COVID-19 RNA Vs 2

Multiplex Real-Time RT-PCR for detection of SARS-CoV-2 - Lyophilised Format

4BShoplab

Rev. 3

COVID-19 RNA Vs 2

A. INTENDED USE

The **Covid-19 RNA Vs 2** Multiplex Real-Time RT-PCR kit coded **COV19RNALYO.CE** is intended for the specific qualitative detection of SARS-CoV-2 in human samples (see chapter H) by simultaneous retrotranscription and amplification of specific target region of Sars-CoV-2 genome (RdRp gene and N gene). In the same reaction tube an endogenous human gene is used as extraction/amplification reaction control (Internal Control).
The kit has been adapted for the use on the Real-Time Thermacyclers and ABI 7500 Sequence Detection System® (Software SDS version 1.3.1, Applied Biosystems™*) and CFX96 Real-Time System (Software CFX manager version 1.7, Biorad™**)

* Applied Biosystems is a registered trademark and ABI PRISM® is a trademark of Applera Corporation or its subsisdiaries in the US and/or certain other countries.
** Biorad is a registered trademark.

B. INTRODUCTION

Coronaviruses (CoV) are a large family of viruses that cause illness ranging from the common cold to more severe diseases.
Common signs of infection include respiratory symptoms, fever, cough, shortness of breath and breathing difficulties. In more severe cases, infection can cause pneumonia, severe acute respiratory syndrome, kidney failure and even death.
Respiratory disease caused by the novel coronavirus, first detected in Wuhan City, China, has been named *coronavirus disease 2019* (COVID-19) and the virus has been named SARS-CoV-2.
The SARS-CoV-2 virus is a betacoronavirus, like MERS-CoV and SARS-CoV; they are enveloped, positive-sense, single-stranded RNA viruses of zoonotic origin.
COV19RNA.CE RT-PCR assays is able to detect viral RNA reducing sample handling steps, providing fast results and a really low risk of cross-contamination for the sample under evaluation.

C. PRINCIPLE OF THE TEST

The **COV19RNALYO.CE** Kit is based on a Real Time chemistry which uses specific Primers and Probes.
The SARS-CoV-2 RNA**,** recovered from the biological sample under investigation through an extraction step, is retrotranscribed and amplified using the Real Time amplification system. The Multiplex RT-PCR is based on a one-tube reaction performed for each sample. The multiplex assay is specifically targeting two regions of the SARS-CoV-2 (RdRp and N) and an endogenous (GAPDH) Internal Control (IC). The Endogenous IC extracted, retrotranscribed and amplified meanwhile, serves as an Collection/Extraction/Amplification control for each individually processed specimen aiming to the identification of good respiratory sample collection and the eventually identification of reaction inhibitors.

Important Note:
The Primers and Probe set for SARS-CoV-2 genomic sequence (N gene) is recommended by Center for Disease Control and Prevention (CDC).

D. COMPONENTS

The standard format of the product code COV19RNALYO.CE contains reagents for 100 tests.

Component	Contents	COV19RNALYO.CE 100 Reactions
A **CODED: ALL/MM-8** **COLOR CODE: BLUE**	5x Master mix	N° 1 vial (Dissolve with the volume of ALL/RB indicated on the vial label)
RB **CODED: ALL/RB** **COLOR CODE: BLUE**	Master Mix Reconstitution Buffer	N° 1 vial/0.40 ml
B **CODED: COV19/CB** **COLOR CODE: YELLOW**	Lyophilized Primers/Probes for RdRp gene, N gene and GAPDH gene	N°2 vial (Dissolve with the volume of ALL/C indicated on the vial label)
C **CODED: ALL/C** **COLOR CODE: RED**	MG Water	N° 1 vials/1.5 ml
NTC **CODED: ALL/NTC** **COLOR CODE: WHITE**	Negative Control	N° 1 vial/1.5 ml
CTRL Positive Control RdRp/N **CODED: COV19/CTRL** **COLOR CODE: PINK**	Lyophilized DNA Positive Control for RdRp gene, N gene	N°2 vial (Dissolve with the volume of ALL/C indicated on the vial label)
Package Insert	Instruction for Use	1

