quanty COVID-19

REF: RT-25

Detection and quantification of the COVID-19 genome with *Real Time PCR*

INTRODUCTION AND PURPOSE OF USE

The quanty COVID-19 system is a quantitative test that allows the quantification, by means of *Real Time PCR*, of the *N* region (nucleocapsid phosphoprotein) of novel Coronavirus (COVID-19).
The Procedure allows the detection of the RNA target by means a retro-amplification reaction in a microplate.
The analysis of the results is made using a Real Time PCR analyzer instrument (thermal cycler integrated with a system for fluorescence detection).

CONTENT

The kit contains reagents enough to perform 48 amplification tests.

	Quantity	Description
R1	3 x 180 µl	**Amplification mMix** dNTPs, Tris-HCl, KCl, $MgCl_2$, Taq Polymerase, *AmpErase* Uracil N-Glycosylase (*UNG)* Nuclease-free water, ROX (Pink Cap)
R2	3 x 280 µl	**N1-N2 probe Mix** Upstream primer, downstream primer, Target probe N1 (FAM), Target probe N2 (VIC), Nuclease-free water, (White Cap)
R3	3 x 280 µl	**N3-RP probe Mix** Upstream primer, downstream primer, Target probe N3 (VIC), Inhibition Control (RP) probe (CY5), Nuclease-free water (Yellow Cap)
R4	3 x 35 µl	**N1 Positive Control** synthetic RNA corresponding to N1 region at the concentration of 10^5 copies/µl (Blue Cap)
R5	3 x 35 µl	**N1 Positive Control** synthetic RNA corresponding to N1 region at the concentration of 10^4 copies/µl (Green Cap)
R6	3 x 35 µl	**N1 Positive Control** synthetic RNA corresponding to N1 region at the concentration of 10^3 copies/µl (Yellow Cap)
R7	3 x 35 µl	**N1 Positive Control** synthetic RNA corresponding to N1 region at the concentration of 10^2 copies/µl (White Cap)
R8	3 x 35 µl	**N2 Positive Control** synthetic RNA corresponding to N2 region (Red Cap)
R9	3 x 35 µl	**N3 Positive Control** synthetic RNA corresponding to N3 region (Pink Cap)
R10	1 x 50 µl	Negative Control

Instruction for use: **ST. RT25-ENG.3**

MATERIALS AND STRUMENTATION REQUIRED BUT NOT SUPPLIED

Disposable latex powder-free gloves or similar material;
Bench microcentrifuge;
Micropipettes and Sterile tips with aerosol filter;
Vortex;
Sterile plastic materials (microplate and optical adhesive cover);
Heat block (only for manual extraction)

Reagents

The **quanty COVID-19** kit was developed and validated to be used with the following extraction method:

Manual Extraction

Ref. 52906. QIAmp Viral RNA Mini Kit
The kit allows the manual RNA extraction from tested samples. The kit contains reagents for 250 samples. (QIAGEN)

Instruments

The **quanty COVID-19** kit was evaluated to be used with the following instruments:

Real Time PCR
- 7500 Fast from Lifetechnologies
- Rotor Gene Q MDx from QIAGEN
- CFX96 from Biorad

Please ensure that the instruments have been installed, calibrated, checked and maintained according to the manufacturers instruction and recommendations.

SAMPLES AND STORAGE

The **quanty COVID-19** kit must be used with extracted RNA from biological samples: **Nasopharyngeal/oropharyngeal swabs, sputum and serum**. Collected samples must be shipped and stored at +2 - +8°C and used within 3 days from the collected data.
Store the sample at -20°C if used after 3 days.

PRECAUTIONS USE

This kit is for in *vitro* diagnostic (IVD), for professional use only and not for in vivo use.
After reconstitution, the amplification master mix must be used in one time. Repeat thawing and freezing of reagents (more than twice) should be avoided, as this might affect the performance of the assay. The reagents should be frozen in aliquots, if they are to be used intermittently. At all times follow Good Laboratory Practice (GLP) guidelines.
Wear protective clothing such as laboratory coats and disposable gloves while assaying samples.
Avoid any contact between hands and eyes or nose during specimen collection and testing.
Handle and dispose all used materials into appropriate bio-hazard waste containers. It should be discarded according to local law.
Keep separated the extraction and the reagents preparation.
Never pipette solutions by mouth.
Avoid the air bubbles during the master mix dispensing. Eliminate them before starting amplification.
Wash hands carefully after handling samples and reagents.
Do not mix reagents from different lots.
It is not infectious and hazardous for the health (see Material Safety data Sheet – MSDS).
Do not eat, drink or smoke in the area where specimens and kit reagents are handled.
Read carefully the instructions notice before using this test.
Do not use beyond the expiration date which appears on the package label.
Do not use a test from a damaged protective wrapper.

