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EliGene® COVID19 Omicron RT

CE

REF **90087-RT (for 100 samples)**
90087-RT-500 (for 500 samples)

Kit components:

90087-RT (for 100 samples):	90087-RT-500 (for 500 samples):
5 x 300 µl **COmic Mix**	5 x 1450 µl **COmic Mix**
2 x 55 µl **Enzyme Mix**	2 x 280 µl **Enzyme Mix**
2 x 260 µl **IC RNA**	2 x 1300 µl **IC RNA**
1 x 150 µl **PC CoVIR**	1 x 150 µl **PC CoVIR**
1 x 150 µl **PC COmic**	1 x 150 µl **PC COmic**
1 x **Instruction for Use**	1 x **Instruction for Use**

Storage and shelf life:

All components of the kit must be transported and stored at -20 °C. Kit and remaining MasterMixes must be stored at -20 °C in a dark.

Intended use

EliGene® COVID19 Omicron RT Kit is intended for qualitative RNA detection of seven types and subtypes of viruses. In a single reaction is detected SARS-CoV-2 virus, Influenza A virus (H1N1, H3N2, H5N1), Influenza B virus, Respiratory Syncytial Virus A and Respiratory Syncytial Virus B. Simultaneously, the EliGene® COVID19 Omicron RT allows to identify Omicron variant of SARS-CoV-2 virus, also denoted as B.1.1.529 by the detection of a specific mutation E484A.

Principle of the method

This diagnostic kit is based on reverse transcription of viral RNA of SARS-CoV-2, Influenza A/B and Respiratory Syncytial Virus A/B, and subsequent one-step qPCR analysis. An innovative mixture of 10 sets of primers and 4 TaqMan probes mixed in the ready-to-use COmic Mix is used. SARS-CoV-2 detection is carried out by amplifying two independent loci targeting RdRp gene and E gene (FAM channel). Influenza A and B are detected in the Cy5 channel and targets genes for M1 and NS1 proteins. Respiratory Syncytial Virus A and B are detected in the TexasRed channel and targets nucleoprotein gene fragments. The uniquely designed and highly specific internal control utilizes HEX labelled probe. Increased sensitivity and specificity of this kit is based on the amplification of multiple independent targets for each virus in a single qPCR reaction. Amplification in four separate channels can distinguish between **coronavirus SARS-CoV-2** (one channel), **influenza virus** (Influenza A virus - H1N1, H3N2, H5N1 and Influenza B virus in one channel), **RS virus** (Respiratory Syncytial Virus A and B in one channel) and **internal control** (separate channel). The identification of the Omicron variant of the SARS-CoV-2 virus is facilitated by the amplification of the targeting E484A site and the signal is visualized in TexasRed and Cy5 channels. The presence of the Omicron variant is therefore characterized by the presence of fluorescence signal in FAM, TexasRed and Cy5 channels with comparable Cq values.

Introduction

In late December 2019, an outbreak of an unknown disease called “pneumonia of unknown cause” occurred in Wuhan, Hubei Province, China. The causative virus has been named severe acute respiratory syndrome coronavirus 2 (**SARS-CoV-2**), and the relevant infection disease has been named coronavirus disease 2019 (COVID-19). Coronaviruses were discovered in the 1960s, and they were classified under the family *Coronaviridae* that is the largest family within the order *Nidovirales*. SARS-CoV-2 is a spherical positive single-stranded RNA virus that is characterized by spike proteins projecting from the virion surface. It is an enveloped virus (envelope is a lipid bilayer derived from the host cell membrane) with the viral structure formed primarily of structural

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proteins such as spike (S), membrane (M), envelope (E), nucleocapsid (N), and hemagglutinin-esterase (HE). For replication and transcription, a multi-protein replicase-transcriptase complex is used. This complex contains conserved RdRp (RNA-dependent RNA polymerase) as the main replicase-transcriptase protein for the synthesis of negative-sense subgenomic RNA strands from viral RNA and transcription of negative-sense subgenomic RNA molecules from corresponding positive-sense mRNAs. The RNA genome of coronaviruses is the second largest of all RNA viruses; SARS-CoV-2 has 29,9 kilobases in size.

Influenza is an infectious disease of birds and mammals caused by RNA viruses of the family *Orthomyxoviridae*, the influenza viruses. Influenza virus is an enveloped virus that contains a segmented negative-strand RNA genome. The eighth RNA segments of the influenza virus genome encode 11 different proteins, of which 8 are packaged into the infectious enveloped virion. On the viral surface are the two main antigenic determinants of the virus - the spike glycoproteins: hemagglutinin and neuraminidase. A third integral membrane protein M2 plays role in virus entry as well as in assembly and budding. Inside the viral envelope, the matrix protein (M1) provides structure to the virion and bridges interactions between the viral lipid membrane and the ribonucleoprotein (RNP) core. The RNP core is composed of the RNA polymerase complex proteins, PB1, PB2 and PA, and the nucleocapsid protein (NP) which mediates binding and packaging of the viral genome. During virus replication, three other proteins are expressed that are not incorporated into the mature virion. Non-structural protein 1 (NS1) is a multi-functional protein with a major role in the evasion of the host immune system. NS2 (NEP) plays a crucial role in mediating the export of viral RNPs from the cell nucleus during replication. Influenza A viruses are divided into subtypes based on hemagglutinin (H) and neuraminidase (N). There are 18 different hemagglutinin subtypes and 11 different neuraminidase subtypes (H1 through H18 and N1 through N11, respectively). Current subtypes of influenza A viruses that routinely circulate in people include: A (H1N1) and A (H3N2). Currently circulating influenza A (H1N1) viruses are related to the pandemic 2009 H1N1 virus that emerged in the spring of 2009 and caused a flu pandemic.

