FLU-COVID RT-PCR

Kit for the detection of Influenza A (FluA), Influenza B (FluB) and SARS-CoV-2 viruses by One-Step Real-Time RT-PCR

REF **Ref. MAD-003942M-L** **100 determinations**

For in vitro diagnostic use only
Directive 98/79/EC

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TABLE OF CONTENTS

1 INTENDED USE 3
2 PRINCIPLE OF THE METHOD 3
3 COMPONENTS 4
4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED 5
4.1 Reagents and materials 5
4.2 Equipment 5
5 STORAGE AND STABILITY CONDITIONS 5
6 WARNINGS AND PRECAUTIONS 6
7 PREPARATION OF THE CLINICAL SAMPLE FOR ANALYSIS 7
7.1 Sample taking 7
7.2 Extraction of nucleic acids from bronchoalveolar lavages and nasopharyngeal and oropharyngeal exudates 8
8 PCR PROTOCOL 8
8.1 Preparation of the Reaction mix 8
8.2 Configuration of the instrument for real-time PCR 9
9 INTERPRETATION OF RESULTS 10
10 PERFORMANCE CHARACTERISTICS 12
10.1 Analytical sensitivity 12
10.2 Analytical specificity 13
10.3 Clinical sensitivity and specificity 14
11 LIMITATIONS OF THE TEST 14
12 BIBLIOGRAPHY 15
13 LABEL AND BOX SYMBOLS 16
14 CHANGELOG 16

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1 INTENDED USE

The **FLU-COVID RT-PCR** kit is an in vitro diagnostics kit for the qualitative simultaneous detection and differentiation of the RNA of the Influenza A[1] (FluA), Influenza B[1] (FluB) and/or SARS-CoV-2 viruses, starting from purified RNA from human clinical samples of different origin, such as nasopharyngeal and oropharyngeal exudates and bronchoalveolar lavages (BAL). It is based on the multiplex One-Step RT-PCR technique, using primers and fluorescent probes for the target genes: Matrix protein FluA (Mp), Matrix protein FluB and SARS-CoV-2 specific N gene (independent regions N1 and N2) following the guidelines recommended by the WHO (11).

[1]Types of influenza virus identified:

- Influenza A
  - Universal
  - Subtype H1N1 (pandemic 2009)
  - Subtype H3
- Influenza B: Victoria and Yamagata lineages.

Specific primers and fluorescent probe are included for the simultaneous detection of the human RNaseP gene as internal quality control of the starting material and amplification. The detection channels of the different targets are:

Target	Fluorophore
SARS-CoV-2	FAM
Influenza A	ROX
Influenza B	JOE
RNasaP	Cy5

Table 1. Detection channels for the different targets of the FLU-COVID RT-PCR kit.

Microbiological status: Non-sterile product.

2 PRINCIPLE OF THE METHOD

The **FLU-COVID RT-PCR** Kit is a multiplexed assay based on reverse transcription and Real Time polymerase chain reaction. The Master mix contains three sets of primers and probes designed to detect the RNA from Influenza A virus, Influenza B and SARS-CoV-2 viruses. It also includes primers and probes to detect the human RNaseP gene in clinical samples or control samples. The oligonucleotides used as primers and probes were selected in evolutionarily conserved regions. With this test it is possible to differentiate between Influenza A virus, Influenza B virus and SARS-CoV-2 virus in a single assay.

In a first reverse transcription step, the RNA regions complementary to the primers are converted to cDNA, which is subsequently amplified by the polymerase chain reaction (PCR). Detection of the different amplicons produced is based on TaqMan probe technology.
If the target nucleic acids are present, they are amplified and during the PCR process the probes will specifically

anneal in the complementary regions between the primers forward and reverse. During the PCR extension phase, the 5' nuclease activity of DNA polymerase degrades the probes specifically bound to their targets, resulting in the separation between the reporter and the quencher and a fluorescent signal will be generated.