LIMIT OF THE METHOD

The extreme sensitivity of gene amplification may cause false positives due to cross-contamination between samples and controls. Therefore, you should:
- physically separate all the products and reagents used for amplification reactions from those used for other reactions, as well as from post-amplification products;
- use tips with filters to prevent cross-contamination between samples;
- use disposable gloves and change them frequently;
- carefully open test tubes to prevent aerosol formation;
- close every test tube before opening another one.

The proper functioning of the retro-amplification mix depends on the correct collection, correct transportation, correct storage and correct preparation of a biological sample.
As with any diagnostic device, the results obtained with this product must be interpreted taking in consideration all the clinical data and other laboratory tests done on the patient.
A negative result obtained with this product suggests that the RNA of COVID-19 was not detected in RNA extracted from the sample, but it may also contain COVID-19-RNA at a lower titre than the detection limit for the product (detection limit for the product, see paragraph on Performance Characteristics); in this case the result would be a false negative.
As with any diagnostic device, with this product there is a residual risk of obtaining invalid, false positives or false negatives results.

STORAGE AND STABILITY

Store the product **quanty COVID-19** at –20°C.
The **quanty COVID-19** kit is shipped on dry ice. The kit components should be frozen.
If one or more components are not frozen upon receipt or if the tubes have been compromised during transport, contact Clonit srl for assistance.
An intact and well stored product has a stability of 12 months from the date of production. Do not use beyond the expiration date which appears on the package label.
Repeat thawing and freezing of reagents (more than twice) should be avoided, as this might affect the performance of the assay. The reagents should be frozen in aliquots, if they are to be used intermittently.

ANALYTICAL PROCEDURE

Manual Extraction

Ref. 52906. QIAmp Viral RNA Mini Kit
Follow the instructions inside the kit *QIAmp Viral RNA Mini Kit.*
Elute the sample in 50 µl of buffer AVE.

SOFTWARE SETTING

Lifetechnologies 7500 fast

Turn the instrument and the computer on and open the control software. Click on "**Advance Setup**": by default the software will shows the page "**experiment properties**". Write in the "**experiment name**" the file name, choose the type of instrument (**7500 o 7500fast**), the type of reaction (**quantitation standard curve**), the type of used reagent (**Taqman®Reagents**) and the reaction time of analysis (**Standard ≈ 2 hours to complete a run**).
Open the page named "**page setup"** (sheet **Define Target and Samples**).

In the window **Define Targets** set:

Target mix N1-N2	Reporter	Quencher
N1	FAM	NONE
N2	VIC	NONE
Target mix N3-RP	**Reporter**	**Quencher**
N3	VIC	NONE
RP (Inhibition Control)	CY5	NONE

Set the samples' name in the window "**Define Samples**".
In the same page "**plate setup**" select the sheet "**Assign Target and Samples**". On the screen you will see the microplate draft.

Select an area of the plate where the N1-N2 mix will be placed and set both targets.
Select "**Assign target to selected wells**" in the blank, the "**task Standard (S)**" for N1 target and set the controls' concentration.
Select "**Assign target to selected wells**" in the blank, the "**Positive control**" for the target N2.
Choose an area in the plate where negative control will be placed for each mix: select "**Assign target to selected wells**" in the blank, the "**task Negative (N)**" for the targets N1 and N2.
Select an area of the plate where the N3-RP mix will be placed and set both targets.
Select "**Assign target to selected wells**" in the blank, the "**Positive control**" for the target N3.
Choose an area in the plate where negative control will be placed: select "**Assign target to selected wells**" in the blank, the "**task Negative (N)**" for the targets N3 and RP.
Select an area of the plate where samples will be placed in each mix: select the wells and set targets (N1 and N2 for the first mix – N3 and RP for the second mix). Link every well to a sample, through the window "**Assign samples to selected wells**".
For each sample, select in the blank "**Assign targets to selected wells**" the "**task UnKnown (U)**" for the targets.
Set ROX as passive reference, using it as normalizer of detecting fluorescence.
Open "**Run Method**" (sheet **Graphic View**) and set the right thermal cycling with the "collect data" in annealing phase at 55°C.