Respiratory syncytial virus (RSV) is an enveloped, non-segmented, and negative-strand RNA virus belonging Paramyxoviridae family. It is the common cause of lower respiratory tract infections with about 33 million cases and about 160 000 – 190 000 deaths annually. RSV genome contains more than 15 000 nucleotides coding for 11 known proteins. Attachment protein (G) helps attach the virus to host cells, and fusion protein (F) is responsible for viral fusion and syncytium formation. Small hydrophobic (SH) protein influences virus infection. The matrix (M) protein serves as the inner envelope protein. Four nucleocapsid-associated proteins include nucleoprotein (N), phosphoprotein (P), large (L) and M2-1 proteins. M2-2 regulatory protein is responsible for RNA synthesis during virion assembly. NS1 and NS2 proteins are non-structural proteins.

Omicron variant, also known as B.1.1.529 was first reported to the World Health Organization (WHO) from South Africa in November 2021. The variant has an unusually large number of mutations, and 32 of them affect the spike protein, the main antigenic target of antibodies generated by infections and of many vaccines widely administered (A67V, Δ69-70, T95I, G142D, Δ143-145, Δ211, L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F). Many of those mutations had not been observed in other strains. The variant is characterized by 30 amino acid changes, three small deletions, and one small insertion in the spike protein compared with the original virus, of which 15 are located in the receptor-binding domain (residues 319–541). This variant is extremely contagious and spreads in the population very quickly. But it is less able to penetrate deep lung tissue, and perhaps for this reason there is a considerable reduction in the risk of severe disease requiring hospitalization. However, the extremely high rate of spread, combined with its ability to evade both double vaccination and the body's immune system, means the total number of patients requiring hospital care at any given time is still of great concern.

Primary sample collection, handling and storage

Clinical material:	Recommended RNA isolation procedure:
nasopharyngeal swabs,	Manual: **EliGene Viral RNA/DNA FAST Isolation kit** (15 min protocol)

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swabs, saliva, sputum, urine	chemagic Viral DNA/RNA Kit (chemagen - PerkinElmer)
	QIAamp Virus Spin Kit or kits recommended by Qiagen
	Vacuum/centrifugation: **EliGene Viral RNA/DNA FAST 96 Vacuum Isolation Kit** (<40 min/96 samples protocol)
serum, plasma	chemagic Viral DNA/RNA Kit (chemagen - PerkinElmer)
	QIAamp Virus Spin Kit or kits recommended by Qiagen
Automatic isolation:	**ZEPHYRUS Magneto** (ELISABETH PHARMACON)
	actually recommended kits
	chemagic 360 Instrument (chemagen - PerkinElmer)
	chemagic Viral DNA/RNA Kit
	chemagic Viral NA/gDNA Kit
	QIAcube Instrument (Qiagen)
	actually recommended kits

EliGene® COVID19 Omicron RT (90087-RT and 90087-RT-500) is intended for the primary detection of SARS-CoV-2 virus with simultaneous genotyping of the E484A mutations characteristic for the Delta variant of SARS-CoV-2 (B.1.1.529). The kit is complementary with the kits EliGene® COVID19 BASIC A RT (90077-RT), EliGene® COVID19 BASIC A500 RT (90077-RT-500), EliGene® COVID19 CONFIRM RT (90078-RT), EliGene® COVID19 CONFIRM 500 RT (90078-RT-500), EliGene® COVID19 Triple RIC RT (90079-RT and 90079-RT-500), EliGene® COVID19 UKV RT (90082-RT and 90082-RT-500) and UKV/SAV RT (90083-RT and 90083-RT-500). Internal controls of all EliGene® kits for the detection of SARS-CoV-2 virus are identical, therefore, RNA isolated with internal control from BASIC, CONFIRM, Triple RIC, UKV and UKV/SAV kits can be analyzed by the EliGene® COVID19 Omicron RT and vice versa.

RNA is recommended to be eluted in water for molecular biology. Due to the composition of the elution buffers of some manufacturers, inhibition of PCR reaction by elution buffer compounds may occur. Elution buffer of EliGene Viral RNA/DNA FAST Isolation kit can be used with no fear of PCR inhibition, as well as elution buffers of isolation kits recommended above. If you intend to use isolation kits from other manufacturers, internal control of amplification (RNA) included in this kit must be added to RNA isolation to ensure that inhibition by elution buffer is excluded.

