1 Intended use

RealCycler CORO-U / CORO-G v.5 is an *in vitro* diagnostic kit of reagents which allows the detection of Coronavirus SARS-CoV-2 and Sarbecovirus RNA in clinical samples. It is recommended to interpret the results with the *BioVisor RC* software that includes the validation criteria of samples and controls.

2 Principle of the test

The polymerase chain reaction (PCR) is based on the amplification of a specific region of the DNA/RNA by using complementary primers to the target sequence. Real-time PCR uses Taqman® probes labelled with fluorophores that emit fluorescence in the case of amplification. The cycle of the PCR protocol in which appears significant fluorescence is proportional to the DNA/RNA quantity present in the sample. This value is called *Cycle Threshold* (Ct).

The system includes an internal control CHIC *(Competitive Heterologous Internal Control*) to prevent false negatives due to reaction inhibition.

The amplification of Coronavirus SARS-CoV-2 is detected with FAM fluorophore, CHIC with HEX and Sarbecovirus E gene with ATTO647N.

3 Technical specifications

Sensitivity

Coronavirus SARS-CoV-2: 1 copy/µL.
Sarbecovirus: 1 copy/µL.

The analytical sensitivity has been determined by limit dilution. This sensitivity has been showed in repeated assays with reproducibility over 95% using a T-COR 8® (Cirrus Dx®) instrument.

Specificity

Coronavirus SARS-CoV-2: ORF8 region.
Sarbecovirus: E gene.

Specificity validation has been performed according to experimental assays and bioinformatics analysis.

4 Contents

RealCycler CORO-U / CORO-G v.5 includes the **AmpliMix CORO v.5** and a **CORO Positive Control**, which consists of a DNA artificial construction of Coronavirus.

All reagents are ready to use without adding or rebuilding any component.

Component	Vials		Volume
	CORO-U	CORO-G	
AmpliMix CORO v.5 (Retrotranscriptase included)	1	2	430 µL
CORO Positive Control	1	2	120 µL

Number of determinations: *RealCycler* CORO-U v.5 allows to perform 30 reactions of 20 µL and 24 reactions of 25 µL. *RealCycler* CORO-G v.5 allows to perform 60 reactions of 20 µL and 48 reactions of 25 µL.

5 Stability and storage

- Stability assays have shown that this product is stable for 6 months between -18 °C and -25 ºC. See the expiration date included in the external label.
- *RealCycler* kits must be stored frozen between -18 °C and -25 ºC.
- The experimental assays performed have shown that *RealCycler* reagents are stable for at least 15 cycles of freezing / thawing.

6 Additional materials and equipment required and not supplied

- Real-time PCR instrument.
- Microcentrifuges.
- RNA purification system.
- Disposable gloves.
- Calibrated pipettes.
- Pipette tips with filter.
- Freezer (between -18 °C and -25 ºC).
- Negative control (RNA from negative samples, water or elution buffer).
- *BioVisor RC* (provided free of charge at the user's request).

7 Warnings and Precautions

- All components of the kit must be kept cold while you are working.
- Load tubes in the Real-time PCR instrument immediately after adding RNA.
- Do not expose tubes with AmpliMix to light for a long time.
- Repeated freezing and thawing of the reagents can decrease the sensitivity of the kit.
- Use disposable gloves.
- Use adequate and calibrated pipettes and pipette tips with filter.
- The tests must be carried out by qualified personnel and following good laboratory practices.
- It is recommended to use positive and negative controls whenever an analysis is performed.
- Do not use the kit after the expiry date.
- The presence of polymorphisms in the binding sequences of probes or primers to pathogen DNA/RNA can lead to erroneous results in a sample. If discordances appear between results and clinical observations it is recommended to check the results obtained using alternative methods.
- Negative results do not exclude an infection caused by the pathogen. The results obtained with this diagnostic kit should be used and interpreted within the context of the clinical history of the patient. Clinical decisions should not be made using solely the results of this kit.
- When the samples used have a pathogen concentration lower to the limit of detection, the results obtained have a reproducibility less than 95%.
- For *in vitro* diagnostic use.
- It is recommended to interpret the results with the *BioVisor RC* software that includes the validation criteria of samples and controls.

8 Clinical samples

- Collect samples in sterile tubes.
- Storage and transportation frozen at -20 °C until use.
- The samples can be kept refrigerated during transport up to a maximum of 48 h.
- The kit is potentially compatible with any sample from which the pathogen's RNA can be extracted in enough quantity and quality, although the user must assess the suitability of non-validated samples through laboratory tests.

Validated clinical samples:

- Coronavirus SARS-CoV-2: nasal exudate, nasopharyngeal exudate, oropharyngeal exudate and rectal exudate.
- Sarbecovirus: nasal exudate, nasopharyngeal exudate and oropharyngeal exudate.

