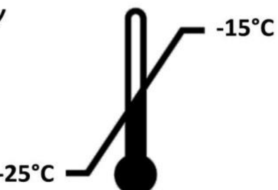
INOGene-SCoV-2 RT-qPCR Detection Kit

Instructions For Use

(For Professional Use Only)

For in Vitro Diagnostic Use Only



INOSCoV-2100
INOSCoV-2500

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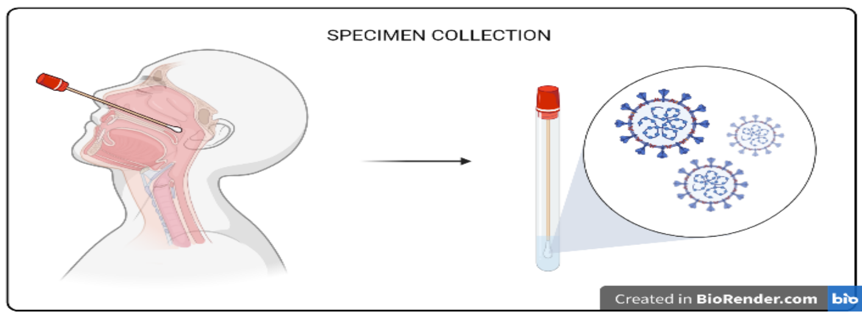
1. Purpose of Use

INOGene-SCOV2 qPCR Detection Kit includes M-MLV Reverse Transcriptase, Taq Polymerase enzymes, Ribonuclease inhibitor, and oligonucleotides specifically designed for this kit, targeting the areas with the lowest mutation risk, (This enables the detection of all variants). It detects SARS-COV-2 viral RNA in qPCR with high sensitivity even in small quantities of RNA. This kit allows for examination of materials acquired by RNA extraction from nasopharyngeal and oropharyngeal swabs, saliva, and sputum.



WHO recommends upper respiratory tract samples such as nasopharyngeal (NP), oropharyngeal (OP) swabs in ambulatory patients, and lower respiratory tract samples such as sputum, endotracheal aspirate, or bronchoalveolar lavage in patients with more severe disease when measuring the level of CoV-2 RNA by real-time reverse transcription polymerase chain reaction (Rt-qPCR) [1].

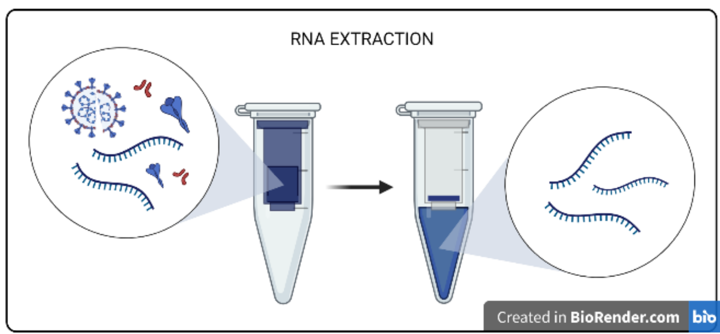
2. Principle and Additional Information

2.1 Specimen Collection and Pre-qPCR

Some specific solutions are needed to transport the sample intact to the facility where it will be evaluated.

- Virus Transport Medium (VTM)
- Viral RNA Transport Medium (VNAT)
- Universal Transport Medium (UTM)
- Phosphate Buffered Saline (PBS) or Saline solution
- Sterile nuclease-free water, may be.

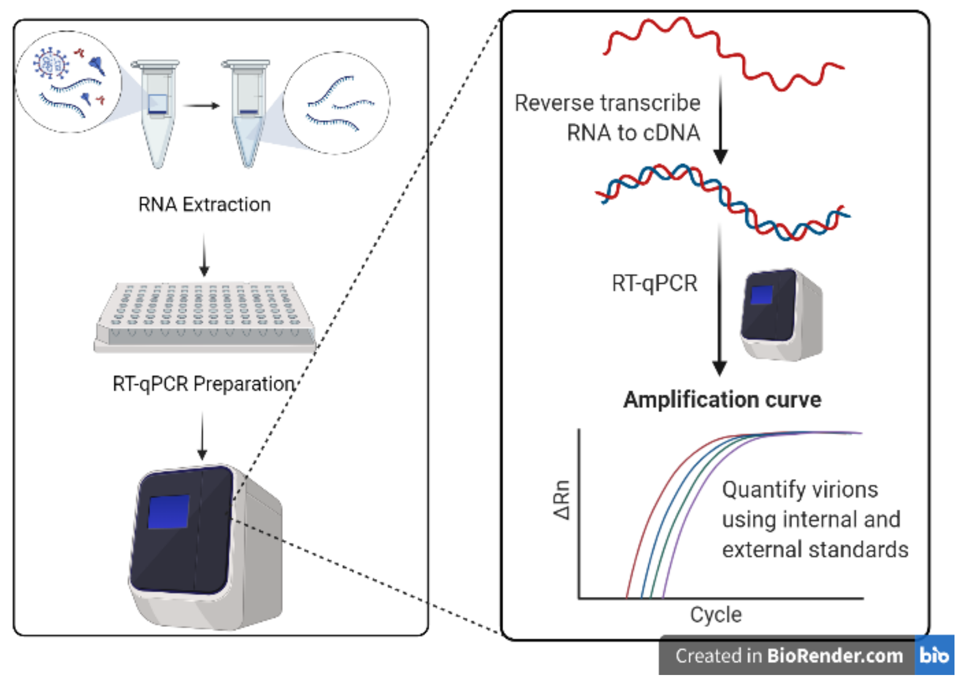
The sample preparation procedure varies depending on the extraction method to be used in the facility.