The specific probes of each virus will generate a fluorescent signal at different wavelengths, so that the real-time PCR equipment can specifically differentiate that signals. Along the denaturation-extension cycles, the breakdown of new reporter molecules is added with the consequent increase in the intensity of the fluorescent signal. At each one the cycles fluorescence intensity is monitored in the Real time PCR equipment, and the data are analyzed with specific analysis software.

The detection of viral RNA, in addition to be helpful in the diagnosis of the disease, provides valuable information for epidemiological and surveillance studies.

3 COMPONENTS

The **FLU-COVID RT-PCR** kit is commercialized as a lyophilized Master Mix which includes all the necessary reagents to perform the real-time RT-PCR.

Furthermore, in order to avoid contamination with previous PCR products, the Mix contains the enzyme Uracil-DNA Glycosylase (Cod-UNG), which degrades PCR products containing dUTP.

A positive control (PC) and a reconstitution solution to reconstitute the lyophilized Master Mix and to include it in the negative controls (NTC) are supplied along with the RT-PCR Mix.

Components of the kit for 100 tests:

REFERENCE (DESCRIPTION)		CONTENT	AMOUNT
MAD-003942M-100-L (FLU-COVID MMIX)	**MAD-003942-MIX-L* (FLU-COVID MMIX)**	Reverse transcriptase, Hot Start DNA Polymerase, Uracil DNA glycosylase, primers, fluorescent probes, reaction buffer, dNTPs (dATP, dCTP, dGTP, dTTP, dUTP)	**2 vials with 50 test/vial Lyophilized**
	MAD-LYO-SOL (Reconstitution solution)	---	**1 vial (1700 µl)**
MAD-FLU-COVID (FLU-COVID PC)		Synthetic non-infectious DNA/RNA containing part of the genome of FluA, FluB, SARS-CoV-2 and human DNA	**1 vial (100 µl)**

Table 2. Reagents supplied in the FLU-COVID RT-PCR kit.

*__Before the first use__, open the glass vial containing the lyophilized Master Mix, shake the **Reconstitution Solution (MAD-LYO-SOL)** in vortex and hydrate the vial by adding **660 µl** of the Reconstitution Solution per vial.

The reconstituted mix is stable if stored at -20 °C in the same glass vial, by closing it with the stopper and the screw cap, for a maximum of 4 months. It is recommended do not freeze-thaw the vial once reconstituted more than five times.

4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1 Reagents and materials

- Disposable gloves.
- DNase/RNase-free filtering pipette tips.
- RNA extraction kit.
- Tube strips/plates/optical adhesive films specific for each equipment of Real-Time PCR

4.2 Equipment

- Laminar flow cabinet
- Microcentrifuge for tubes of 1.5ml.
- Microcentrifuges of PCR tube strips or 96-well plates.
- Vortex.
- Automatic micropipettes: P1000, P200, P20 and P2.
- Real Time-PCR instrument.

5 STORAGE AND STABILITY CONDITIONS

The **FLU-COVID RT-PCR** kit must be transported at a temperature between 2 and 8 °C*, and long-term storage must be as indicated on the label for each of its components: FLU-COVID MMIX (mastermix and reconstituent) at 2 and 8 °C and the FLU-COVID PC (positive control) at -10°C to -30°C.

Once reconstituted, the reaction mix **FLU-COVID MMix** is stable for 4 months frozen stored at -10 °C to -30 °C and supports up to five freezing/thawing cycles. If a run is performed with a low number of samples, it is recommended to aliquot the reagent in advance. The mix contains fluorescent molecules and it must be kept away from direct light.

The positive control is sensitive to physical state changes and it must not undergo more than eight freeze-thaw cycles. It is advisable to handle the positive control vial separately from the clinical samples to avoid potential contamination which might yield false positives.

If stored at recommended temperature, the PCR reagents are stable until the expiration date indicated. The PCR reagents must be stored in areas free of contamination by DNA or PCR products.

*A temperature indicator is included in the package to control the conditions during the shipment. In case the cold chain is interrupted, it is recommended to contact the manufacturer before using the reagents.