cycles	UNG	Retrotrascription	Denaturation	Annealing
1	25°C 2 min	50° C 15 min		
1			95°C 2 min	
45			95° C 3 sec	55° C 30 sec

In the window "**Reaction volume plate per well**" set a volume of 25 µl.
After preparing the plate, and correctly inserting it in the instrument, press the button "**Start Run**".

Rotor Gene Q MDx

The experiments can be **set using the Quick Start Wizard or the** Advanced Wizard, which appears when the software is started.
Select the wizard "**Advanced**". As a first step, select the model "**Two Step Reaction**" with a double click in the "**New Run**".
In the next window, select the type of rotor installed on the instrument from the list that appears. Check the "**Locking Ring Attached**", check the checkbox and then click "**Next**".
Enter the name of the operator and the reaction volume of 25 µl, and then click "**Next**".
In the next window click on "**edit profile**". Set the following thermal cycle:

cycles	UNG	Retrotrascription	Denaturation	Annealing
1	25°C 2 min	50° C 15 min		
1			95°C 2 min	
45			95° C 3 sec	55° C 30 sec

Select the annealing from the thermal profile and click on "**Acquiring A to cycling**."
In the next window, select Yellow and Red from the available channels and add it to **acquiring channel** along with the Green channel and click "**OK**".
Click on "Edit Gain" button and set the following values for each channel:

Target	Reporter	Gain
N1	Green	5
N2 e N3	Yellow	5
RP	Red	5

In the next window click on "**OK**" and then click "**Next**".
To begin the course, click on the button "**Start Run**". You can save the model before you begin your run by clicking on "**Save Template**".
After clicking on the button "**Start Run"** window appears "**Save As**". The stroke can be saved in the desired position by the user.
Once the run started, the window **"Edit Samples"** allows you to set the name of samples and controls in the positions in which they were loaded on the instrument.
Select the locations where they were positioned the controls at a known concentration and name them as **N1 Standard**.
Clicking on the box next to "**Type**" correspondent, in the dropdown menu "**Samples**" you can select the type of sample being analyzed. Select "**Standards**". **Insert the controls' concentration.**
Select the locations where they were positioned the controls N2 for the first mix and N3 for the second mix and name them.
Clicking on the box next to "**Type**" correspondent, in the dropdown menu "**Samples**" you can select the type of sample being analyzed. Select "**Positive Control**".
Select the location where you placed the Negative Control for each mix and name it as **Negative Control**. Clicking on the box next to "**Type**" correspondent, in the dropdown menu "**Samples**" you can select the type of sample being analyzed. Select "**Negative Controls**".
Select the location of each sample for each mix and enter the name or code of the patient. Clicking on the box next to "**Type**" correspondent, in the dropdown menu "**Samples**" you can select the type of sample being analyzed. Select "**UnKnown**".
At the end of the operation click "**OK**" in the "**edit samples**" and wait until the end of the race for the analysis (see "**Interpretation of Results**").

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CFX96 Real Time PCR

Turn the instrument and the computer on and start the control software. In the principal screen will appear the window "**Startup wizard**": select "**CFX96"** and press **"ok".** In the next window push "**create new**" and set the thermal protocol and the reaction volume (25µl):

cycles	UNG	Retrotrascription	Denaturation	Annealing
1	25°C 2 min	50° C 15 min		
1			95°C 2 min	
45			95° C 3 sec	55° C 30 sec

Save the protocol and click "**Next**". The software will open in default the sheet "**plate**". Click "**create new**", select **"Fluorophores button"** to choose fluorophores (FAM, VIC and CY5).