Important note: Upon request, Dia.Pro can supply reagents for 200 tests, as reported below**:**

1. Component A	n°2 vials
2. Component RB	n°2 vials/0,04 ml
3. Component B	n°4 vials
4. Component C	n°2 vials/1.5 ml
5. NTC	n°1 vial/1.5 ml
6. CTRL	n°4 vials
7. Pack. insert	n°1
Number of tests	**200**
Code	**COV19RNALYO.CE.200**

E. STORAGE AND STABILITY

The kit COV19RNALYO.CE upon arriving must be stored at +2°C/+8°C.
Once dissolved Component B (coded COV19/CB) and Component CTRL (coded COV19/CTRL) are stable for up to 90 days at -20°C. If the components are to be used only intermittently, it should be frozen in aliquots, repeated thawing and freezing should be avoided. Only three thawing process is allowed.
Once dissolved component ALL/MM-8 is stable for up to 90 days at -20°C. If the component is to be used only intermittently, it should be frozen in aliquots, repeated thawing and freezing should be avoided. Only five thawing process are allowed.

F. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (0.5 µl < volume <1000 µl)
2. RNA extraction kit
3. MG EtOH
4. Thermal Block
5. Microcentrifuge
6. Tube racks
7. Sterile filtered tips with aerosol barrier
8. Nuclease-Free Microtubes
9. 0,2 ml Microtubes or PCR Microplates recommended from the Real-Time PCR instruments manufacturers
10. Disposable gloves, powder-free
11. Real-Time PCR Thermalcycler (*)
12. Absorbent paper tissues.
13. Vortex or similar mixing tools.

(*) Attention: A valid calibration of the pure dyes (Pure Spectra Component File) and of the background (Background Component File) must be done routinely.

G. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.
2. The technical personnel must be deeply trained in the use of Real-Time thermalcyclers, in the manipulation of Molecular Biology reagents and skilled in the Real-Time PCR amplification protocols.
3. The kit has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.
4. All the personnel involved in performing the assay have to wear protective laboratory clothes, powder-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
5. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
6. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial agents, when opening kit vials and the Components and when performing the test.
7. Components A/CB are light sensitive. Protect them from strong light exposition.
8. Avoid vibration of the bench surface where the test is undertaken.
9. Upon receipt, store the kit at +2°C/+8°C into a temperature-controlled fridge.
10. Do not interchange components between different lots of the kits. It is recommended that components between kits of the same lot should not be interchanged.
11. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.
12. Avoid cross-contamination between samples by using disposable tips and changing them after each sample.
13. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
14. Do not use the kit after the expiration date stated on the external container label.
15. Treat all specimens as potentially infective. All human specimens should be handled according the more recent CDC and WHO Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019.
16. Store and extract specimens separately from the other reagents and use a separate room for their handling
17. Carry on all the working operations as quickly as possible maintaining the components on ice or in a cooling block.
18. The laboratory Workflow must proceed in an unidirectional way, beginning in the Extraction Area and moving to the Amplification and Data Analysis Areas. Do not return samples, equipment and reagents to the area where previous steps have been performed.
19. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring of components into automated workstations, in order to avoid cross contamination.
20. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from sample extraction procedures, has to be treated as potentially infective material and inactivated before waste. Do not put in contact the extraction waste with bleach.
21. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.
22. Other waste materials generated (example: tips used for samples) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

H. SPECIMEN: PREPARATION AND RECOMMENDATIONS

For initial diagnostic testing for COVID-19 the Center for Disease Control, Atlanta, U.S. (CDC) recommends collecting and testing **upper respiratory** (nasopharyngeal and oropharyngeal swabs), and **lower respiratory** (sputum, if possible) for those patients with productive coughs. Induction of sputum is not recommended. Specimens should be collected as soon as possible once a Person Under Investigation (PUI) is identified, regardless of the time of symptom onset.