6 WARNINGS AND PRECAUTIONS

- Read the instructions for use before using this product.
- The kit must be handled by qualified technicians in molecular biology techniques applied to diagnosis.
- Do not use any component of the kit after the expiration date.
- **The mix FLU-COVID MMix must be reconstituted before first use.** Once reconstituted, it must be handled in ice or cold plate and away from light. Mix the solutions by inverting the tubes several times without shaking in vortex, and centrifuge briefly.
- The positive control must be thawed at room temperature, mixed well and centrifuged briefly before use.
- The safety and disposal precautions are described in the Safety Data Sheet of this product. This product is only intended for professional laboratory purposes, and it is not intended for pharmacological, home or any other type of use. The current version of the Safety Data Sheet of this product can be downloaded in the web page www.vitro.bio or requested at regulatory@vitro.bio.
- The SARS-CoV-2 RT-PCR kit uses nucleic acids previously extracted and purified as starting material. It is the client's responsibility to include the necessary controls to verify that the system of extraction of the used genetic material works properly.
- General considerations to avoid RNA degradation with ribonucleases (RNases)

  RNases are very stable enzymes, hard to inactivate, that get to degrade RNA quickly. The introduction of RNases in the test sample and the reagents used for the RT-PCR must be avoided by taking the following precautions:

  - Work in a clean RNase-free area. The main RNase contamination source comes from skin and dust particles, which are bacterial and fungal carriers.
  - Always use disposable gloves to prevent RNase contamination from the skin.
  - Change the gloves frequently and keep the tubes closed.
  - Use RNase-free tubes and pipette tips.
  - Work quickly to avoid RNA degradation by residual and endogenous RNases during the whole preparation process of the sample to be amplified.

- **General considerations to avoid the contamination with PCR product**

  The most important contamination source is usually the same amplified PCR product. Therefore, it is recommended to carry out the amplification and handling of the amplified products in a different area to the one where the RNA extraction and PCR preparation are performed. It is recommended to work in different pre- and post-PCR areas where the handling of the test RNA and preparation of the PCR tubes (pre-PCR), and the amplification and handling of the amplified products (post-PCR) are performed. These areas must be physically separated, and different laboratory material must be used (laboratory coats, pipettes, tips, etc.) to avoid the contamination of the samples with the amplified DNA, which could lead to false positive diagnoses. The workflow must always go in a single direction, from the pre-PCR area to the post-PCR area and never the opposite way. The material and personal flow from the post-PCR area to the pre-PCR area must be avoided. Furthermore, in order to avoid the contamination with previous PCR products, the enzyme *Uracil-DNA Glycosylase (Cod-UNG)*, which degrades the PCR products containing

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dUTP, is included in the kit.

It is recommended to include negative amplification controls replacing the RNA sample with RNase/DNase-free water, in order to detect and control any possible contamination of the reagents with test samples or amplified products.

- **Waste disposal:**
  The handling of wastes generated by the use of the products commercialized by Vitro S.A. must be performed according to the applicable law in the country in which these products are being used. As reference, the following table indicates the classification of wastes generated by this kit according to the European Law, specifically according to the European Commission Decision of December 18, 2014 amending decision 2000/532/EC on the list of waste pursuant to Directive 2008/98/EC of the European Parliament and of the Council:

POTENTIAL WASTES GENERATED AFTER USING THIS PRODUCT	ELW CODE*	TYPE OF WASTE ACCORDING TO ELW*
1. Liquid waste disposal	161001	"Aqueous liquid wastes containing dangerous substances" after adding 10% of the total volume of a disinfectant agent. If the disinfection is not carried out, these wastes must be considered as "wastes whose storage and disposal is subjected to special requirements in order to prevent infection"
2. Perishable material (tubes, tips, etc.) 3. Any element that has been in contact with the starting genetic material	180103	"Wastes whose collection and disposal is subject to special requirements in order to prevent infection"
4. Container for reagents used classified as dangerous (according to the Safety Data Sheet)	150110	"Containers having residues of or contaminated by dangerous substances"

Table 3. Classification of wastes generated by this kit according to the European Legislation. *ELW: *European Legislation of Waste.*

*Note: This classification is included as a general guideline of action, being under the final responsibility of the user the accomplishment of all the local, regional and national regulations on the disposal of this type of materials.