Serum or plasma:

According to standard protocol, take the sample of serum into a sterile tube. Transport samples at 4 °C to the laboratory. Serum samples are stable for a maximum of 4 days under these conditions. For longer storage, freeze the sample at -70 °C.

We recommend using volume 200 µl of serum and elution volume of 50 µl of PCR water. Before the isolation, 5 µl of Internal Control RNA (IC RNA) must be added to the sample after the addition of lysis buffer.

Swabs:

These specimens should be collected according to standard protocol in collection tubes.

Recommended swabs:

Flocked swabs (swabs made by the flocking technique) are inserted into the virus transport medium after swabbing.

Do not use cotton swabs due to possible inhibition of the PCR reaction. Do not use dry swabs for transport!

Samples should be transported to the laboratory at 4 °C (blue ice). Oropharyngeal and nasopharyngeal swabs are stable at 4 °C in virus transport medium for at least 3 days after sampling. For longer storage, freeze the sample at -70 °C.

Another option is to use inactivating transport media. Each inactivation medium must be validated for the used RNA isolation method!

In the case of sampling in transport medium, 200 µl or quantity recommended by instruction manual of used isolation kit should be used for RNA isolation. 5µl of Internal Control RNA (IC RNA) must be added to the sample used for RNA isolation after the addition of lysis buffer.

Additional required equipment

- Automatic pipettes 1-1000 µl and sterile tips with filter DNA-, RNA- free, DNase-, RNase- free (we recommended plastic with CE certificate for diagnostic purposes).
- Sterile plastic (strips, plates, tubes) DNase-, RNase- free compatible with given qPCR system. Always use only original plastic or plastics recommended by the manufacturer of the respective qPCR system. **The utilization of non-original plastic can lead to difficulties with the fluorescence readout and determination of the threshold. We cannot guarantee a correct interpretation of the results when non-original or disapproved plastics are used.**
- Sterile stand DNA-, RNA- free, DNase-, RNase- free.
- Equipment for qPCR – the kit is designed for qPCR instruments LightCycler® 480 (Roche; **Color compensation provided by Elisabeth Pharmacon is required!!!, please order EliGene® 4-channel Color Compensation Kit, cat. No. 90080-CC**), QuantStudio 5 (ThermoFisher Scientific), Rotor-Gene Q (Qiagen), and CFX96 (Bio-Rad). The RT-qPCR for the detection of SARS-CoV-2, Influenza A/B, and RSV RNA utilizes TaqMan technology (FAM, HEX, Texas Red, and Cy5 probes) and can be performed on other instruments that can work with these channels.
- Lab safety gloves and respirators FFP3. Please work in appropriate biohazard boxes. Also, the centrifugation of samples must be performed in biohazard boxes. Keep in mind that also viral RNA can cause infection.
- As it is a serious pathogen, please follow actual WHO recommendations for BSL2+ or BSL3 laboratories.

Configuration of qPCR instrument

- For detection of target sequences of SARS-CoV-2, two probes labeled with FAM are used (exc. 494 nm – em. 518 nm)
- For detection of Internal control, the probe labeled with HEX is used (exc. 520 nm – em. 548 nm)
- For detection of Respiratory Syncytial Virus, the probe labeled with TexasRed is used (exc. 589 nm – em. 615 nm)
- For detection of Influenza A/B, the probe labeled with Cy5 is used (exc. 650 nm – em. 670 nm)
- For detection of E484A, the probe labeled with TexasRed is used (exc. 589 nm – em. 615 nm) and Cy5 is used (exc. 650 nm – em. 670 nm)

LightCycler® 480 (Roche):

Please, use white plates only intended for LightCycler® 480 II. The usage of natural plates can lead to decreased sensitivity of the kit. Do not reuse plates; the contamination of your laboratory could occur during the manipulation with plates.

Creation of the detection profile:

Open "Toolbox" in the "Main menu" (icon with a wrench), select "Detection formats". Select "New" detection format and assign it a name according to your choice. In the excitation and emission spectra matrix on the top right corner, click on boxes with the following combinations:

Excitation Filter	Emission Filter	Name	Melt Factor	Quant Factor	Max Integration Time
465	510	FAM	1	10	2
533	580	HEX	1	10	2
533	610	TexasRed	1	10	2
618	660	Cy5	1	10	2

In option Detection format, choose the format you have created

Set up the following temperature profile:

Step 1 - Analysis mode "None", 1 Cycle

55°C 15 min Ramp rate (4.4°C/s) Acquisition mode “None”

Step 2 - Analysis mode "None", 1 Cycle

95°C 2 min Ramp rate (4.4°C/s) Acquisition mode “None”

Step 2 - Analysis mode "Quantification", 45 Cycles

95°C 5 s Ramp rate (4.4°C/s) Acquisition mode “None”

55°C 15 s Ramp rate (2.2°C/s) Acquisition mode “Single”

67°C 15 s Ramp rate (4.4°C/s) Acquisition mode “None”

Step 3 - Analysis mode "None", 1 Cycle

40°C 20 s Ramp rate (2.2°C/s) Acquisition mode “None”

The complete temperature profile can be uploaded from Run Template "EliGene COVID19 Omicron RT_LC480.ixo". The Run Template can be imported to the software in the menu "Navigator" by clicking to icon "Import" from the CD included in the kit.