9 Procedure

a) Nucleic acids purification

RNA should be purified from the clinical sample using an appropriate procedure. There are many nucleic acids purification systems available in the market. Please carry out the purification according to the manufacturer's instructions and using the recommended volume.

Validated purification systems:

- QIAamp Viral RNA Mini kit (references 52904, 52906). Qiagen.
- MagCore® Automated Nucleic Acid Extractor (references MVN400-03, MVN400-04). RBCBioscience.
- Maxwell® 16 Viral Total Purification kit (reference AS1150). Promega Corporation.

RealCycler kits are compatible with most purification systems, among them:

- Arrow/LIAISON® IXT. DiaSorin.
- BioRobot EZ1. Qiagen.
- QIAcube. Qiagen.
- NucliSENS® easyMAG®. bioMérieux.

b) Thermal profile

Programme the "CORO" amplification protocol according to the following specifications:

Time	Temperature	Cycles	Fluorescence
10:00	45 °C	1	OFF
2:00	95 °C	1	OFF
0:15	95 °C		OFF
0:30	55 °C	45	ON
0:30	72 °C		OFF

c) PCR reaction set-up

- Thaw **AmpliMix CORO v.5** and **CORO Positive Control**. Use as negative control RNA from negative samples, water or RNA elution buffer (not supplied).
- Prepare the necessary amplification tubes for samples and controls.

c.1) SmartCycler® (Cepheid®)

- Pipette **17,5 µL** of AmpliMix into each amplification tube.
- Add **7,5 µL** of RNA sample or control to each reaction tube.
- Spin tubes to transfer AmpliMix to optical area of tube. Check there are no bubbles in the optical area.
- Load tubes in the SmartCycler®.
- *Create Run* > *Dye Set* > FATA25.
- *Add/Remove Sites*: assign both the "CORO" protocol and sites to each sample including controls.
- *Start Run*.

c.2) CFX96™ (Bio-Rad)

- Pipette **14 µL** of AmpliMix into each amplification tube.
- Add **6 µL** of RNA sample or control to each reaction tube.
- Spin tubes. Check there are no bubbles.
- Load tubes in the Real-time PCR instrument.
- Open *CFX™ Manager* software (version 1.6).
- Select *File* > *New Plate* > Select the whole plate or the used wells.
- Select *Sample type* > *Unknown* > OK.
- Select channels: FAM, HEX, TxR and Cy5.
- On the window *Experiment Setup* > *Protocol* > *Select Existing* > select CORO protocol (indicate 20 µL of volume) > *Save* > *Start Run*.

c.3) Mic qPCR Cycler (Bio Molecular Systems)

- Open micPCR software (version 2.4.0).
- *New* > *Assay* > *Assay Setup* > *Information* > On *Chemistry Type* select *Hydrolysis Probes*.
- Click on *Target* until there are 4 *Targets* available. Enter fluorophore names according to the following table:

Target Name	*Reporter*	*Quencher*
FAM	BHQ1	*None*
HEX	BHQ1	*None*
TxR	BHQ2	*None*
Cy5	BHQ2	*None*

- *Assay Setup* > *Profile*.
- On *Temperature Control* select *Standard TAQ (v3)* and on *Volume* Indicate reaction volume 20 µL.
- Define CORO thermal profile.
- *Analysis* > not indicate any analysis method > Save.
- Pipette **14 µL** of AmpliMix into each amplification tube.
- Add **6 µL** of RNA sample or control to each reaction tube.
- Spin tubes. Check there are no bubbles.
- Load tubes in the Real-time PCR instrument.
- *New* > *Run Setup* > *Assays* > assign "CORO" protocol and on *Samples* indicate the name of the samples.
- *Start run*.

c.4) T-COR 8® (Cirrus Dx®)

- Pipette **17,5 µL** of AmpliMix into each amplification tube.
- Add **7,5 µL** of RNA sample or control to each reaction tube.
- Centrifuge the tubes. Check that there are no bubbles and that the volume of the tube is located at its base.
- Load tube in the corresponding well.
- Select *New run.* In *Advanced,* select on the top of the screen the wells you want to analyse > *Continue*.
- Select *Barcode*. Bring the *Sample* QR code to the scanner.
- If it is the CORO Positive Control select its well > *Barcode* > bring its QR code to the scanner.
- Select *Start run*.

c.5) Other instruments

- Pipette **14 µL** of AmpliMix into each amplification tube.
- Add **6 µL** of RNA sample or control to each reaction tube.
- Load tubes in the Real-time PCR instrument.
- Select the corresponding fluorophores.
- Select "CORO" protocol.
- Start run.

d) Adjust the fluorescence threshold

Once the run is concluded, it is necessary to set the fluorescence threshold for each channel according to the indicated values hereafter. This adjust is indispensable for the correct results interpretation.