Target mix N1-N2	Reporter	Quencher
N1	FAM	NONE
N2	VIC	NONE
Target mix N3-RP	**Reporter**	**Quencher**
N3	VIC	NONE
RP (Inhibition Control)	CY5	NONE

Select the locations where they were positioned the controls of N1 target of known concentration and choose the **"Sample Type" Standards.** Click "**Load check boxes**" to load fluorophores and type or select Target Name.
In the box "**load concentration"** set the concentrations of the 4 calibrators, following the instructions indicated in the paragraph **Analysis of the results**.
Select the locations where they were positioned the controls of N2 target and N3 target. Choose the **"Sample Type" Positive Control.** Click "**Load**" check boxes to load fluorophores and type or select Target Name.
Select the location where you placed the Negative Control. Choose the **"Sample Type" NTC.** Click "**Load check boxes**" to load fluorophores and type or select Target Name.
Select the location of each sample and enter the name or code of the patient. Choose the **"Sample Type" Unknown.** Click "**Load check boxes**" to load fluorophores and type or select Target Name.
Save the plate by clicking the "Next" **button and** after preparing the plate, and correctly inserting it in the instrument, press the button "**Start Run**".

PREPARATION OF THE REACTIONS:

Defrost a tube of **Amplification mMix**;
Defrost a tube of **N1-N2 probes Mix;**
Defrost a tube of **N3-RP probes Mix;**
Mix carefully by vortex **85µl** of the **Amplification mMix and 255 µl** of **N1-N2 probes Mix** (the mix as produced is enough to prepare **16 reactions** of amplification: **4 Standard Controls (N1), 1 Positive Control (N2), 1 Negative Control and 10 samples**).
Mix carefully by vortex **85µl** of the **Amplification mMix and 255 µl** of **N3-RP probes Mix** (the mix as produced is enough to prepare **16 reactions** of amplification: **1 Positive Control (N3), 1 Negative Control and 14 samples**).
Distribute, in the amplification plate, **20µl of just reconstituted mix** in chosen positions as already set on the instrument software for each mix.
Distribute, in the negative control position, **5 µl** of solution taken by the negative control vial for each mix.
Distribute, in chosen position for each sample, **5 µl** of corresponding sample for each mix.
Distribute, in chosen positions for the positive controls, **5µl** of the following solutions: **10^2 copies/µl, 10^3 copies/µl, 10^4 copies/µl and 10^5 copies/µl** for **N1** target**,** distribute **5µl positive control** for **N2** target and distribute **5µl positive control** for **N3** target in the corresponding mix**.**
Seal up accurately the plate using an optical adhesive film and verify that there aren't air bubbles in the mix, to avoid the amplification interferences.
For the Rotor-gene Q 5/6 plex, seal each tube with the appropriate cap.
The air bubbles presence is not influent; the rotor centrifugal force will allow automatic deletion.
Transfer the plate in the instrument and select "**Run**" button.

QUALTITATIVE ANALYSIS

Lifetechnologies 7500 Fast

At the end of the PCR run, the software automatically opens the "**Analysis**" window in the "**Amplification plot**" sheet on the menu on the left.
Select the wells corresponding to the positive control, negative control and samples for analysis.
Select in the "**Option**" window inside the "**Target**" pop-up menu the **N1 target**. Check the correct setting of the threshold.
Select in the "**Option**" window inside the "**Target**" pop-up menu the **N2 target**. Check the correct setting of the threshold.
Select in the "**Option**" window inside the "**Target**" pop-up menu the **N3 target**. Check the correct setting of the threshold.
Select in the "**Option**" window inside the "**Target**" pop-up menu the **RP target**. Check the correct setting of the threshold.

The analysis of the results is made selecting from the menu in the left the page **"Analysis"**. From the page "**Standard Curve**", maintaining open the sheet "**view well plate**" in the right side of the software select the wells containing the points of the curve and verify the parameters described in the paragraph "**Interpretation of the Results**" (coefficient of correlation, slope etc).
From the page "**Amplification Plot**" verify the amplification plot for every single sample.
Opening the sheet "**view well table**" in the right side of the software it is possible to verify the data obtained from experiments: Threshold Cycles and emitted fluorescence.
Clicking from the menu file and selecting the box export, the window "**export properties**" will open. Indicate the file name, select the position to save it (**Browse**) and click on button **"Start export".** In this way the software will permit to save a excel file with all the data corresponding to selected experiment.