QuantStudio 5 (ThermoFisher Scientific):

Use the Experiment type," Presence/Absence", Chemistry "TaqMan Probes", and Run Mode "Standard". As a reporter, dyes use FAM (SARS-Cov2), VIC (IC RNA), ROX (RSV, E484A) and Cy5 (Inf A and B, E484A). **Do not use any passive reference dye!**

Set up the following temperature profile:

Holding stage

55°C 15 min Ramp rate (1.6°C/s)

Holding stage

95°C 2 min Ramp rate (1.6°C/s)

Cycling stage – 45 cycles

95°C 5 s Ramp rate (1.6°C/s)

55°C 15 s Ramp rate (1.6°C/s) Data collection ON

67°C 15 s Ramp rate (1.6°C/s)

Post-Read Stage

40°C 20 s Ramp rate (1.6°C/s)

Collect the emission signal at the second step at 55 °C

The complete temperature profile can be uploaded from Run Template "EliGene COVID19 Omicron RT_QS3.edt" or "EliGene COVID19 Omicron RT_QS5.edt". The Run Template can be copied from the CD included in the kit.

RotorGene Q (Qiagen):

In the "New Run" window, choose "Three Step" run

Choose the appropriate "Rotor Type" and click "Next".

Set up the following temperature profile:

Holding stage

55°C 15 min

Holding stage

95°C 2 min

Cycling stage – 45 cycles

95°C 5 s

55°C 15 s Acquiring in channels "Green", "Yellow", "Orange" and "Red"

67°C 15 s

Holding stage

40°C 20 s

For the Gain optimization in all channels, select the option "Automatic gain optimization before first acquisition". The complete temperature profile can be uploaded from Run Template "EliGene COVID19 Omicron RT_Q-GENE.ret". The Run Template can be copied from the CD included in the kit.

CFX96 Touch (Bio-Rad):

In Startup Wizard Create a new Experiment for CFX96 instrument and Create New Protocol.

Set up the following temperature profile:

Step 1	55°C	15 min
Step 2	95°C	2 min
Step 3	95°C	5 s
Step 4	55°C	15 s + Plate Read
Step 5	67°C	15 s
Step 6	*GOTO Step 3*	*44x*
Step 7	40°C	20 s

Enter the Sample Volume 20ul

Collect the emission signal at Step 4 at 55° C.

For filter settings, use the "Scan Mode" All Channels, and in Plate Manager, select for the samples only fluorophores FAM, HEX, TexasRed, and Cy5. Then assign the samples with positions and Targets as an Unknown sample (Samples) or Standard.

MIC PCR (Bio Molecular Systems Pty Ltd):

In Run Setup Create a new Run Profile for the MIC instrument. Enter the Sample Volume to 20 µl and

Temperature Control “Fast TAQ (v3)”.

Set up the following temperature profile:

Hold

55°C 15 min

Hold

95°C 2 min

Cycling – 45 cycles

95°C 5 s

55°C 15 s Acquiring in channels "Green", "Yellow", "Orange" and "Red"

67°C 15 s

Hold after cycling

40°C 20 s

Reagent preparation

- To avoid contamination, keep all tubes closed and follow the instructions.
- All reagents must be completely thawed before the usage, briefly mixed on vortex, and shortly spun.
- Add 5 µl of Internal Control RNA (IC RNA) to the sample with lysis buffer. Never add Internal Control RNA to isolated RNA before starting PCR!

WARNING: Contamination in laboratory space is possible. Use separate pipette for Master Mixes, separate pipette for positive controls, and separate pipette for samples! Follow all recommendations for laboratories providing RNA analyses.

Preparation of Master Mix

1. Take the COmic Mix tube and the Enzyme Mix tube, and then thaw at room temperature. Immediately after thawing, spin briefly in a centrifuge. Prepare the Master Mix by mixing 14 µl COmic Mix and 1 µl Enzyme Mix per reaction and spin briefly.

2. Detection: Add 15 µl of the Master Mix to the amplification tubes or plates and add 5 µl of the isolated RNA sample. Be careful when pipetting the sample to avoid cross-contamination of the samples. The prepared Master Mix should be used within 30 minutes and cannot be reused. Do not freeze the prepared Master Mix.

3. Positive Control: Pipette 15 µl of the Master Mix separately into the amplification tube or plate. Then add 5µl of PC CoVIR. Be careful when pipetting the positive control to avoid contamination of samples. **Use a different micropipette for pipetting, only positive controls.**

4. Positive Control Omicron: Pipette 15 µl of the Master Mix separately into the amplification tube or plate. Then add 5µl of PC COmic. Be careful when pipetting the positive control to avoid contamination of samples. **Use a different micropipette for pipetting, only positive controls.**

Insert the microtubes or plate into the qPCR instrument and run the program as described in Configuring the qPCR Instrument above.