It is not necessary to set up these values when *BioVisor RC* software is used. These values are included on its database.

d.1) SmartCycler® (Cepheid®)

- *Analysis settings* > *Manual Thresh Fluor Units* > 30.0.

d.2) CFX96™ (Bio-Rad)

Adjust the fluorescence threshold for each channel:

- FAM: *Settings* > *Baseline Threshold* > *User defined* > 250 > OK.
- HEX: *Settings* > *Baseline Threshold* > *User defined* > 250 > OK.
- Cy5: *Settings* > *Baseline Threshold* > *User defined* > 250 > OK.

d.3) Mic qPCR Cycler (Bio Molecular Systems)

- *Analysis* > *Cycling.*
- For each channel on *Method* select *Dynamic* and on *Ignore Cycles Before* indicate 0.
- Indicate on *Threshold level* the following thresholds:

Green: 0,2.
Yellow: 0,1.
Red: 0,2.

- On *Exclusion* select *Extensive* and on *Fluorescence Cutoff Level* select 5,0%.

d.4) T-COR 8® (Cirrus Dx®)

For this instrument the fluorescence thresholds are defined by the QR code and it is not necessary to adjust them.

d.5) Other instruments

It is recommended to perform assays with samples of known result (positive and negative) in order to stablish the basal signal and fix the fluorescence thresholds.

e) Control results interpretation

- Valid control results

Control	Channels			
	Ch1	Ch2	Ch3	Ch4
	FAM	HEX	TxR	ATTO647N
	ORF8 region	CHIC	-	E gene
POS	POS (Ct within the range)	Indifferent	-	POS (Ct within the range)
NEG	NEG	POS (Ct within the range)	-	NEG

- Invalid control results

In case of obtaining a negative result in any channel of the **Positive Control** (excepting for CHIC) the result is invalid. The results obtained in the samples included in the working series must be discarded (not assessable).

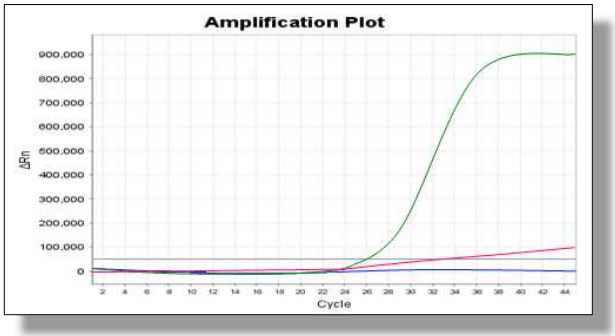
In case of obtaining a positive result (Ct > 0) in any channel of the negative control (excepting for CHIC) the result is invalid. The results obtained in the samples included in the working series must be discarded (not assessable).

f) Sample results interpretation

Interpret the result obtained in each sample by the combination of signals indicated in the following table:

Channels				Interpretation
Ch1	Ch2	Ch3	Ch4	
FAM	HEX	TxR	ATTO647N	
ORF8 region	CHIC	-	E gene	
POS	Indifferent	-	NEG	POSSIBLE POSITIVE (Inconclusive)
NEG	Indifferent	-	POS	POSSIBLE POSITIVE (Inconclusive)
POS	Indifferent	-	POS	POSITIVE Coronavirus SARS-CoV-2
NEG	Ct within the range	-	NEG	NOT DETECTED
NEG	Ct out of range	-	NEG	NOT ASSESSABLE
NEG	0,00	-	NEG	NOT ASSESSABLE

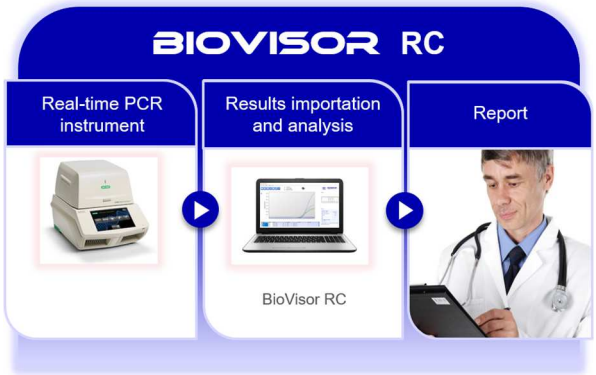
g) Example result



Result obtained when processing a positive sample (green) and a negative sample (blue). Positive sample: exponential curve. Negative sample: signal below the threshold. Other curves with different shapes must be considered abnormal and be evaluated in an individual way, such as linear signal (pink) above the threshold.

