DIRECT SARS-COV-2 REALTIME PCR KIT

For *in vitro* diagnostic use

RTPCR002: Real Time RT-PCR kit to detect nucleic acid from SARS-CoV-2 in human respiratory samples. The test is a qualitative assay to aid in the diagnosis of 2019 novel coronavirus disease (COVID-19). 96 tests. Lyophilized.

INTRODUCTION:

Coronaviruses (CoVs) are large enveloped positive-sense RNA viruses. SARS-CoV-2 (Severe acute respiratory syndrome coronavirus 2) was identified as the cause of an outbreak of respiratory illness first detected in Wuhan, China in December 2019. Coronaviruses are a large family of viruses that are common in many different species of animals, rarely, animal coronaviruses can infect people and spread such as with MERS, SARS, and SARS-CoV-2. This new virus shares 80% sequence identity to previously isolated human SARS-CoV and it is >96% identical to a SARS-related bat coronavirus. The disease produced by SARS-CoV-2 is called Covid-19—CoronaVirus Disease, 2019 and it is associated with lower respiratory tract infections, symptoms reported for patients with SARS-CoV-2 include mild to severe respiratory illness with fever, cough, and difficulty breathing.

PRINCIPLE OF THE TEST:

It is based on the reverse transcription (RT) and amplification in the same reaction well of specific fragments of SARS-CoV-2 (2019-nCoV) and SARS-related coronaviruses by real time PCR.

One lyophilized master mix is provided for screening and confirmation using two independent targets of the virus. The assays do not cross react with common human respiratory coronavirus or MERS.

PCR mix targets a specific fragment of the *N* gene for SARS-CoV-2 and a generic fragment of the *E* gene which is positive for SARS-CoV-2 and also for others SARS-related Coronavirus.

An amplification control is included associated to the extraction of the sample (human *RNAse P* gene) to check the absence of carry-over of amplification inhibitors and the correct reverse transcription and amplification set-up.

The technique is divided into 2 main steps: RNA extraction and reverse transcription and amplification/detection with specific oligo pairs and probes. Coronavirus RNA is detected in FAM (*N*) and Cy5 (*E*) channels while the internal control is labelled with HEX/VIC (human *P RNAse*).

KIT FEATURES:

This kit is based on reverse transcription and amplification and detection using real time PCR.

It is recommended to use conventional RNA purification kits, however the RT-PCR Mix is formulated with enzymes partially resistant to inhibitors so it allows using a quick extraction kit RTEXT001 (DIRECT EXTRACTION KIT) which VIRCELL also offers as an alternative RNA extraction reagent (see "Preliminary preparation of reagents" section).

The design of the mix targets conserved regions, however considering the variability of RNA virus, a result should only be considered positive for SARS-CoV-2 if both targets are positive.

RT-PCR Mix and positive control reagents are lyophilized. It is necessary to reconstitute them before use (see "Preliminary preparation of reagents" section). The rest of the reagents are ready to use.

KIT CONTENTS:

1 VIRCELL CoV-2 RT-PCR MIX: 6 vials containing reverse transcriptase, Taq polymerase, buffer and specific primers/probe for *N* gene of nCoV and for *E* gene of SARS-related coronaviruses. Also, as internal control, primers/probe for human *RNAse P* gene. 16 reactions per vial. Lyophilized.

3 VIRCELL CoV-2 POSITIVE CONTROL: 1 vial containing a mixture of lyophilized non-infectious nucleic acids to be used as positive control. Red cap.

4 VIRCELL NEGATIVE CONTROL: 1 vial containing 200 µl of deionized water to be used as negative control. Green cap.

5 VIRCELL PCR MIX RECONSTITUTION SOLUTION: 2 vials with 1 ml of aqueous solution to reconstitute the PCR mix. Yellow cap.

6 VIRCELL POSITIVE CONTROL RECONSTITUTION SOLUTION: 1 vial with 500 µl of aqueous solution to reconstitute the positive control. Brown cap.

Store at 2-8°C and check expiration date.

Materials required but not supplied:

Microbiological safety cabinet
RNA extraction kit (see recommendations in "Test procedure")
qPCR thermocycler
Precision micropipettes
Sterile tips with aerosol barrier
Microcentrifuge
PCR cabinet (recommended)
Vortex

STORAGE REQUIREMENTS:

Store at the recommended temperature indicated. Do not use the kit reagents beyond the expiration date. This will be valid only if reagents are capped and stored at the indicated temperature.

STABILITY OF REAGENTS ONCE OPENED:

Reagent	Stability
Reconstituted VIRCELL POSITIVE CONTROL	Store below -20°C and use until expiration date. Avoid multiple freeze-thaw cycles.
Reconstituted RT-PCR MIX	Store below -20°C and use until expiration date. Avoid multiple freeze-thaw cycles.
Rest of the components	Store at 2-8°C and use until expiration date

STABILITY AND HANDLING OF REAGENTS:

The kit is stable until the expiration date at the indicated temperature. After the reconstitution of VIRCELL POSITIVE CONTROL, this should be stored below -20°C.