1. Collect 2-3 ml bronchoalveolar lavage and tracheal aspirate sample into a sterile, leak-proof, screw-cap sputum collection cup or sterile dry container. Refrigerate specimen at 2-8°C (≤ 48h).
2. Have the patient rinse the mouth with water and then expectorate deep cough sputum directly into a sterile, leak-proof, screw-cap sputum collection cup or sterile dry container. Refrigerate specimen at 2-8°C (≤ 48h).
3. For Nasopharyngeal (NP) and Oropharyngeal (OP) specimen use only synthetic fiber swabs with plastic shafts. Do not use calcium alginate swabs or swabs with wooden shafts, as they may contain substances that inactivate some viruses and inhibit PCR testing. Place swabs immediately into sterile tubes containing 2-3 ml of viral transport media. NP and OP specimens should be kept in separate vials. Refrigerate specimen at 2-8°C (≤ 5 days).
4. Collect 2-3 ml nasopharyngeal wash/aspirate or nasal aspirate into a sterile, leak-proof, screw-cap sputum collection cup or sterile dry container. Refrigerate specimen at 2-8°C (≤ 48h).
5. Avoid any addition of preservatives to samples.
6. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results.
7. Samples who want to be stock for longer period must be store at -70°C. Avoid repeated freezing / thawing cycles.
8. When using frozen samples, thaw the samples just before the extraction in order to avoid cases of nucleic acid degradation.
9. Serum samples collection (acute sample and convalescent sample) are recommended for further serological test.
10. All specimens collected for laboratory investigations should be regarded as potentially infectious.
11. The Health Care Workers (HCWs) who collect, or transport clinical specimens should adhere rigorously to infection prevention and control guidelines and national or international regulations for the transport of infectious substances to minimize the possibility of exposure to pathogens.

I. PREPARATION OF COMPONENTS AND WARNINGS

Master Mix:

Component A

- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized Component A with the volume of Component RB (Code: ALL/RB) indicated on the vial label.
- Keep it on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Mix well

WARNING: Component A is light sensitive. Protect it from strong light exposition.

Component RB

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap
- Transfer 400ul of component RB (code: ALL/RB) to the Component A vial (code: ALL/MM-8)

Primers/Probes for RdRp gene, N gene and GAPDH gene

Component B

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized Component B with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

WARNING: Component B is light sensitive. Protect it from strong light exposition.

MG Water:

Component C. Ready to use.

Negative Control:

NTC. Ready to use.

Positive Control for RdRp and N gene:

Component CTRL

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized Component B with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

L. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. **Micropipettes** have to be calibrated and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-5%.
2. **Extraction Device:** The COV19RNALYO.CE Kit is intended to be used in combination only with QIAamp Viral RNA Mini kit Coded:52904 (QIAGEN), NucleoSpin Virus Kit Coded: FC140983 (Macherey-Nagel) and QIAamp MinElute Virus Spin kit Coded: 57704 (QIAGEN).The end users must strictly follow the Instruction for use supplied by the manufacturers.
3. **Real-Time Thermocyclers.** The COV19RNALUO.CE Kit is intended for the use in combination only with the Real Time Thermal cyclers ABI 7500, software SDS version 1.3.1 (Applied Biosystems), and CFX96 Real-Time System, Software CFX manager version 1.7, Biorad™
4. The end users must strictly follow the Instruments Instruction for use supplied by the manufacturers.

M. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the components are not contaminated by naked-eye visible particles or aggregates. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box.
3. Turn the Thermalcyclers on, check settings and be sure to use the right assay protocol.
4. Follow strickly the Instruments Manual supplied by the manufacturers for the correct setting of the Real-Time Thermalcyclers.
5. Check that the micropipettes are set to the required volume.
6. Check that all the other equipment is available and ready to use.
7. In case of problems, do not proceed further with the test and advise the supervisor.

N. ASSAY PROCEDURE

The assay has to be carried out according to what reported here below.