Rotor Gene Q MDx

At the end of the PCR run open the "**Analysis**" window. Select the "**Quantification**" sheet and click on "**cycling A (green)".**
Select from the menu **"Dynamic Tube"** and subsequently **"Slope correct"**.
Check the correct setting of the threshold in the space provided "**CT calculation – Threshold"**.
Open the "**Analysis**" window. Select the "**Quantification**" sheet and click on "**cycling A (yellow)".**
Select from the menu **"Dynamic Tube"** and subsequently **"Slope correct"**.
Check the correct setting of the threshold in the space provided "**CT calculation – Threshold"**.
Open again the "**Analysis**" window. Select the "**Quantification**" sheet and click on "**cycling A (red)".**
Select from the menu **"Dynamic Tube"** and subsequently **"Slope correct"**.
Check the correct setting of the threshold in the space provided "**CT calculation – Threshold"**.

Also in this case, you can print a report of the analysis by clicking on the **"Report"** window and selecting the file in the first **Quantification cycling A (green)** and then the file **cycling A (yellow) and cycling A (red).**

CFX96 Real Time PCR System

At the end of the PCR, select the "**quantitation**" sheet. On the top of the screen, select "**settings**" from the menu and choose "**Baseline Threshold**" for each fluorophore.
For more accurate results, is essential to set threshold for each fluorophore separately of the different amplification mix.
You can export the report by selecting the paper block figure on the top of the screen.

INTERPRETATION OF RESULTS

In the amplification reaction for each sample, the Ct values of each specific probe for N1, N2, N3 are used to detect the presence of the Target in analysis.
The instrument software is able to analyze the fluorescence emitted by specific probes and the fluorescence increase of the specific probe for N1 (FAM) or N2 (VIC) and for N3 (VIC) indicate a positive sample for the target in question.
A sample is defined positive for COVID-19 if fluorescence is detected for all three targets (N1, N2 and N3). If one or more targets are found negative the sample is considered inconclusive.

N1	N2	N3	RP	Result
Positive	Positive	Positive	< 35	COVID-19 Positive
Negative	Negative	Negative	< 35	COVID-19 Negative
Negative	Negative	Negative	>35	Result invalid
Only one or two, out of three targets, positive			<35	Result inconclusive

As with any diagnostic device, the results obtained with this product must be interpreted taking in consideration all the clinical data and the other laboratory tests done on the patient.
As with any diagnostic device, with this product there is a residual risk of obtaining invalid, false positives or false negatives results.
The use of positive and negative controls in each amplification session allow to verify the correct functioning of the amplification mix and the absence of any contamination.
The instruments' software is able to analyze the emitted fluorescence of specific probes for N1 (FAM), N2 (VIC), N3 (VIC) and the specific probe of the internal control RP (Cy5).
The Ct values obtained from the amplification of the 4 controls at known concentrations, for the target N1, are used by the software to calculate the calibration curve where the unknown samples are interpolated.
Correct functioning of the amplification mixture can be verified by analyzing the following parameters:

Parameter	Reference
RTS conc. 10^5 copies/µl (FAM)	Ct ≤ 25
Correlation Coefficient	0.990 ≤ r^2 ≤ 1
Slope	-3,6 ≤ Slope ≤-3.2
PCR efficiency	90 ≤ Efficiency ≤ 100

If the results of the amplification reaction of the 10^5 copies/µl of RTS produce a Ct> 25 or undetermined, the session cannot be considered valid and must be repeated.
Verify that the correlation coefficient value (r^2), the slope and therefore the reaction efficiency fits to the limited indicated in the above table or do not deviate much from them, which represent the ideal range for a proper PCR reaction.
A further correct functioning of the amplification mixture must be verified by analyzing the following parameters:

Parameter	Reference
N2 positive Control	Ct ≤ 33
N3 positive Control	Ct ≤ 33

If the results of the amplification reaction of the positive controls produce a Ct> 33 or undetermined, the session cannot be considered valid and must be repeated.

The results obtained with this product must be interpreted taking in consideration all the clinical data and the other laboratory tests performed on the patient.