Reconstituted VIRCELL MIX VIALS should be used immediately after reconstitution maintaining in a cold rack protected from light. Store reconstituted mix not used immediately below -20°C until use.

Handle reagents in aseptic conditions to avoid microbial contaminations.

VIRCELL, S.L. does not accept responsibility for the mishandling of the reagents included in the kit.

RECOMMENDATIONS AND PRECAUTIONS:

1. For *in vitro* diagnosis use only. For professional use only.
2. The product should be limited to personnel who have been trained in the technique.
3. The user of this kit is advised to carefully read and understand the package insert. Strict adherence to the protocol is necessary to obtain reliable test results.
4. Use only protocols described in this insert. Conditions other than specified may give erroneous results.

5. Wear personal protective equipment when handling samples. Wash hands properly after handling the samples. All procedures must be carried out in accordance with the approved safety standards.
6. Do not use the kit after expiration date.
7. Specimens should be handled as in the case of infectious samples using safety laboratory procedures. Thoroughly clean and disinfect all work surfaces with a freshly prepared solution of 0.5% sodium hypochlorite in deionized or distilled water.
8. Testing of all the samples at the earliest interval following collection will help ensure the most accurate test results. Variation in storage times during specimen shipment has not been assessed.
9. It is recommended to have two different areas to perform the test: Pre-Amplification and Amplification areas.
10. Due to the high analytical sensitivity of this test, extreme care should be taken to preserve the purity of kit reagents or amplification mixtures. All reagents should be closely monitored to purity.
11. Reagents in this kit could include genetic material or substances of animal and/or human origin. Although that material is not infectious, it should be handled as potentially infectious. All material should be handled and disposed as potentially infectious. Observe the local regulations for clinical waste disposal.
12. Dispose of unused reagents and waste in accordance with all applicable regulations.
13. Any serious incident that occurs in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

SPECIMEN COLLECTION AND HANDLING:

The most used samples are nasopharyngeal / oropharyngeal swab. Alternatively, tracheal aspirates, bronchoalveolar lavage or saliva can be used.

Do not delay transport and laboratory investigations. Specimens could be stored at 2-8°C for up to 72 hours after collection, if delay is expected storage below -70°C is recommended.

PRELIMINARY PREPARATION OF THE REAGENTS:

All reagents supplied are ready to use, except for the VIRCELL PCR MIX VIALS 1 and the VIRCELL POSITIVE CONTROL 3.

If the reference RTEXT001 (DIRECT RNA EXTRACTION KIT) is used for RNA extraction, the volumes indicated below are not optimal. It is necessary to follow the instructions in the product manual for the correct performance of the RT-PCR assay.

1 VIRCELL PCR MIX VIALS. For reconstitution add 240 µl of VIRCELL PCR MIX RECONSTITUTION SOLUTION 5 per vial. Mix thoroughly using a vortex for 2-3 seconds.

⚠ The reconstituted PCR MIX must be used right after adding the reconstitution solution and it should be kept in a freeze rack until use protected from light.

The excess of reconstituted PCR mix can be frozen at temperature below -20°C protected from light to be used in subsequent reactions.

3 VIRCELL POSITIVE CONTROL. Follow the next steps to reconstitute it:
- Centrifuge the corresponding tube for 5 seconds at 5000 g.
- Add 100 µl of VIRCELL POSITIVE CONTROL RECONSTITUTION SOLUTION 6.
- Mix with vortex for 1-2 seconds.
- Centrifuge the tube for 5 seconds at 5000 g.

After reconstitution, the VIRCELL POSITIVE CONTROL 3 can be frozen at temperature below -20°C to be used in subsequent reactions.

TEST PROCEDURE:

1. RNA extraction (performed in the Pre-Amplification area):
It is recommended to use a commercial extraction kit for RNA extraction. If not available, the reference RTEXT001 DIRECT RNA EXTRACTION KIT (VIRCELL) has been validated for the extraction of SARS-COV-2 from respiratory samples in virus transport media. This kit provides a fast extraction of RNA with hardly any manipulation. For further information please consult the user's manual.

In order to use commercial extraction kits, follow the manufacturer instructions for RNA extraction. Consult with Customer Service.

2. Amplification using RT-PCR (performed in the Amplification area):
VIRCELL PCR MIX VIALS 1 are lyophilized. Each vial contains the necessary components to perform 16 RT-PCR reactions.