2.2 RT-qPCR Assay

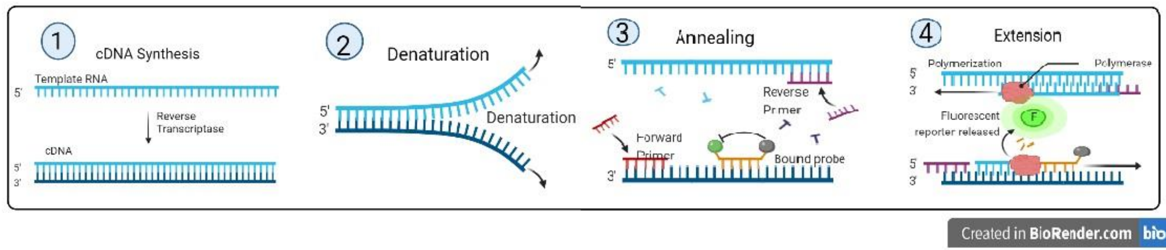
Polymerase chain reaction (PCR) is a highly sensitive and specific method used for the amplification and detection of deoxyribonucleic acid (DNA). Its conceptual simplicity has made it the most widely used technique in molecular biology and can, in theory, detect even a single copy of DNA. Therefore, it is widely used as a diagnostic test for a wide variety of bacterial, fungal, viral and parasitic pathogens. However, the genome of coronaviruses consists of ribonucleic acid (RNA) rather than DNA. Although RNA is similar to DNA, the result is unsatisfactory as Taq polymerase, the standard enzyme used for DNA amplification, transcribes it very inefficiently. For this reason, RNA is detected by a variant of the PCR test called reverse transcription (RT)-PCR. RT-PCR typically involves a two-step method involving two enzymes; The first step uses an RNA-linked DNA polymerase, also known as reverse transcriptase, to transcribe RNA to DNA (cDNA), while the second step involves amplification using Taq polymerase, which amplifies the cDNA as in a standard PCR test.

While it is most appropriate to carry out RT and PCR reactions in a single test tube for diagnostic purposes; For research use, the two steps are usually performed in separate tubes. Most diagnostic tests use a specific version of the RT-PCR test called fluorescence-based quantitative RT-PCR (RT-qPCR) [2].



2.3 TaqMan RT-qPCR Assay

Real-time PCR using 5′ nuclease or hydrolysis probes, also known as TaqMan qPCR, is an important and powerful tool used in various fields of life science. It also has great potential in diagnostic microbiology where TaqMan qPCR is often the first-line screening method for the detection of many viral or bacterial pathogens in human, animal or plant samples.



TaqMan qPCR uses a pair of primers and a non-extendable probe. The probe is a short, sequence-specific oligonucleotide that binds within the region bounded by the primers. One end of the probe, usually the 5' end, is labeled using a fluorescent dye, while the other end, the 3' end, is labeled with a quencher. As amplification progresses, the TaqMan probe hybridizes to the sequence on the downstream target of one of the primers. These two oligonucleotides form an effective unit. As the upstream primer begins to extend, Taq polymerase-mediated 5′→3′ hydrolysis of the probe takes place, leading to disruption of the pair formed by the quencher and fluorescent dye. Irradiation in the reaction tube results in detectable fluorescence with dye-specific wavelength. The emitted fluorescent signal is proportional to the amount of amplified PCR product [3].

3. Kit Contents

The Kit comes in two different package sizes: 100 and 500 reactions. Table 1 lists the components of both packages.