N.1 RNA extraction

The extraction step of the Wuhan COVID-19 genomic RNA has to be carried out exclusively in combination with the following kits:

Material	Description	Kit code	manufacturer
Respiratory specimens/Serum	Nucleospin Virus kit	740983	MN™
	QIAamp Viral RNA mini kit®	52904	Qiagen™
	QIAamp Virus MinElute kit®	57704	Qiagen™

The RNA isolation must be carried out only according to the Instruction Manual supplied by the Manufacturer (QIAGEN™; MN™).

Important Note: The following volumes have to be strictly used in the extraction procedures:

Description	Sample volume µl	Elution volume µl
NucleoSpin Virus kit	400	60
QIAamp Viral RNA mini kit®	140	60
QIAamp Virus MinElute kit®	200	100

The RNA collected from the samples, not used in the run, has to be stored frozen (-18°C/-22°C).

Important note:

The Internal Control Ct value for the negative sample is used to evaluate if the sample collection and the RNA extraction procedures have been performed correctly (see section Q).

N.2 Setting up of the reaction

The COV19RNALYO.CE Kit is intended for the use in combination only with the Real Time Thermal cyclers ABI 7500, software SDS version 1.3.1 (Applied Biosystems), and CFX96 Real-Time System, Software CFX manager version 1.7, Biorad™

N.2.1 Preparing the RT-PCR

Important: *An example of dispensation scheme is reported in Section O. Please, refer to it before starting the operations described here below.*

- Prepare the components as described in Section I;
- Prepare the required number of reaction tubes or a 96-well reaction plate for the samples under evaluation and for the Positive control (prepared as described in section I).

Important note:
1- Use only optical tubes or microplates suggested by the Real-Time thermalcyclers manufacturers.

- Consider that the samples, if possible, should be tested in duplicate;
- Include at least 1 tube for the NTC (negative control)
- Prepare the **Reaction Mix** for **Samples, NTC and positive control (CTRL)** as table below:

Preparation of the Reaction Mix

Number of Reactions		x1	x12
A	Master mix	4 µl	48 µl
B	Primers/probes	2 µl	24 µl
C	MG Water	4 µ	48 µl
Tot vol.		**10 µl**	**120 µl**

- Dispense 10 ul of the amplification mix in each reaction tube or microplate well
- Add 10 ul of the **Samples, NTC, CTRL** to the reaction tubes.
- Close firmly the reaction tubes
- Centrifuge briefly the reaction tubes at 2000 rpm
- Don't leave the reaction tubes at room temperature (RT) for more than 30 minute and at light exposure (cover the tubes).
- Load the reaction tubes in the Real-Time Thermacycler Thermoblock Holder.
- After the setting operations described in the Sections N3 (Instrument Programming) start the Thermacycler run.

N.3 Instrument programming

For programming the instrument refer to the Instrumentation Instruction Manual provided by the manufacturers.

N.3.1 RT-PCR Thermal Profile

The thermal profile is reported in the table below:

Step	Cycle	Temp.	Time
1	1	50°C	20 min
1	1	95°C	10 min
2	45	95°C	15 sec
		58°C (*)	45 sec

IMPORTANT NOTE: (*) step for the real time data collection.
WARNING: Keep attention to set up the Real-Time Thermacycler with the correct Thermal Profile following the Instrument Manual supplied by the Instrument manufacturer.

N.3.2 Selection of the Detectors

Following the Instruction manuals of the Real-Time thermocyclers suggested (CFX96 and ABI7500) select the Detectors reported in the table here below:

RdRp gene / N gene / Internal Control Detection

Detection	Reporter	Quencher
POL (RdRp gene)	FAM	Non Fluorescent
NUC (N gene)	Cy5	Non Fluorescent
Internal Control (IC)	JOE/VIC	Non Fluorescent
Passive Reference (ABI7500)	ROX	

WARNING: Keep attention to set up the Real-Time Thermacycler with the correct settings following the Instruments Manual supplied by the manufacturer.