Result reading

LightCycler® 480 (Roche):

In the "Sample Editor" menu, choose "Abs Quant" workflow.

In the menu, "Analysis" chooses "Abs Quant/2nd Derivative Max" option.

It is compulsory to perform the analysis of the data with active Color Compensation. Otherwise, data from HEX and the Texas Red channel could not be interpreted. Select a Color Compensation file for FAM, HEX, TexasRed, and Cy5.

The positive result for SARS-CoV-2: The positive result is characterized by the growth of the fluorescence signal in the FAM channel (465-510). In the case of negative results, the amplification will not occur.

The positive result for SARS-CoV-2 Omicron variant: The positive result is characterized by the growth of the fluorescence signal in the FAM (465-510), TexasRed (533-610) and Cy5 (618-660) channel. The cycle number value between the individual channels must not differ by more than two cycles.

The positive result for RSV: The positive result is characterized by the growth of fluorescence signal in the TexasRed channel (533-610). In the case of negative results, the amplification will not occur.

The positive result for Influenza A/B: The positive result is characterized by the growth of fluorescence signal in the Cy5 channel (618-660). In the case of negative results, the amplification will not occur.

The Internal Control must be amplified in each sample. The Internal Control amplification is characterized by a growth of signal in the HEX channel (533-580).

QuantStudio 5 (ThermoFisher Scientific):

In "Analyse Settings" choose "Automatic Treshold" and "Automatic Baseline" options and analyze results.

The positive result for SARS-CoV-2: The positive result is characterized by the growth of the fluorescence signal in the FAM channel. In the case of negative results, the amplification will not occur.

The positive result for SARS-CoV-2 Omicron variant: The positive result is characterized by the growth of the fluorescence signal in the FAM, ROX and Cy5 channel. The cycle number value between the individual channels must not differ by more than two cycles.

The positive result for RSV: The positive result is characterized by the growth of the fluorescence signal in the TexasRed (ROX) channel. In the case of negative results, the amplification will not occur.

The positive result for Influenza A/B: The positive result is characterized by the growth of fluorescence signal in the Cy5 channel. In the case of negative results, the amplification will not occur.

The Internal Control must be amplified in each sample. The Internal Control amplification is characterized by the growth of the fluorescence signal in the HEX (VIC) channel.

Rotor-Gene Q (Qiagen):

Click to "Analysis" icon in the menu and choose the Analysis option "Quantitation". In the "Quantitation Analysis" window, select "Dynamic Tube" and "Slope Correct" options. In the option "Outlier Removal" setup NTC Threshold value for 10 %.

The positive result for SARS-CoV-2: The positive result is characterized by the growth of the fluorescence signal in the FAM channel (Green). In the case of negative results, the amplification will not occur.

The positive result for SARS-CoV-2 Omicron variant: The positive result is characterized by the growth of the fluorescence signal in the FAM (Green), TexasRed (Orange) and Cy5 (Red) channel. The cycle number value between the individual channels must not differ by more than two cycles.

The positive result for RSV: The positive result is characterized by the growth of the fluorescence signal in the TexasRed channel (Orange). In the case of negative results, the amplification will not occur.

The positive result for Influenza A/B: The positive result is characterized by the growth of fluorescence signal in the Cy5 channel (Red). In the case of negative results, the amplification will not occur.

The Internal Control must be amplified in each sample. The Internal Control amplification is characterized by the growth of the fluorescence signal in the HEX channel (Yellow).

CFX96 Touch (Bio-Rad):

In the Data Analysis window, choose "Quantification". In "Settings" menu, choose "Baseline Setting" option, and select "Baseline Subtracted Curve Fit" and "Apply Fluorescence Drift Correction".

In the Data Analysis window, select a single fluorophore (FAM, HEX, TexasRed, Cy5) by clicking the box next to the fluorophore name located under the amplification chart and read the results for individual samples.

The positive result for SARS-CoV-2: In "Settings" select "Baseline Threshold" and set the "Single Threshold" baseline to “Auto Calculated”. The positive result is characterized by the growth of the fluorescence signal in the FAM channel. In the case of negative results, the amplification will not occur.

The positive result for SARS-CoV-2 Omicron variant: The positive result is characterized by the growth of the fluorescence signal in the FAM, TexasRed and Cy5 channel. The cycle number value between the individual channels must not differ by more than two cycles.

The positive result for RSV: In "Settings" select "Baseline Threshold" and set the "Single Threshold" baseline to “Auto Calculated”. The positive result is characterized by the growth of the fluorescence signal in the TexasRed channel. In the case of negative results, the amplification will not occur.