Table 1 INOGene-SCOV-2 RT-qPCR Detection Kit components

Reagents	100 reactions	100 rxn Vials	500 reactions	500 rxn Vials
Reaction Mix (Taq Polymerase,RT, RI, Buffer)	*1050 ul*	*1.5 ml screw tube*	*5250 ul*	*5x1.5 ml screw tube*
Primer-Probe Mix (Specially synthesized oligonucleotides for E, RDRP and Rnase P control)	*325 ul*	*0.5 ml screw tube*	*1550 ul*	*2 ml screw tube*
Negative Template Control (Nuclease-free water)	*400 ul*	*0.5 ml screw tube*	*1650 ul*	*2 ml screw tube*
Positive Control (Plasmid mix containing Covid-19 genome)	*40 ul*	*0.5 ml screw tube*	*200 ul*	*0.5 ml screw tube*

The kit uses reporter fluorophores to target the E and RdRp genes as SARS-COV-2 controls, as well as the Rnase P gene as an internal control, as indicated in Table 2.

Table 2 Targeted genes and reporter fluorophores

TARGET GENE	FLUOROPHORE
E Gene	*CY5 (RED)*
RdRp Gene	*FAM (GREEN)*
Internal Control (Rnase P)	*HEX (YELLOW)*

4. Storage Conditions

✓ The kit is shipped to the user with dry ice.
✓ All elements of the kit should be stored between -15 and -25 ℃. Aliquoting is recommended if thawing is to be done very frequently.
✓ The expiry date of the product is indicated on the boxes and tubes. As long as it is stored in suitable conditions, it can be used without any problems until the expiration date.
✓ The Primer-probe mix, in particular, should not be exposed to light.

5. Materials not provided

- 0.1-0.2 ml white or transparent PCR tubes
- PCR Optical lid, film or membrane
- Pipette tips (sterile, nuclease-free filters)
- Micropipettes
- Vortex
- Mini centrifuge

6. RT-qPCR Protocol

1. Reaction setup in the qPCR device is done by selecting the appropriate program for the device used.
2. The environment in which the reaction preparation will be made is made suitable for use by performing the necessary sterilization processes.
3. After the reagents are thawed on ice, they are gently vortexed, centrifuged briefly and placed back on ice.
4. Master mix according to the number of tests to be done It is prepared according to the quantities specified in the Table 3.

Table 3 Quantities of Reagents that will be used in reaction

Reagents are required for a reaction	Amounts
Reaction Mix (Taq Polymerase, RT, RI, buffer)	10 ul
Primer-Probe Mix (Primer and probes of E, RDRP ve Rnase P genes)	3 ul
Nuclease-free water	3 ul
Total volume	16 ul

5. 16 µl of the master mix is distributed to the previously prepared PCR tubes or 96-well plates.
6. Patient samples are loaded into the wells with 4 µl of the negative and/or positive control.

*A negative control must be included in each analysis for contamination research. Also, when the accuracy of the results is in doubt, enzyme activity or the operation of the primer-probes can be checked using a positive control.

7. Tubes or 96-well plates are tightly sealed using caps or film and placed in the instrument.
8. The program is run in the software and the results are waited for.

7. RT-qPCR program settings

The kit is optimized in Bio Rad CFX96, Roche Lightcycler 480 and Inovialab GenX-4. The program can be set as shown in Table 4. The program takes between 45-55 minutes depending on the device.The cDNA synthesis and Taq polymerase activation stages each take one cycle, whereas the Amplification segment's two phases take 40 cycles. In devices, the data acquisition step should be set to End Point Only Runs (Biorad,CFX96), Quantification (Roche, LC480), and End Point (Inovialab, GenX-4) for the second phase of amplification.

Table 4 RT-qPCR program

Steps	Temperature	Time	Cycles
cDNA Synthesis	48 °C	5 min	1 cycle
Taq polymerase Activation	95 °C	2 min	1 cycle
Amplification	95 °C	5 s	40 cycles
	60 °C	10 s	

8. Analysis of Results and Interpretation

8.1 Threshold Setting

When setting the threshold value, after adding an NTC well in each study and adjusting the threshold value for Rnase P to not give Ct in NTC, Rnase P Ct values are checked in patient samples, if the results are positive, RNA extraction is considered successful and Cts are checked for other genes. If patient samples do not test positive for Rnase P, the study may be contaminated or the RNA extraction was not successful. The test is retried with a new NTC. Ct values considered positive for genes are as in the Table 5.

Table 5 Negative-Positive Ct values for genes

Gene	**Fluorophore**	**Ct Value**	**Result**
RDRP	FAM	<36	Positive (+)
		≥36	Negative (-)
E	CY5	<36	Positive (+)
		≥36	Negative (-)
RNASE P	HEX	<28	Positive (+)
		≥28	Negative (-)

8.2 Analysis of Results

When analyzing results, the threshold value of the Rnase P internal control must first be set correctly. If the RNA extraction is successful and no contamination is detected, the positive-negative status of the E and RDRP genes are checked and the results are analyzed according to the Table 6.

