data analysis, analyze the PCR file for the three fluorophores separately. Using the instruments CFX96, Rotorgene Q 5-plex, ABI 7500, Quant Studio 5, Light Cycler 480II refer respectively to appendices **A, B, C, D, E** for the set-up of the analysis parameters

4.1 Run validity

Before interpreting the results relating to the samples, it is necessary to check the validity of the PCR analysis. Proceed with checking the controls based on the following table:

Table 5: Check reaction Controls

Control ID	Detection Channel		
	FAM 465-510 (Green channel)	VIC/Cal Fluor orange 560 533-580 (Yellow channel)	Quasar670/Cy5 618-660 (Red channel)
PCR Positive Control	Positive	Positive	Positive
PCR Negative Control (NTC)	No amplification signal	No amplification signal	No amplification signal*

*the presence of aspecific amplification signal (Ct value \geq 45) can appear but it can be considered as trascurable

4.2 Interpretation of results

If the run is valid, continue with the interpretation of the sample results according to the table 6. Alternatively, if the run is not valid please repeat before to proceed.

Table 6: Interpretation of results

Ct value			Interpretation of Result
RdRp	E	RNaseP (IC)	
Positive	Positive	\leq 45*	SARS-CoV-2 RNA Positive
Positive	N/A	\leq 45*	
N/A	Positive	\leq 45	SARS-CoV-2 RNA Negative**
N/A	N/A	\leq 45	SARS-CoV-2 RNA Negative
N/A	N/A	N/A	Invalid result (re-test)

*A sample is considered positive if the Ct value obtained is equal or less than 45. However, sometimes, this target cannot be amply due to preferential amplification of RdRp or E.

** Repeat the test. Possible infection with others Coronavirus

5. INSTRUMENT COMPATIBILITY

The kit has been validated and can be used with the following thermal cyclers:

- CFX96 Biorad
- ABI 7500 Applied Biosystem
- Rotor gene Q 5-plex Qiagen
- Quant Studio 5 Applied Biosystems
- Life Cycler 480 II Roche

For use with other Real-Time PCR instruments, contact Diatheva.

6. PERFORMANCES CHARACTERISTICS

ANALYTICAL SPECIFICITY

The specificity of primers and probes can be ensured through the use of specific sequences evaluated by *in silico* analysis and amplification under stringent conditions.

DIAGNOSTIC SENSITIVITY

A significant number of samples positive for SARS-CoV-2 were tested with the COVID-19 DIATHEVA Detection kit and simultaneously with another device. The results obtained made it possible to calculate a diagnostic sensitivity of **98.2%**.

DIAGNOSTIC SPECIFICITY

A significant number of negative samples for SARS-CoV-2 negativity were tested with the COVID-19 DIATHEVA Detection kit and simultaneously with another device. The results obtained made it possible to calculate a diagnostic specificity of **100%**.

7. REFERENCES

WHO Laboratory testing for coronavirus disease 2019 (COVID-19) in suspected human cases, 2 March 2020

Corman, V.M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D.K., Bleicker, T., Brünink, S., Schneider, J., Schmidt, M.L. and Mulders, D.G., 2020. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eurosurveillance*, 25(3).

Appendix A-CFX96 Analysis parameters

For each fluorophore select "settings", "baseline threshold" and "single threshold-user defined", entering the following values and click OK:

Target/Fluorophore	Threshold value
RdRp /FAM	30
E/Cal Orange 560	40
RNaseP/Quasar 670	30

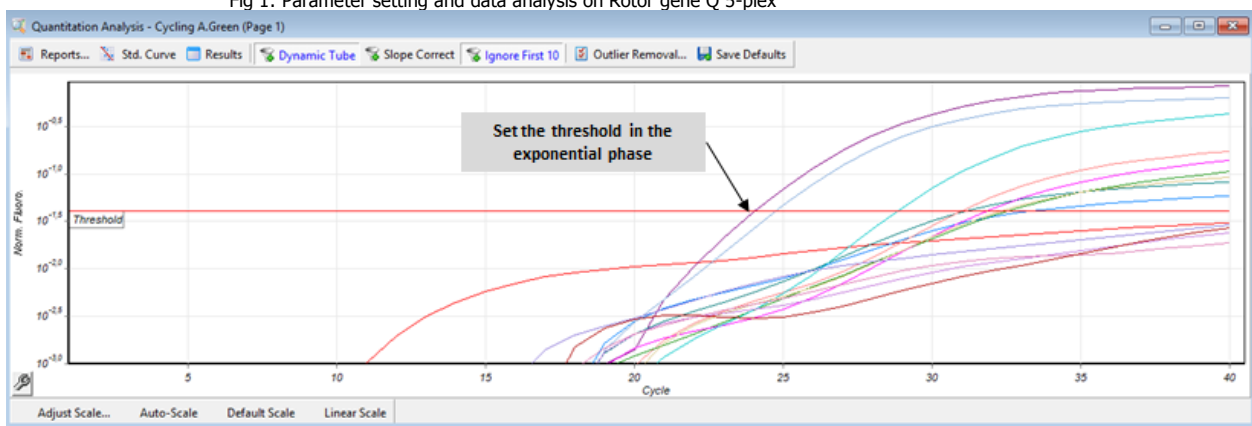
ATTENTION: for a correct interpretation of the results, pay attention to the amplification curves.