The positive result for Influenza A/B: In "Settings" select "Baseline Threshold" and set the "Single Threshold" baseline to “Auto Calculated”. The positive result is characterized by the growth of the fluorescence signal in the Cy5 channel. In the case of negative results, the amplification will not occur.

The Internal Control must be amplified in each sample. In "Settings" select "Baseline Threshold" and set the "Single Threshold" baseline to "Auto Calculated". The Internal Control amplification is characterized by the growth of the fluorescence signal in the HEX channel.

MIC Cycler (Bio Molecular Systems):

To view and analyze acquired data, click the small cross next to Cycling under Analysis module and then select the target – Non-Assay Green (FAM), Non-Assay Yellow (HEX), Non-Assay Orange (TexasRed) and Non-Assay Redn (Cy5). In the down right corner **increase** “**Fluorescence Cut-off Level**” **to 20 %**. A quantification cycle (Cq) value is displayed for each positive well. This is the cycle number at which the sample fluorescence rises above the threshold (the red horizontal line near the bottom of the amplification curve chart). In the Samples panel on the right, click sample names to hide or display the associated amplification curves and result data.

The positive result for SARS-CoV-2: The positive result is characterized by the growth of the fluorescence signal in the FAM channel (Green). In the case of negative results, the amplification will not occur.

The positive result for SARS-CoV-2 Omicron variant: The positive result is characterized by the growth of the fluorescence signal in the FAM (Green), TexasRed (Orange) and Cy5 (Cy5) channel. The cycle number value between the individual channels must not differ by more than two cycles.

The positive result for RSV: The positive result is characterized by the growth of the fluorescence signal in the TexasRed channel (Orange). In the case of negative results, the amplification will not occur.

The positive result for Influenza A/B: The positive result is characterized by the growth of fluorescence signal in the Cy5 channel (Red). In the case of negative results, the amplification will not occur.

The Internal Control must be amplified in each sample. The Internal Control amplification is characterized by the growth of the fluorescence signal in the HEX channel (Yellow).

Interpretation of results

Negative result:

If the increase of amplification signal in FAM, TexasRed, and Cy5 channels does not appear before cycle number

40 with appropriate threshold settings applied, the result of the test should be interpreted as probably negative or with a concentration of RNA below the detection limit of this kit (5 genomic RNA/reaction). The signal for Internal Control must be positive – see article Quality control.

This result does not exclude the occurrence of SARS-CoV-2, Influenza A/B and RSV in a sample because the results of this test are dependent on proper sample collection and processing. Results are also dependent on an adequate quantity of analyzed RNA. **It has been reported that the viruses can be secreted intermittently, and even in an infected patient, the virus level in clinical specimens may be below the detection limit of any RT-qPCR method each day. For this reason, it is recommended to perform at least two, ideally more RT-qPCR examinations in a single patient over several days.**

Positive result:

If the amplification signal in FAM, TexasRed, and Cy5 channel appears before cycle number 40 at the appropriate threshold baseline, RNA of the respective virus is detected in the sample. When the Omicron variant of the SARS-CoV-2 virus is present, the amplification signal in FAM, TexasRed (ROX) and Cy5 channels must be present. The detailed interpretation criteria are following:

CHANNEL			INTERPRETATION
FAM	**TexasRed**	**Cy5**	
+	-	-	SARS-Cov-2 detected
+	+	+	SARS-CoV-2 Omicron variant detected*
-	+	-	RSV detected
-	-	+	Influenza A/B detected
+	+	-	SARS-CoV-2 and RSV detected
+	-	+	SARS-CoV-2 and Influenza A/B detected
-	+	+	RSV and Influenza A/B detected

* The cycle number value between the individual channels must not differ by more than two cycles!

The fluorescence signal in the FAM channel is stronger (has higher absolute fluorescence) than in the remaining channels! It must be noted that the amplification signal for E484A mutation in both Texas Red and Cy5 channels can be indistinguishable from the background in weakly positive samples (Cycle number higher than 30) even if the mutation E484A is present. The cycle number values in both FAM, Texas Red and Cy5 channels must lie within 2 cycle difference if E484A mutation is present!

IMPORTANT!

Due to the rapid evolution of the SARS-CoV-2 virus, new mutations can occur in time and the definition of the variants can change. We advise users to check the newest information about the SARS-CoV-2 classification to prevent possible misclassification of the SARS-CoV-2 virus. It is strongly recommended to perform sequencing of samples in a particular time and area (whole genome or just part of the SARS-CoV-2 virus genome) to identify the SARS-CoV-2 virus variant circulating in the area precisely!

This SARS-CoV-2 virus detection and the genotyping kit is intended for the routine screening of large sets of samples and does not serve for the full genotyping of the SARS-CoV-2 virus variants. The usage of this kit does not replace sequencing.

Inhibited sample:

In the case that increase of the amplification signal is observed in none of the channels, including the HEX channel for the IAC, the analysis should be repeated, preferably with newly isolated RNA samples. Make sure the elution buffer does not inhibit the qPCR reaction. In this case, it is recommended to perform elution into the water for molecular biology.