7 PREPARATION OF THE CLINICAL SAMPLE FOR ANALYSIS

7.1 Sample taking

The **FLU-COVID RT-PCR** kit has been validated for its use starting from purified genetic material from bronchoalveolar lavage and nasopharyngeal and oropharyngeal exudates.

The samples from bronchoalveolar lavage are taken from hospital patients with a bronchoscope through the installation and subsequent aspiration of liquid from one or two pulmonary segments or sub-segments.

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In the case of nasopharyngeal and oropharyngeal exudates, these samples are taken with swabs. The swab is introduced carefully into the posterior part of the nasal cavity or the pharynx. The tip of the swab must be of polyester, rayon or nylon, with a soft and flexible handle of plastic (swabs with tip of calcium alginate or cotton must not be used). Once inserted, the swab is held in the same place for about 10 seconds and, after that, it is placed in a dry sterile tube, or preferably in a tube with transport medium (for example, Universal Transport Medium UTM) to preserve the integrity of the sample.

The samples are collected in a sterile recipient and kept at 2-8 °C for a maximum of 5 days. Once the samples are classified or for longer storage periods, they are stored at -80 °C in order to preserve the viral viability. The nucleic acids extracted must be stored at -80 °C.

7.2 Extraction of nucleic acids from bronchoalveolar lavages and nasopharyngeal and oropharyngeal exudates

The **FLU-COVID RT-PCR** kit has been tested with purified genetic material from human bronchoalveolar lavages and nasopharyngeal and oropharyngeal exudates. This kit has been validated with starting genetic material from the following DNA/RNA purification kits and extraction equipment* from 200 µl of clinical sample and eluting in 100 µl of elution buffer:

EXTRACTION KITS	EXTRACTION EQUIPMENT
MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics)	MagNA Pure Compact Instrument. Version 1.1.2 (Roche Diagnostics)
QIAsymphony Certal Kits (Qiagen)	QIAsymphony SP (Qiagen)
RNeasy Mini QIAcube Kit (Qiagen)	QIAcube (Qiagen)
PureLink Viral RNA/DNA extraction mini kit (Invitrogen)	Manual system
Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promega)	Maxwell® 16 (Promega)

Table 4. Extraction kits and instruments used for the purification of DNA/RNA from clinical samples.

*Note: The system has not been validated with other DNA/RNA extraction systems. Therefore, if any other purification system is used, this must be verified beforehand.

8 PCR PROTOCOL

8.1 Preparation of the Reaction mix

Before the first use, open the glass vial containing the lyophilized Master Mix, shake the **Reconstitution Solution (MAD-LYO-SOL)** in vortex and reconstitute the vial by adding **660 µl** of that Reconstitution Solution

per vial. Homogenize through 8 – 10 suctions with micropipette. The liquid reconstituted mix must be used as follows:

1. Mix in each PCR tube the following volumes for each sample:

Reagent	V/test
FLU-COVID MMix	12 µl
Sample	8 µl

2. Include a negative control by adding 8 µl of the Reconstitution Solution included in the kit.
3. Include a positive control by adding 8 µl of the positive control FLU-COVID PC included in the kit.
4. Centrifuge briefly to make sure there are no air bubbles in the wells.

Excess of reconstituted mix can be stored at -20 °C in the same glass vial, by closing with the stopper and the screw cap for a maximum of 4 months, to be used in subsequent assays.

It is recommended to keep the MMix on cold plate during the preparation of the samples and do not freeze-thaw the vial once reconstituted more than five times.

For the next uses, the RT-PCR reaction will be prepared as follows:

1. Thaw FLU-COVID MMix and homogenize through several suctions-dispensations with micropipette (do not use vortex).
2. Mix in each PCR tube the following volumes for each sample:

Reagent	V/test
FLU-COVID MMix	12 µl
Sample	8 µl

3. Include a negative control by adding 8 µl of the Reconstitution Solution included in the kit.
4. Include a positive control by adding 8 µl of the positive control FLU-COVID PC included in the kit.
5. Centrifuge briefly to make sure there are no air bubbles in the wells.