By correctly setting the standards concentration as a function of the extraction system you can get the quantization of the sample directly in copies / ml:

		Alternative Extraction
	RTS 1	500.000 copies
	RTS 2	50.000 copies
	RTS 3	5.000 copies
	RTS 4	500 copies

When alternative systems are used sample concentration expressed in copies/ml will be obtained using the formula:

$$copie/ml = \frac{1000}{Ve} \times \frac{Ev}{Ea} \times C_{reaz}$$

where:
- **Ve:** extracted sample Volume expressed in µl
- **Ev:** eluted sample Volume during extraction step expressed in µl
  Ea: extracted sample volume used for amplification expressed in µl
- **C_{reaz}:** copies provided by the instrument.

PERFORMANCES

Analytical sensitivity:

It is considered as analytical sensitivity the highest dilution (title) to which a positive sample can be diluted without the system losing the ability to detect with a positivity rate of ≥ 95%. The analytical sensitivity of the system was assessed by analyzing synthetic RNA, quantified by spectrophotometric analysis, containing the regions of interest of the virus in serial dilutions.

Target	Copies/µl
N1	1 cps/µl
N2	1 cps/µl
N3	1 cps/µl

Clinical sensitivity:

It is considered as clinical sensitivity the ability to detect true positive samples in the totality of the samples screened as positive. The analysis was made on COVID-19 positive samples and the test was performed following the method recommendations. Positive samples were confirmed with an other method available on the market.

Obtained results show a clinical sensitivity of 100%: for RNA samples extracted.

Linearity/Proportionality

The system linearity was valued analyzing synthetic RNA, quantified by spectrophotometric analysis, containing the regions of interest N1 of the virus in serial dilutions (1:10) from 500.000.000 copies/reactions to 50 copies/reaction of RNA in 5µl of extracted material added in the retro-amplification reaction.
The evaluation was performed analyzing 10 calibration curves, that showed these parameters:

RTS conc. 10^5 copie (FAM)	Ct ≤ 25	Medium Ct 22.19
Coefficiente correlazione	$0.990 \leq r^2 \leq 1$	Medium r^2 0,999
Slope	-3,6 ≤ Slope ≤-3.2	Medium slope -3,299
Efficienza PCR	90 ≤ Efficiency ≤ 100	Medium Eff 101%

Diagnostic Specificity:

It is considered as diagnostic specificity the ability of the method to detect trues negative samples. The diagnostic specificity of the system was evaluated analyzing human samples tested and confirmed as COVID-19 negative with an other method available on the market.

Diagnostic specificity is 100% for RNA samples extracted.

Analytical Specificity:

Test's specificity was guaranteed by the use of specific primers for COVID-19.
The alignment of the chosen regions for specific primers hybridization for COVID-19 with available sequences of the region present in database, demonstrated: their conservation, the absence of significative mutations for the analysed target.

INTERFERENCES:

Verify that in the RNA extracted from the sample there is no contamination from mucoproteins and haemoglobin, to exclude possible inhibition of PCR reaction. The interference due to contaminants can be detected through a spectrophotometric analysis, verifying the ratio between the absorbance readings at 260 nm (maximum absorbtion of Nucleic Acids) and 280 nm (maximum absorbtion of Proteins). A pure RNA should have a ratio of approximately 2.

QUALITY CONTROL

It is recommended to include in each analytical run, as quality control of every extraction, amplification and detection step, an already tested negative and positive sample, or a reference material with known concentration.
In accordance with the Clonit srl ISO EN 13485 Certified quality Management System, each lot of **quanty COVID-19** is tested against predetermined specification to ensure consistent product quality.

BIBLIOGRAPHY

Laboratory testing for 2019 novel coronavirus (2019-nCoV) in suspected human cases Interim guidance 17 January 2020 WHO/2019 nCoV/laboratory/2020.3

CDC/NCIRD/DVD Effective: 24 Jan 2020

TECHNICAL ASSISTANCE

For any question and support please contact our Technical support:

e-mail: info@clonit.it
phone: +39 02 56814413

IVD	*In vitro* diagnostic device
	Read the instruction's manual
	Range of temperature
	Use within (dd/mm/yyyy: year-month)
LOT	Lot (xxxx)
REF	Code
	Manufacturer
Σ	Contains sufficient for <n> tests

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