O. ASSAY SCHEME

An example of dispensation scheme for the Analysis is reported here below:

Microplate or tubes

	1	2	3
A	**CTRL**		
B	NTC		
C	Sample 1		
D	Sample 2		
E	Sample 3		
F	Sample 4		
G	Sample 5		
H	Sample 6		

Legend: NTC = Negative Control; CTRL = COVID-19 DNA Positive Control for RdRn gene and N gene; Sample 1,2,3,etc.. = Samples under evaluation.

P. INTERNAL QUALITY CONTROL

P.1 Pre - Analysis Settings

Before starting the analysis:

- Set the "Baseline" (the background fluorescence level) as reported below:

"Baseline"	
ABI™PRISM® 7500 SDS	**Auto Baseline**
BIORAD™ CFX96®	**Auto Calculated Baseline**

- Set manually the FAM/Cy5/JOE/VIC fluorescence "Threshold"

"Threshold"	ABI™PRISM® 7500 SDS
FAM (POL)	0.15
Cy5 (NUC)	0.15
JOE (IC)	0.15

"Threshold"	BIORAD™ CFX96®
FAM (POL)	250
Cy5 (NUC)	250
VIC (IC)	150

P.2 Data analysis

A check is carried out on the Positive Control any time the kit is used in order to verify whether their Ct values are as expected and reported in the table below.

Check	Requirements
CTRL	Ct (Threshold Cycle) < 28.5

Q. INTERPRETATION OF THE RESULTS AND TROUBLESHOOTING

For each samples the Internal Control JOE/VIC Ct value is assumed to validate SARS-CoV-2 RNA collection, extraction and detection as described in the table below:

POL - FAM	NUC – CY5	Internal Control – JOE/VIC	Assay Result
SAMPLE POSITIVE	SAMPLE POSITIVE	Ct < 33	CORRECT
		Ct ≥ 33 or undetermined	CORRECT*
SAMPLE NEGATIVE	SAMPLE NEGATIVE	Ct < 33	CORRECT
		Ct ≥ 33 or undetermined	INVALID**
SAMPLE NEGATIVE	SAMPLE POSITIVE	Ct < 33	INVALID***
		Ct ≥ 33 or undetermined	INVALID**
SAMPLE POSITIVE	SAMPLE NEGATIVE	Ct < 33	INVALID***
		Ct ≥ 33 or undetermined	INVALID**

*High Initial concentration of SARS-CoV-2 RNA in the sample can lead to a REDUCED or an ABSENT Fluorescent Signal for the Internal Control IC due to the reagents Competition.

**Problems may be occurred during the collection (initial sample containing an insufficient number of cells) or during the extraction step (presence of inhibitors) or during the amplification step (inefficient or absent retrotranscription/amplification) leading to an incorrect result.
The test procedure must be repeated starting from the Extraction step and/or using a fresh sample coming from the patient.

***Problems may be occurred during the extraction step or during the amplification step (inefficient or absent retrotranscription/amplification) leading to an incorrect result. The test procedure must be repeated starting from the Extraction step and/or using a fresh sample coming from the patient.
If the result is confirmed twice the sample could be positive for other Sarbecovirus.

The results obtained with this product must be interpreted taking consideration of the clinical symptoms and the other laboratory parameters related to the patient conditions.
The following results are possible:

Troubleshooting table

	POL-FAM	NUC-CY5	JOE	Result	CHECK
SAMPLE unknown	+	+	+/-	CORRECT RESULT *Positive*	***IMPORTANT***: High Initial concentration of SARS-CoV-2 RNA can lead to REDUCED or ABSENT Fluorescent Signal of Internal Control I.C. due to the reagents Competition.
SAMPLE unknown	+	-	-/+	ATTENTION! POSSIBILITY OF: Inhibition, error in the collection/amplification procedure	1. That the CY5 dye is selected for the NUC detection and JOE/VIC dyes for the IC detection; 2. that the Analysis has been run with the correct Instrument settings; 3. that the kit has been stored correctly; 4. that no potential PCR inhibitors have been contaminated the well 5. that the Extraction procedure have been executed correctly;
SAMPLE unknown	-	+	-/+	ATTENTION! POSSIBILITY OF: Inhibition, error in the collection/amplification procedure	1. That the FAM dye is selected for the POL detection and JOE/VIC dyes for the IC detection; 2. that the Analysis has been run with the correct Instrument settings; 3. that the kit has been stored correctly; 4. that no potential PCR inhibitors have been contaminated the well 5. that the Extraction procedure have been executed correctly;
SAMPLE unknown	-	-	-	ATTENTION! POSSIBILITY OF: Inhibition, error in the procedure or misfunctioning of the Instruments	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the FAM dye is selected for the POL detection, CY5 dye for the NUC detection and JOE/VIC dyes for the IC detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube 7. that the Extraction procedure have been executed correctly;
SAMPLE unknown	-	-	+	CORRECT RESULT *Negative*	
CTRL	+	+	-	CORRECT RESULT	***IMPORTANT***: NO Internal Control I.C. signal on CTRL because endogenous control.
CTRL	-	-	-	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the FAM dye is selected for the POL detection and CY5 dye dye is selected for the NUC detection 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube
CTRL	-	+	-	ATTENTION ! POSSIBILITY OF: Error in the procedure	1. That the CY5 dye is selected for the NUC detection 2. that the Analysis has been run with the correct Instrument settings; 3. that the kit has been stored correctly; 4. that no potential PCR inhibitors have been contaminated the well
CTRL	+	-	-	ATTENTION ! POSSIBILITY OF: Error in the procedure	1. That the FAM dye is selected for the POL detection 2. that the Analysis has been run with the correct Instrument settings; 3. that the kit has been stored correctly; 4. that no potential PCR inhibitors have been contaminated the well
NTC	-	-	-	CORRECT RESULT	***IMPORTANT***: NO Internal Control I.C. signal on NTC because endogenous control.
NTC	+	-	+/-	ATTENTION ! POSSIBILITY OF: Contamination	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3.that the work space

					and Instruments are decontaminated at regular intervals; 4. that the kit has been stored correctly;
NTC	-	+	+/-	ATTENTION ! POSSIBILITY OF: Contamination	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3.that the work space and Instruments are decontaminated at regular intervals; 4. that the kit has been stored correctly;

If one of more of the problems described in the table above happen, after checking, report any residual problem to the supervisor for further actions.

Important notes:

1. *Interpretation of results should be done under the supervision of the responsible of the laboratory to reduce the risk of judgment errors and misinterpretations.*
2. *When test results are transmitted from the laboratory to an informatics centre, attention has to be paid to avoid erroneous data transfer.*

R. PERFORMANCES

Evaluation of Performances has been conducted in accordance to what reported in the Internal Technical Specifications or ITS.
The performance evaluation was carried out in DiaPro's laboratories on materials supplied by the reference clinical labs.

R.1 ANALYTICAL SENSITIVITY

Analytical sensitivity may be expressed as **Limit of Detection**.
Limit of detection (LOD): it is the lowest amount of target that can be detected by the system with a stated probability.
For the NAT tests it is expressed as the smallest concentration of the **analyte** that tested in multiple repetitions gives a positive result.
The **limit of detection (LOD)** was determined by testing limiting dilutions of a sample quantified on the NIBSC research reagent for Sars-CoV-2 RNA (code 19/304).
The sample dilutions at nominal 750 copies/ml and 500 copies/ml were tested in 20 replicates.
For the kit COV19RNALYO.CE the LOD value was defined as the amount of the target resulted positive 19 out of 20 replicates.
On the basis of the results obtained the Limit of detection at the 100% of the system has been calculated in 750 copies/ml.
The Limit of detection at the 85% of the system has been calculated in 500 copies/ml.