8.2 Configuration of the instrument for real-time PCR

Enter the different targets and detection channels for each of them in the instrument's software. Create the samples, the positive control (PC), the negative control (NTC) and allocate the positions of the samples in the PCR plate.

Set the real-time PCR instrument following the steps below:

PCR PROGRAM		
25 °C	5 min	1 cycle
50 °C	15 min	1 cycle
95 °C	5 min	1 cycle
95 °C	15 sec	45 cycles
56 °C*	40 sec	

Table 5. PCR program of the FLU-COVID RT-PCR kit.

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*The fluorescence data must be collected during the extension stage (*) by means of the FAM (SARS-CoV-2), ROX (FluA), HEX, JOE or VIC (FluB) and Cy5 (Internal Control) channels.

This kit has been validated with the platforms:

- QuantStudio™ 5 Real-Time PCR System (Applied Biosystems)
- 7500 Real-Time PCR System (Applied Biosystems)
- StepOne Plus™ Real-Time PCR System (Applied Biosystems)
- CFX96™ Real-Time PCR Detection System (Bio-Rad)
- Rotor-Gene Q (Qiagen)

For its use in other platforms, it is recommended to verify the compatibility of the fluorochromes with the detection channels of each instrument. Although the fluorochromes included in the kit are compatible with the majority of the most-used real-time instruments available on the market.

In the thermal cyclers Applied Biosystems 7500 Fast Real-Time PCR System, Applied Biosystems StepOne Plus™ Real-Time PCR System, Applied Biosystems QuantStudio™ 5 Real-Time PCR System and Stratagene Mx3005P™ Real Time PCR System, the option of passive control ROX must be disabled.

In the thermal cyclers Applied Biosystems QuantStudio™ 5 Real-Time PCR System and Applied Biosystems 7500 Fast Real-Time PCR System, select Ramp Speed Standard in the menu "Select New Experiment/Advanced Setup/Experiment Properties".

9 INTERPRETATION OF RESULTS

Before interpreting the results of the clinical samples, it is necessary to follow the interpretation guide of the positive and negative controls as in the table below:

	RESULT	INTERPRETATION
Positive Control FLU-COVID	Signal for the channels FAM, ROX, JOE and Cy5*	The control/reaction is correct
	No signal for FAM and/or ROX and/or JOE and/or Cy5	Problem in the amplification: repeat analysis
Negative control	Signal for the channels FAM and/or ROX and/or JOE and/or Cy5	Contamination, repeat analysis
	No signal	The control/reaction is correct

*The amplification signal must be determined by a rapid and steady increase in the fluorescence values and not by peak phenomena or gradual increase of the background signal (irregular background or increased background noise) (Fig 1).

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The run is considered valid when adequate results have been obtained for all the reaction controls.