If the reference RTEXT001 (DIRECT RNA EXTRACTION KIT) is used for RNA extraction, the volumes indicated below are not optimal. It is necessary to follow the instructions in the product manual for the correct performance of the RT-PCR assay.

2.1 Preparation of the RT-PCR tubes: Label and allocate in freeze rack the number of tubes/strips of tubes needed. One tube will be required for each sample, plus one tube for the negative control and another one for the positive control.
2.2 Reconstitution of PCR mix: Add 240 µl of VIRCELL PCR MIX RECONSTITUTION SOLUTION 5 per vial. Mix thoroughly using a vortex for 2-3 seconds. Maintain cold when thawed.
2.3 Pipet 15 µl of Mix to a PCR tube.
2.4 Addition of the sample: Add 5 µl of each extracted RNA sample to each tube. Add 5 µl of VIRCELL POSITIVE CONTROL 3 and VIRCELL NEGATIVE CONTROL 4 to the corresponding tubes. The negative control is water. Secure tube/strip of tubes caps.

2.5 RT-PCR program: Insert the PCR tubes/strip of tubes in the real time thermocycler and run the following program*:

1 cycle	51ºC 20 minutes
1 cycle	95ºC 2 minutes
45 cycles	95ºC 15 seconds
	58ºC 45 seconds *

*Fluorescence data (FAM, Cy5 and HEX/VIC) should be collected.

INTERNAL QUALITY CONTROL:

Each batch is subjected to internal quality control testing before releasing, complying with strict specifications.

INTERPRETATION OF RESULTS AND VALIDATION PROTOCOL FOR USERS:

It is recommended to include one negative control in each run performed. Negative control is water, therefore no amplification in any channel should be detected. The negative control will monitor reagent or environmental contamination.
The positive control is recommended to be included on each run. The positive control monitors for reagent failures and for correct operation of essential procedure.

The controls result interpretation is as follows:

CONTROL	N (FAM)	E (Cy5)	IC (HEX/VIC)	Interpretation
VIRCELL CoV-2 POSITIVE CONTROL	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 40)	Correct
	No amplification (>40 or N/A)	No amplification (>40 or N/A)	No amplification (>40 or N/A)	Invalid
VIRCELL NEGATIVE CONTROL	No amplification	No amplification	No or late amplification (Ct>38)	Correct
	Amplification (Ct < 40)	Amplification (Ct < 40)	Amplification (Ct < 38)	Invalid

The result interpretation is described in the tables below:

RESULT	N (FAM)	E (Cy5)	IC (HEX/VIC)	Interpretation
1	**No amplification**	**No amplification**	**No amplification**	**Invalid (sample/kit/setup related)**
2	**No amplification**	**No amplification**	**Amplification (Ct < 40)**	**SARS-CoV-2 Negative sample**
3	**Amplification (Ct < 40)**	**Amplification (Ct < 40)**	**Amplification (Ct < 40)**	**SARS-CoV-2 Positive**
4	**Amplification (Ct < 40)**	**Amplification (Ct < 40)**	**No amplification**	**SARS-CoV-2 Positive**
5	**Amplification (Ct < 40)**	**No amplification**	**Amplification (Ct < 40)**	**Inconclusive**
6	**No amplification**	**Amplification (Ct < 40)**	**Amplification (Ct < 40)**	**SARS-related Coronavirus Positive –* Presumptive SARS-CoV-2 Positive**

*A sample result positive only for E target could be considered presumptive SARS-CoV-2 positive, probably due to low RNA, close to the limit of detection, or different amplification efficiency for *E* and *N* targets, however the presence of a SARS-related Coronavirus (other Sarbecovirus) could not be discarded. Sample might be retested for confirmation.

In case of invalid or inconclusive result, it is recommended to re-extract RNA from original specimen and re-test it. In the case of failure of amplification of internal control, improper extraction of nucleic acids or absence of sufficient human cellular material could be assumed. Testing a new sample is recommended.

LIMITATIONS:

1. This kit is intended to be used with human respiratory samples. The performance with other types of samples has not been evaluated.
2. Detection of the virus nucleic acids depends on the number of virus load present in the specimen and may be affected by specimen collection methods, patient factors, stage of infection and/or strain. False negative results may also occur if amplification inhibitors are present in the specimen, validated nucleic acids extraction methods for RNA virus should be used.
3. The results of samples should be used in conjunction with clinical evaluation and other diagnostic procedures.
4. The test provides qualitative results. No correlation can be drawn between the magnitude of a positive result and the number of microorganisms in the sample.
5. The test only works within the limits of the genomic regions from which the primers and probes have been chosen. The test targets highly conserved regions, however due to the high variability of RNA genomes it is possible that certain sub-types might not be detected. At design time, mutations of the target regions were not detected.
6. A negative test result does not exclude the presence of the target organism at levels below the detection limit of the assay.
7. A positive test does not rule out the possibility that other pathogens may be present.
8. The performance results showed correspond to comparative studies with commercial predicative devices in a defined population sample. Small differences can be found with different populations or different predicative devices.