The results are reported in the tables below:

750 copies/ml		RdRp gene		
		Positive	**Negative**	**total**
N gene	**Positive**	18	1	**19**
	Negative	1	0	**1**
		19	**1**	**20**

500 copies/ml		RdRp gene		
		Positive	**Negative**	**total**
N gene	**Positive**	16	0	**16**
	Negative	1	3	**4**
		17	**3**	**20**

R.2 DIAGNOSTIC SPECIFICITY AND SENSITIVITY

R.2.1 Diagnostic Specificity:

The Diagnostic specificity is the probability that the device gives a negative result in the absence of the target marker. So the **true negative** sample is a specimen known to be negative for the target marker and correctly classified by the device.
This parameter was studied by examining 22 SARS-CoV-2 RNA negative nasopharyngeal swab samples:

SARS-CoV-2 RNA Negative samples

TRUE NEGATIVES	22
FALSE POSITIVES	0
TOTAL SAMPLES	22
SPECIFICITY %	**100**

On the basis of the results obtained Diagnostic Specificity of the system has been calculated in the 100%.

R.2.2 Diagnostic Sensitivity

Diagnostic sensitivity is the probability that the device gives a positive result in the presence of the target marker. So the **true positive** sample is a specimen known to be positive for the target marker and correctly classified by the device.
For the kit code COV19RNALYO.CE the parameter was studied by examining 27 SARS-CoV-2 RNA nasopharyngeal swab samples:

SARS-CoV-2 RNA Positive samples

TRUE POSITIVES	27
FALSE NEGATIVES	0
TOTAL SAMPLES	27
SENSITIVITY %	**100**

On the basis of the results obtained the Diagnostic Sensitivity of the system has been calculated in the 100%.

Diagnostic Sensitivity	100 %
Diagnostic Specificity	100 %

R.2.3 Proficiency Panel

The performances of the Covid-19 RNA Vs 2 Multiplex Real-Time RT-PCR kit coded **COV19RNALYO.CE** have been tested with the **QCMD 2020 SARS-CoV-2 EQA Programme** (cod. SCV2_20 – C1).
The panel contain positive, negative and interfering samples in a matrix swab-like (Transport Medium - TM).
Samples have been purified using the QIAamp Viral RNA mini kit following the manufacturer procedure.
The results obtained are reported in the table below:

code	target	Sample correlation	Ct POL	Ct NUC	Ct IC
SCV2_101C1-01	**Coronavirus 229E**	**3.93dPCR cp/ml**	**Und**	**Und**	30.13
SCV2_101C1-02	**SARS-CoV2**	**4.12dPCR cp/ml**	29.46	28.43	29.16
SCV2_101C1-03	**SARS-CoV2**	**3.15dPCR cp/ml**	32.45	31.41	30.09
SCV2_101C1-04	**SARS-CoV2**	**2.82dPCR cp/ml**	33.29	32.31	29.19
SCV2_101C1-05	**SARS-CoV2**	**2.82dPCR cp/ml**	33.44	32.33	29.44

R.2.4 Sensitivity on SARS-CoV-2 Variants
The kit COV19RNALYO.CE ability to detect the new SARS-CoV-2 Variants was studied by examining serial dilutions of the available RNA Controls supplied by International Reference Suppliers (as for example the AMPLIRUN from Vircell, SPAIN). The Variants Controls have been tested from 1E+04 copies/ul to 5E-01 copies/ul keeping as the reference the AMPLIRUN SARS-CoV-2 RNA control (code MBC137-R).
The kit code COV19RNALYO.CE showed an equal sensitivity on all the Variants RNA controls tested being able to detect successfully both the target regions (N and RdRp) up to 1E+00 copies/ul.
Due to the lack of International Reference Preparations for all the SARS-Cov-2 Variants today circulating worldwide, Dia.Pro has performed a bioinformatical study evaluating the sequence homology of the target regions amplified.
Thanks to the design done on highly conserved sequences of structural highly stable SARS-Cov-2 Genes the Bioinformatical Study showed a strong homology of the Primers and Probes used in the kit with all the Virus Variants circulating and today deposited in the International most important Databases.
The results obtained are deeply described in the Product Technical File.