Control procedure

EliGene® COVID19 Omicron RT kit involves Internal Control. Internal Control follows the quality of RNA isolation and detects the inhibition of reverse transcription and amplification. The internal control must be added directly to the sample with lysis buffer before the viral RNA isolation. In the case that no amplification occurs in FAM, TexasRed, and Cy5 channels, there must be present an amplification in the HEX channel (internal amplification control) with a cycle number value lower than 32.

Additionally, **cycle number values of all the samples undergoing the same procedure of RNA isolation must have a cycle number value of IAC in the HEX channel within the range of 3 cycles.** The higher fluctuation of the cycle number values in a single qPCR run indicates non-standard conditions in the RNA isolation. However, this condition does not apply to highly positive samples (cycle number in FAM channel < 20).

Reference material:

To monitor the whole examination process covering RNA isolation and qPCR detection is possible to use reference viral material positive for SARS-CoV-2, Influenza A/B, and RSV. The positive commercial material is not available. Do not use artificial RNA or DNA or positive controls from other manufacturers.

Troubleshooting:

1. If there is no amplification of Internal Control, there is some problem in the isolation of RNA or the kit is after the expiration date or there is qPCR instrument malfunction.
2. If the cycle number range of the Internal Amplification Control is higher than 3 cycles, there is a non-homogeneity in the RNA isolation caused probably by the partial inhibition of the qPCR. Repetition of the RNA isolation should be considered in this case. Another possibility is to dilute the isolated RNA twice, alternatively more times.
3. If there is no amplification of Positive Control, the kit is after the expiration date or there is qPCR instrument malfunction. It may also be a failure to follow the recommended procedure for sample preparation and analysis.

Performance characteristics

Analytical performance characteristics:

The analytical sensitivity of the EliGene® COVID19 Omicron RT Kit, defined as the lowest number of genomic RNA copies present in an amplification reaction, that can be successfully amplified in 3 independent replicates, is 5 SARS-CoV-2 and Influenza A/B genomic RNAs and 50 RSV genomic RNAs added to the Master Mix. The sensitivity of the RT-qPCR procedure depends on the RNA isolation method. The sensitivity of the method was verified as follows. Positive control dilution series of known concentration were prepared. They were tested three times. SARS-CoV-2 detection was 100% successful in all samples containing 5 or 50 or more RNAs in the Master Mix.

The analytical sensitivity is 5 copies of SARS-CoV-2 and Influenza A/B and 50 copies of RSV viral RNA in the reaction mix.

The analytical specificity of the method is 100%. All primers and probes were taken from the literature and/or

approved by authorities such as WHO, CDC or ECDC. In addition, the analytical specificity of the method was analysed by comparing primer and probe sequences to all known RNA and DNA sequences in the GenBank database and no cross-reaction was found. No cross-reaction with the human genome was found.

Clinical specificity and sensitivity were tested on a total of 1531 clinical samples, of which 600 SARS-CoV-2 samples (of which 500x Omicron variant), 613 positive for RSV virus and 218 positive for influenza virus and 100 negative samples. A combination of oropharyngeal and nasopharyngeal swabs were used as reference material samples. Samples were independently tested by the reference method with the EliGene® COVID19 Triple RIC RT kit. The presence of the specific E484A mutation in the spike protein was analyzed by NGS sequencing of all positive samples that had sufficient RNA concentration.

Sensitivity and specificity of SARS-CoV-2 detection

A = 600 True positive	B = 0 False positive
C = 0 False negative	D = 100 True negative

Sensitivity = A/(A+C) = 600/600+0) = 100%
Specificity = D/(D+B) = 100/(100+0) = 100%

Sensitivity and specificity of Influenza A/B detection

A = 218 True positive	B = 0 False positive
C = 0 False negative	D = 100 True negative

Sensitivity = A/(A+C) = 218/(218+0) = 100%
Specificity = D/(D+B) = 100/(100+0) = 100%

Sensitivity and specificity of RSV detection

A = 613 True positive	B = 0 False positive
C = 0 False negative	D = 100 True negative

Sensitivity = A/(A+C) = 613/(613+0) = 100%
Specificity = D/(D+B) = 100/(100+0) = 100%

The clinical specificity and sensitivity of the EliGene® COVID19 Omicron RT kit is 100% for all three targets. Compared to samples sequenced by NGS, the kit showed 100% specificity for the E484A mutation at a minimum Ct of 30.

Diagnostic performance characteristics:

Measuring interval

The kit enables the detection of 5×10^{1} - 5×10^{8} of viral RNA molecules in Reaction Mix.

Internal control of quality

As an internal control of quality, the Internal Control (IC RNA) for checking the process of RNA isolation, reverse transcription, and DNA amplification is used. Positive control for functional control of Master Mix and as a reference sample is used.