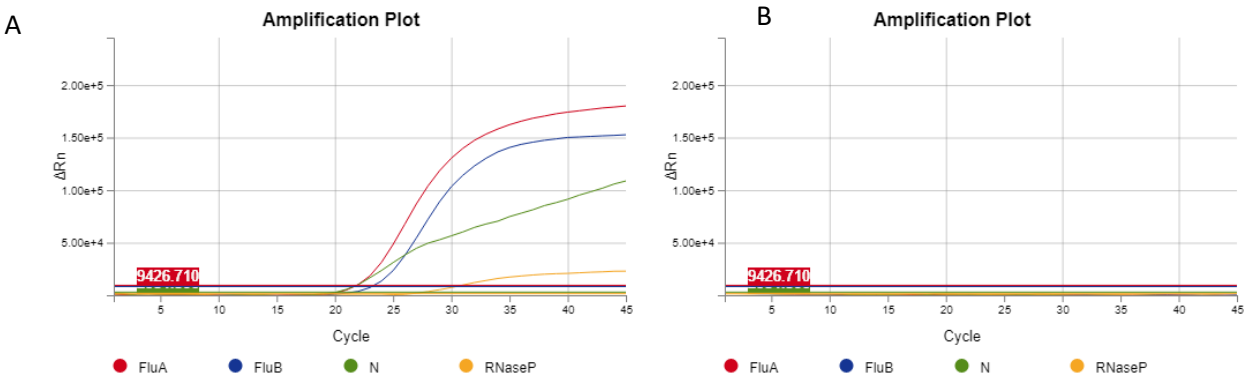

Figure 1: Graphs of amplification of the positive control PC (A) and of a negative control NTC (B). Experiment performed in Applied Biosystems QuantStudio™ 5 Real-Time PCR System.

If an amplification graph is observed in the negative control or there is a lack of amplification in the well of the positive control the result is considered invalid. In this case, it is recommended to repeat the test.

If the run has been validated, interpret the results of the clinical samples according to the following table:

FLU-COVID RT-PCR				INTERPRETATION
SARS-CoV-2 (FAM)	**FluA (ROX)**	**FluB (JOE)**	**RNasaP (Cy5)**	
Signal	No signal	No signal	Signal	Positive sample for SARS-CoV-2
			No signal	
No signal	Signal	No signal	Signal	Positive sample for influenza A
			No signal	
No signal	No signal	Signal	Signal	Positive sample for influenza B
			No signal	
No signal	Signal	Signal	Signal	Positive sample for influenza A and influenza B
			No signal	
Signal	Signal	No signal	Signal	Positive sample for SARS-CoV-2 and influenza A
			No signal	
Signal	No signal	Signal	Signal	Positive sample for SARS-CoV-2 and influenza B
			No signal	
Signal	Signal	Signal	Signal	Positive sample for SARS-CoV-2, influenza A and influenza B
			No signal	

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No signal	No signal	No signal	Signal	Negative result [1]
			No signal	Invalid result [2]: extraction or amplification problem

[1] Negative or below the limit of detection of the kit.
[2] It is recommended to repeat the RNA extraction and/or repeat the RT-PCR, if in the repetition of the analysis is still invalid, we recommend starting from a new sample taking.

It is recommended to use the automatic threshold adjustment made by the default software of each instrument and if necessary the threshold can be adjusted manually ensuring that it falls within the exponential phase of the fluorescence curve and that the background noise is below the threshold line.

A sample is positive if the Ct value obtained is ≤38, although the internal control does not show an amplification graph. Sometimes, it might occur that the internal control is not amplified correctly due to the presence of a high initial number of copies of target viral nucleic acid, which can cause a preferential amplification of the latter.

A sample is negative if an amplification curve is not detected over the threshold value, and if the internal control does show it. The inhibition of the PCR reaction can be excluded by the amplification of the internal control.

10 PERFORMANCE CHARACTERISTICS

10.1 Analytical sensitivity

The analytical sensitivity of the FLU-COVID RT-PCR RT-PCR kit was determined with six replicas of serial dilutions of synthetic fragments from each of the targets, ranging from 10^7 copies/rxn hasta 10^1 copies/rxn.

It has been established that the FLU-COVID RT-PCR kit has a limit of detection (LoD) of 10 copies/reaction for Influenza A, Influenza B and SARS-CoV-2 (Figure 2).