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

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. 1).

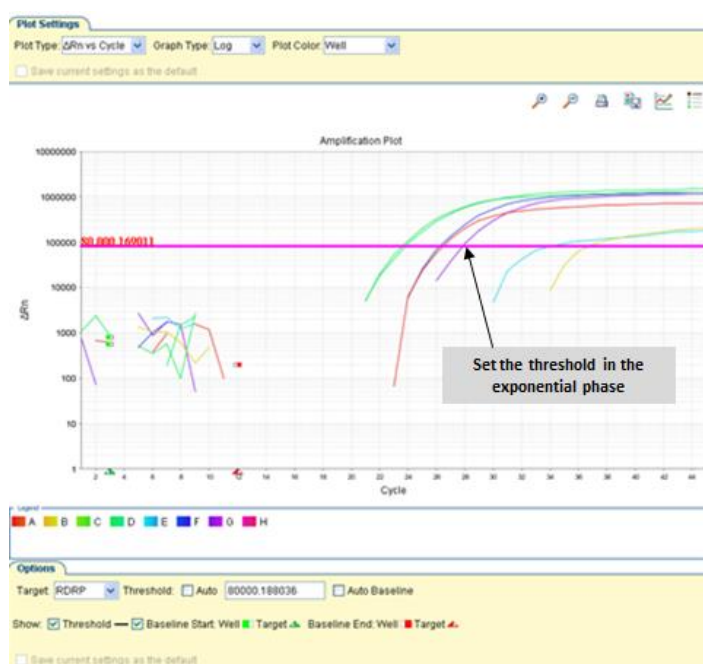
Fig 1: Parameter setting and data analysis on Rotor gene Q 5-plex



Appendix C-ABI 7500 Analysis parameters

For analysis, in the screen "Amplification plot" and function "Options", deselect "Auto Baseline" and "Auto" respectively at the threshold. To set the threshold, manually move the line in the exponential phase of the fluorescence curves (Fig. 2).

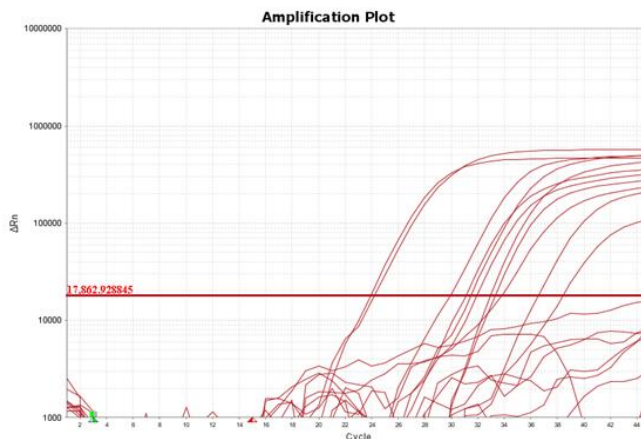
Fig 2: Parameter setting and data analysis on ABI 7500



APPENDIX D - Quant Studio 5 Analysis parameters

For the analysis, in the "Results" screen select "Amplification Plot" from the drop-down menu. Select one target at a time. Click the "Show Plot Settings" symbol and deselect "Auto Baseline" and "Auto" respectively at the threshold. 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 Quant Studio 5



APPENDIX E – LightCycler 480 Roche (LC-480) Analysis parameters

Click the "Analysis" button. Select "Abs Quant / Fit Points" from the drop-down menu and select the checkbox in the "Create new analysis" window. Click "Noise Band" and then on "Filter combination". To set the threshold, manually move the line in the exponential phase of the fluorescence curves for each channel (Fig. 4).

Fig 4: Parameter setting and data analysis on LC-480 Roche