Limitation of the examination procedure

The sensitivity of the kit depends on handling the specimen (isolation of RNA). It is strictly recommended to use isolation kits and procedures recommended in this manual.
A negative result does not exclude the occurrence of viral infection. The results of this test are dependent on proper sample collection and elaboration. Results are also dependent on enough quantity of analyzed RNA. The presence of any of the viruses detected RNA in clinical samples of infected persons is dependent on the infection phase and could be intermittent. The attending physician must give a conclusion on the diagnosis and treatment of patients.

Biological reference intervals

Not applicable information for this kit.

Warning

After the preparation, the Master Mix is stable for 30 minutes. Do not freeze tubes with Master Mix repeatedly! Do not mix components of the kits of different lots!

Warnings and general precautions

This kit is intended for *in vitro* use only.

- Lab safety gloves and respirators FFP3 are necessary for work. Please work in appropriate biohazard boxes. Also, centrifugation of samples must be performed in biohazard boxes. Keep in mind that also viral RNA can cause infection.
- **As SARS-CoV-2 is a serious pathogen, please follow actual WHO recommendations for BSL2+ or BSL3 laboratories!**
- Handle and dispose of all biological samples as if they could transmit infective agents. Avoid direct contact with the biological samples. Avoid splashing or spraying. The materials that come into contact with biological samples must be treated with 3% sodium hypochlorite for at least 30 minutes or autoclaved at 121 °C for one hour before disposal.
- Handle and dispose of all reagents and all assay materials as if they could transmit infective agents. Avoid direct contact with the reagents. Avoid splashing or spraying. Waste must be treated and disposed of in compliance with the appropriate safety standards. Disposable combustible materials must be incinerated. Liquid waste containing acids or bases must be neutralized before disposal.
- Wear suitable protective clothing and gloves and protect your eyes/face.
- Never pipette solutions by mouth.
- Do not eat, drink, smoke or apply cosmetic products in the work areas.
- Wash hands carefully after handling samples and reagents.
- Dispose of leftover reagents and waste in compliance with regulations in force.
- Read all the instructions provided with the kit before running the assay.
- Follow the instructions provided with the kit while running the assay.
- Do not use the kit after the expiry date.
- Only use the reagents provided in the kit and those recommended by the manufacturer.
- Do not mix reagents from different batches.

- Do not use reagents from other manufacturer's kits.
- Do not change recommended protocol for PCR analysis!

Warnings and precautions for molecular biology

- Molecular biology procedures, such as extraction, reverse transcription, amplification and detection of nucleic acids, require qualified staff to prevent the risk of erroneous results, especially due to degradation of the nucleic acids contained in the samples or due to sampling contamination by amplification products.
- It is necessary to have separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never introduce an amplification product in the area designed for extraction/preparation of amplification reactions.
- It is necessary to have lab coats, gloves and tools which are exclusively employed in the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never transfer lab coats, gloves or tools from the area designed for the amplification/detection of amplification products to the area designed for the extraction/preparation of the amplification reactions.
- The samples must be exclusively employed for this type of analysis. Samples must be handled under a laminar flow hood. Tubes containing different samples must never be opened at the same time. Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.
- Reagents must be handled in a PCR box. The reagents required for amplification must be prepared in such a way that they can be used in a single session. The pipettes employed to handle the reagents must be used exclusively for this purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.
- Amplification products must be handled in such a way as to reduce dispersion into the environment as much as possible, to avoid the possibility of contamination. Pipettes used to handle amplification products must be employed exclusively for this specific purpose.

Warnings and precautions specific to components of the kit

The tubes containing COmic Mix and Enzyme Mix are disposable and therefore must be used once only in the preparation of the reaction mixture.

These Mixes carry the following safety warnings (P):

P280 Wear protective gloves/protective clothing/eye protection/face protection.
P281 Use personal protective equipment as required.

The tubes containing IC RNA are disposable and therefore must be used once only in the preparation of the reaction mixture.

In case of any problems, please contact ELISABETH PHARMACON, Ltd.

Literature

He F, Deng Y, Li W. Coronavirus Disease 2019 (COVID-19): What we know? J Med Virol. 2020 Mar 14. doi: 10.1002/jmv.25766.

Khan S, Siddique R, Shereen MA, Ali A, Liu J, Bai Q, Bashir N, Xue M. The emergence of a novel coronavirus (SARS-CoV-2), their biology and therapeutic options. J Clin Microbiol. 2020 Mar 11. pii: JCM.00187-20. doi: 10.1128/JCM.00187-20.

Ashour HM, Elkhatib WF, Rahman MM, Elshabrawy HA. Insights into the Recent 2019 Novel Coronavirus (SARS-CoV-2) in Light of Past Human Coronavirus Outbreaks. Pathogens. 2020 Mar 4;9(3). pii: E186. doi: 10.3390/pathogens9030186.

Symbols

	Catalogue number
	The upper limit of temperature
	Batch code
	Use by (last day of the month)
	*in vitro* diagnostic medical device
	Fulfilling the requirements of European Directive 98\79\EC for *in vitro* diagnostic medical device.
	Contains sufficient for "N" tests
	Attention, consult instructions for use
	Manufacturer

Manufacturer

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