10 Results interpretation using the software *BioVisor RC*

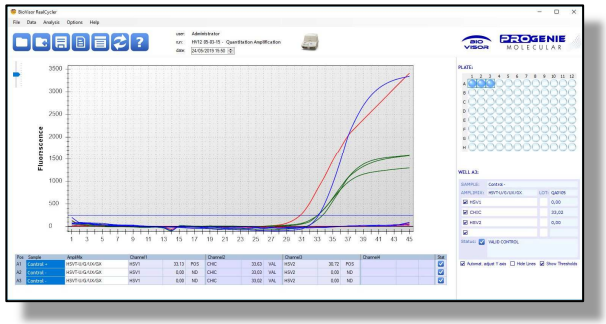
For the correct interpretation of the results obtained with the *RealCycler* products in the Real-time PCR instrument used, it is recommended to use the *BioVisor RC*.



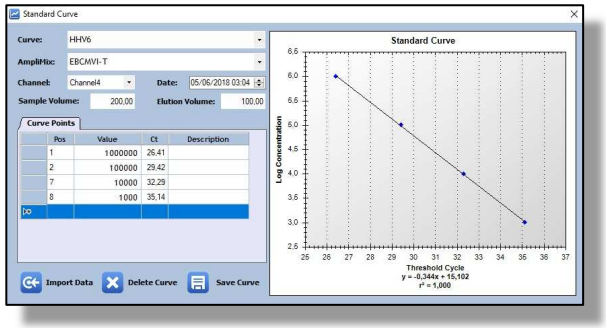
Specifications *BioVisor RC*:

- CE mark.
- Users and licenses management.
- Data importation from PCR instrument.
- Predetermined fluorescence thresholds.
- Normalization of fluorescence values.
- Visualization of all channels simultaneously.
- Interpretation of the result of each sample depending of its CHIC result.
- Series validation depending on the controls result.
- Monitoring of possible inhibitions.
- Allows the simultaneous quantification for several pathogens.
- Automatic calculation of the load of pathogen in the sample.
- Generation of a report with results.
- Conservation of calibration curves.
- Archiving of work series.
- Management of all the runs from the different instruments in a single database.
- Storage of runs obtained with the following validated instruments:
    - SmartCycler® (Cepheid®)
    - ABI 7500 (Thermo Fisher Scientific)
    - CFX96™ (Bio-Rad)
    - Mic qPCR Cycler (Bio Molecular Systems)
    - T-COR 8® (Cirrus Dx®)
- Connectivity with several T-COR 8® instruments in a local area network.

Detection example (all channels):



Quantification example:



The *BioVisor RC* is free for users of *RealCycler* products. To obtain it you can contact Progenie molecular (soporte@progenie-molecular.com) or your distributor.

11 Quality control

To validate the results, Ct values obtained for the positive control and the internal controls of each sample must be within the ranges specified in the internal label of the kit.

Every lot of *RealCycler* CORO-U / CORO-G v.5 kit has been tested according to the specifications of the real-time PCR using the SmartCycler® instrument (Cepheid®) and the validation criteria included in the *BioVisor RC*.

12 Observations

RealCycler reagents include FAM, HEX, TxR and ATTO647N fluorophores, which emit in the wavelengths indicated on the table (considered channels from 1 to 4). If an instrument does not explicitly recognise these fluorophores, it must be set up according to one of the following criteria:

1) Selection of an equivalent wavelength emission to those indicated on the table.

2) Selection of equivalent fluorophores (that emit in the same wavelength as the reagent uses).

Channel	Used fluorophores	Emission (nm)	Equivalent fluorophores
Ch1	FAM	519	——
Ch2	HEX	556	JOE, VIC, Alexa 532, CAL Fluor Orange 560
Ch3	Texas Red	610	ROX, LC Red 610, CAL Fluor Red 610
Ch4	ATTO647N	669	Cy5, Alexa 647, LC Red 670, Quasar 670, Oyster 645

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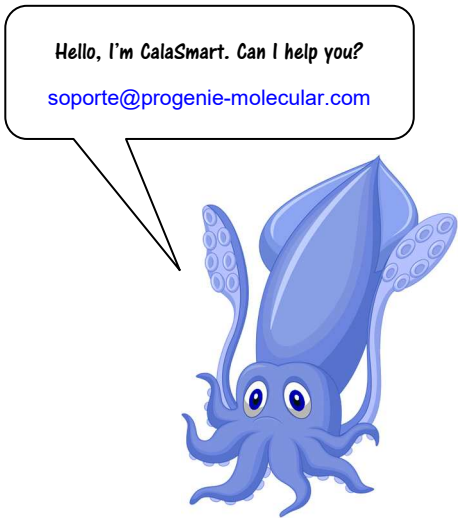

Revision history:

- **Version 5 - April 2020:** Improvement of sensitivity for the detection of Coronavirus SARS-CoV-2 RNA.
- **Version 4 - March 2020:** Target for the detection of E gene of Sarbecovirus.
- **Version 3 - March 2020:** CE mark.
- **Version 2 - February 2020:** Modification of the thermal profile.

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