R.3 ANALITYCAL SPECIFICITY
The Analytical specificity is the ability of the method to detect only the target RNA sequence.
The analytical specificity of COV19RNALYO.CE SARS-CoV-2 RNA assay has been studied as follow:

1. The RdRp gene primer/probe Set has been choose analysing the genome target sequences with appropriate software (BioEdit Sequence Alignment Editor, Oligo Analyzer and Primer Express v.3.0" supplied by Applied Biosystem Inc.).
2. The N gene primer/probe Set has been choose from those published from Centers for Disease Control and Prevention (CDC) Atlanta.
3. The primer/probe Set and the target genome sequence has been controlled by the "BLAST" software, in order to check if any of the nucleotide sequences deposited in the worldwide genomic banks has any homology with Wuhan Covid-19, and by the "ClustalW" software, in order to compare the genome target sequences of the different genotypes of Toxoplasma.
4. The specificity was improved through the selection of stringent reaction conditions.
5. Samples coming from a panel patients suffering infections due to potential interfering organisms were obtained from the European Virus Archive (Marseille, France).

The results are reported in the following table:

Organism	RdRp region	N region
HCoV-NL63	Negative	Negative
HCoV-229E	Negative	Negative
HCoV-OC43	Negative	Negative
MERS-CoV	Negative	Negative
SARS-CoV	Negative	Positive *

*discordant results between the two target region could meaning the homology in the N Region sequence between the SARS-CoV and SARS-CoV-2. The RdRp Region shows a perfect specificity.

An additional titled (1.2E+04 copies/ul) SARS-CoV control has been tested to better verify the analytical specificity of the kit coded COV19RNALYO.CE.
In this case no cross-reactivity has been detected on both target regions meaning a complete specificity of the kit COV19RNALYO.CE for the SARS-CoV-2.

T . LIMITATIONS
The user of this kit is advised to carefully read and understand this package insert. Strict adherence to the protocol is necessary in order to obtain reliable test results. In particular, accurate sample and reagent pipetting, application of a correct workflow along with careful programming of thermalcycling steps are essential for an accurate and a reproducible SARS-CoV-2 RNA detection.
The SARS-CoV-2 RNA determination in a patient sample has extensive medical, social, Psychological and economical implications.
Detection of a possible human case of emerging pathogen causing severe acute respiratory disease should be immediately notified to local and national public health authorities. In line with the International Health Regulation (IHR) 2005 the national health authority must notify WHO within 24 hours of all events that may constitute a public health emergency.
It is recommended that confidentiality, appropriate counselling and medical evaluation be considered as an essential aspect of the testing sequence.

U. BIBLIOGRAPHY

1. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. V.M. Corman et al. www.eurosurvellance.org
2. Real-Time RT-PCR panel for dection 2019-novel coronavirus. Center for disease control and prevention (CDC).
3. Detection of 2019 novel coronavirus (2019-nCoV) in suspected human cases by RT-PCR. HKU ; LKS faculty of medice school of public health.
4. Interim guidelines for collecting, handling and testing clinical specimens from person under investigation (PUIs) for coronavirus disease 2019 (COVID-19). Center for disease control and prevention (CDC).
5. Laboratory testing for 2019 novel coronavirus (2019-nCoV) in suspected human cases. World Health Organization (WHO) 17th of January 2020.
6. Polmonite da nuovo coronavirus (2019-nCoV) in Cina. 0001997-22/01/2020-DGPRE-DGPRE-P. Ministero della Salute, direzione generale della prevenzione sanitaria.
7. Development of a quantitative assay for SARS coronavirus and correlation of GAPDH mRNA with SARS coronavirus in clinical specimens. S.C.C. Wong et al.; J Clin Pathol 2005;58:276-280.

V. SYMBOLS

LEGENDA			
REF	Product code		Storage temperature
IVD	In Vitro Diagnostic Device		See use instructions
LOT	Lot number		Manufacturer
	Expiry date		Number of tests
CE	CE conformity mark		Date of manufacturing

All the IVD Products manufactured by the company are under the control of a certified Quality Management System in compliance with ISO 13485 rule. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

DISTRIBUTOR

4BShop Lab Srls

info@4BShopLab.com

www.4BShopLab.com

+39.0371.18.56.643

MANUFACTURER

Dia.Pro - Diagnostic Bioprobes Srl

MADE IN ITALY

EN ISO 13485:2013 Certified