PERFORMANCES:

- **SENSITIVITY AND SPECIFICITY**

DIRECT SARS-CoV-2 REALTIME PCR KIT has been evaluated with a panel of 87 respiratory samples (nasopharyngeal and/or oropharyngeal swabs in transport medium) from Lozano Blesa University Hospital (Zaragoza, Spain) and San Cecilio University Hospital (Granada, Spain). This panel included 48 positive samples and 39 negative samples previously characterized by hospital reference molecular assay.
The results show 100 % specificity and 96 % sensitivity.

- **WITHIN-RUN PRECISION:**

4 samples (two positive and the positive and negative control) were amplified 5 times in a single assay performed by the same operator in essentially unchanged conditions.
Results: 100 % agreement with CV% < 1.

- **BETWEEN-RUN PRECISION:**

4 samples (two positive and the positive and negative control) were individually amplified on 3 consecutive runs in two different RT-PCR thermocyclers.
Results: 100 % agreement with CV% < 1.

- **CROSS REACTIVITY:**

The specificity of the DIRECT SARS-CoV-2 REALTIME PCR KIT was confirmed by testing a panel consisting of different microorganisms representing the most common respiratory pathogens: HCoV-OC43, HCoV-229E, HCoV-NL63, MERS-CoV, SARS-CoV (2003), Influenza A nH1N1, Influenza A H3N2, Influenza B, Rhinovirus, Enterovirus, Respiratory syncytial virus A, Respiratory syncytial virus B, Parainfluenza 1 virus, Parainfluenza 2 virus, Parainfluenza 3 virus, Parainfluenza 4 virus, Human metapneumovirus, Adenovirus, *Chlamydophila pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Bordetella pertussis*, *Mycoplasma pneumoniae*, *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus epidermis* and a pooled human nasal wash (represent diverse microbial flora in the human respiratory tract). No cross reactivity with these organisms was found.
In addition, an in-silico analysis of the primers/probes sequences comparing to other microorganisms that could be found in a respiratory sample was performed, according to guideline by WHO: Instructions for

Submission Requirements: In vitro diagnostics (IVDs) Detecting SARS-CoV-2 Nucleic Acid.
Emergency Use Listing of IVDs WHO. The sequence of 15 microorganisms that could be found in a respiratory sample was analysed: *Bacillus anthracis*, *Chlamydophila psittaci*, *Corynebacterium diphtheriae, Coxiella burnetii*, Human coronavirus HKU1, Influenza C, Legionella non-pneumophila, Leptospiraceae, *Moraxella catarrhalis*, *Neisseria elongate*, *Neisseria meningitidis*, Parechovirus, *Pneumocystis jirovecii*, *Staphylococcus aureus* and *Streptococcus salivarius*.
A homology greater than 80% was not found in any primered microorganism.

• ANALYTICAL SENSITIVITY:
A preliminary LoD (limit of detection) was determined by testing 5 replicates of 3-fold serial dilutions of quantified SARS-CoV-2 virus sample.
Once an approximated LoD is established, the final concentration was confirmed by testing 20 replicates of 3-fold serial dilutions. The LoD is determined as the lowest concentration where ≥ 95% (19/20) of the replicates are positive.

	Result (copies/ml)	Result (copies/reaction)
LoD N target	700	3.5
LoD E target	700	3.5

• INCLUSIVITY:
An *in-silico* analysis for the primered genes included in the assay was performed to determine the inclusivity for the different SARS-CoV-2 sequences available. SARS-CoV-2 selected sequences representing those deposited from the outbreak initial event to the latest ones available covering worldwide samples matched 100% the sequence of primes/probes used in the assay.

• EXTERNAL CONTROL:
Controls that are required but not provided with the test kit will be the following. As positive extraction control, AMPLIRUN® TOTAL SARS-CoV-2 CONTROL (SWAB) Cat. MBTC030-R (Vircell). As negative extraction control (NEC), RESPIRATORY SWAB MATRIX NEGATIVE CONTROL Cat. MC110 (Vircell), which is a synthetic respiratory matrix, simulating a SARS-CoV-2 negative patient sample collected in UTM. Both controls help monitoring any cross-contamination that occurs during the extraction process, additionally serve as validation tools for extraction reagents.

SYMBOLS USED IN LABELS:

IVD	*In vitro* diagnostic medical device
	Use by (expiration date)
Y°C / X°C	Store at x-y°C
Σ n	Contains sufficient for <n> tests
LOT	Batch
REF	Catalogue number
	Consult instructions for use
RCNS Xµl	Reconstitute in <X> µl
STORE	Storage conditions

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