Table 6 Possible analysis according to results

	E gene(CY5)	RdRp gene(FAM)	Rnase p (IC)(HEX)	Analysis results
Result 1	+	+	-	Covid Positive
Result 2	+	+	+	Covid Positive
Result 3	-	+	+	Covid Positive
Result 4	+	-	+	Covid Positive
Result 5	-	-	+	Covid Negative
Result 6	-	-	-	Invalid

8.3 Potential Problems and Their Solutions

Table 7 includes problems that may occur during the analysis and the solutions for them.

Table 7 Potential problems and their solutions

Problem	Possible Causes	Suggested Solutions
No signal	Incorrect setting of the Rt-qPCR program in the software.	The program is checked and the test is repeated.
	Wrong choice of paint or well.	The program is checked and the test is repeated.
	Reagents may not have been added correctly. (Not adding kit elements to the well, etc.)	The test is repeated.
	Expiration date of the kit, storage in the wrong conditions or decrease in enzyme activity after too much freezing and thawing etc.	The expiry date and the number of freeze-thaws are checked. If necessary, the test is repeated with a new kit.
	Presence of inhibitor in the reaction.	RNA extraction and RT-qPCR analysis are repeated.
	The RNA extraction may not have been successful and there may not be enough RNA or the sample may not have been added to the well.	The RNA sample is checked, if there is not enough RNA, a new sample is taken from the patient, first the extraction and then the Rt-qPCR analysis is repeated.
NTC has signal	There may be contamination.	The test is repeated with new consumables. In case of contamination again, the test is repeated by replacing the reagents with new ones.

9. Kit characteristics

- Kit optimizations were carried out using customized plasmids containing SARS-COV-2 genes. Depending on the number of copies in the positive sample, the test results were evaluated and the results are shown in Table 8.

Table 8 RT-QPCR analyzes with positive controls containing different number of copies

		Analysis Results	
	Positive control #Copy/reaction	**Positive**	**Negatif**
Sample 1	50,1*10^3	20/20	0/20
Sample 2	50,1*10^2	20/20	0/20
Sample 3	50,1*10^1	20/20	0/20
Sample 4	50,1*10^0	20/20	0/20

- Kit was compared to another commercially available kit and the results are shown in Table 9.

Table 9 Comparison Results

		Commercially Available Kit		
		Positive	Negative	Total
INOGene-SCoV-2 RT-qPCR Detection Kit	Positive	78	0	78
	Negative	1	16	17
	Total	79	16	105

- The possibility of false positives of the sequences used was investigated by in silico analysis, and as a result, it was observed that organisms causing similar symptoms were not likely to give false positives in the Covid-19 test. Oligonucleotide sequences were not found in an organism except the Sars-Cov-2 genome.

Table 10 Results of In silico analysis

No	Organism	E gene	RdRp gene
1	Homo sapiens (taxid:9606)	No match.	No match.
2	Influenza A (taxid:11320)	No match.	No match.
3	Influenza B (taxid:11520)	No match.	No match.
4	Middle east respiratory syndrome (taxid:1335626)	No match.	No match.
5	Human respiratory syncytial virus (taxid:11250)	No match.	No match.
6	Human rhinovirus A (taxid:147711)	No match.	No match.
7	Human adenovirus B (taxid:108098)	No match.	No match.
8	Human bocavirus (taxid:573977)	No match.	No match.
9	Epstein barr virus (taxid:10376)	No match.	No match.
10	Mycoplasma (taxid:2093)	No match.	No match.
11	Mycobacteria (taxid:85007)	No match.	No match.
12	Streptococcus (taxid:1301)	No match.	No match.
13	Streptococcus pyogenes (taxid:1314)	No match.	No match.
14	Legionella (taxid:445)	No match.	No match.
15	Bordetella pertussis (taxid:520)	No match.	No match.
16	Pneumocystis jirovecii (taxid:42068)	No match.	No match.

10. References

[1] Harikrishnan, Pandurangan. "Saliva as a potential diagnostic specimen for COVID-19 testing." *The Journal of craniofacial surgery* (2020).

[2] Bustin, Stephen A., and Tania Nolan. "RT-qPCR testing of SARS-CoV-2: a primer." *International journal of molecular sciences* 21.8 (2020): 3004.

[3] Nagy, A., Vitásková, E., Černíková, L. et al. Evaluation of TaqMan qPCR System Integrating Two Identically Labelled Hydrolysis Probes in Single Assay. Sci Rep 7, 41392 (2017) https://doi.org/10.1038/srep41392

11. Explanation of Symbols

REF	**Reference/Catalogue number**		**Expiration date**
LOT	**Lot/Batch number**	-15 °C -25 °C	**Storage Conditions**
CE IVD	**European Conformity For In vitro Diagnostics**		**Do not expose to light!**
Σ	**Package Contents**		**Manufacturer**