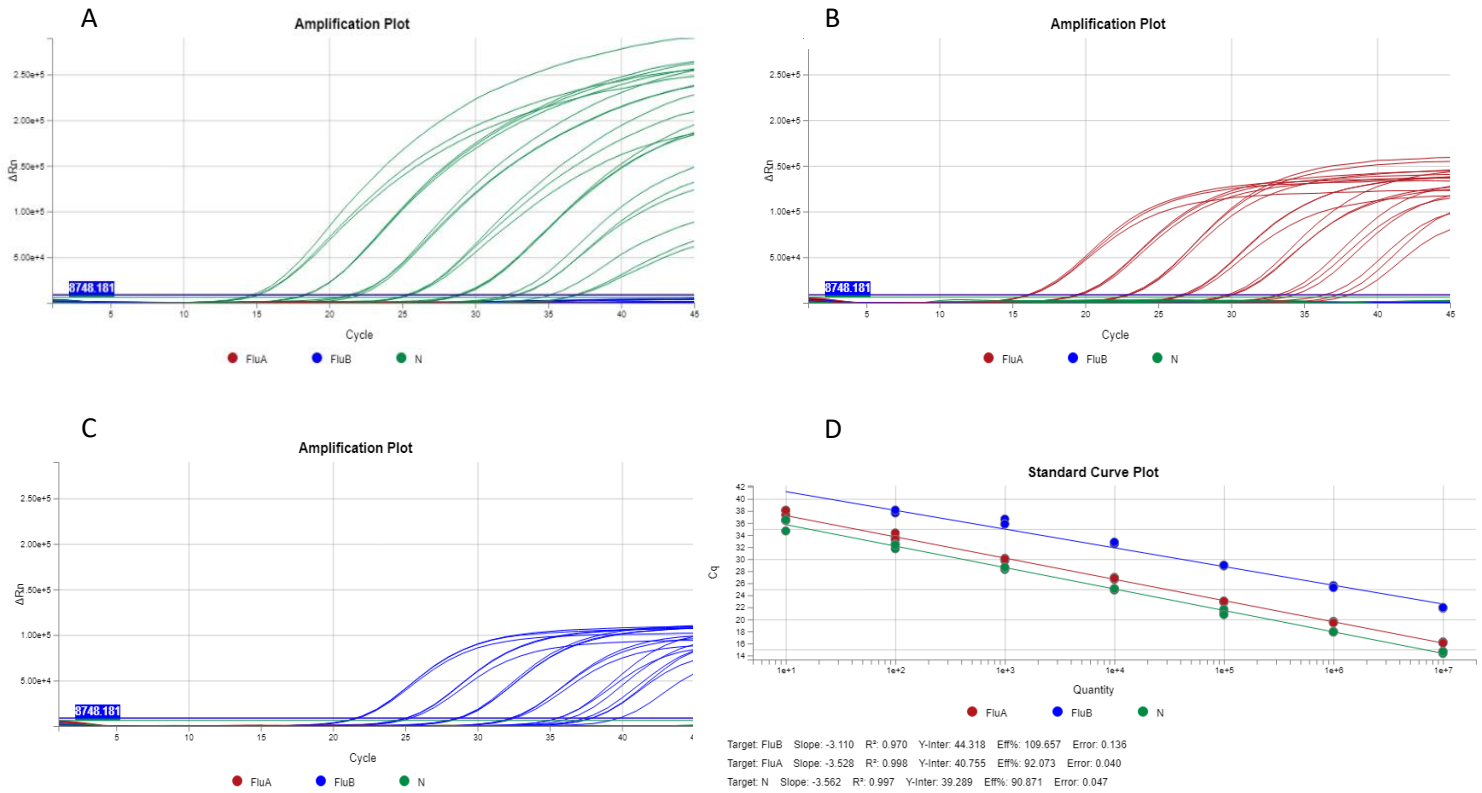

Figure 2: Serial dilutions from 10^7 copies/reaction to 10^1 copies/reaction of synthetic fragments of SARS-CoV-2 (N) in the FAM channel (A), FluA in the ROX channel (B), and FluB in theJOE channel (C). Standard curve for the three targets (D).

By adjusting the Cts data to a line, the amplification efficiency, R^2 and the slope were determined for each of the targets.

The N gene showed an efficiency of 90.87%, an R^2 of 0.997 and a slope of -3.56. The FluA Mp gene showed an efficiency of 92.07%, an R^2 of 0.998 and a slope of -3.52. The FluB Mp gene showed an efficiency of 109.66%, an R^2 of 0.970 and a slope of -3.11.

10.2 Analytical specificity

The specificity of the test of the FLU-COVUD RT-PCR kit was confirmed by testing positive clinical samples for different microorganisms representing the most common respiratory pathogens. No cross-reactions were detected with any of the following pathogens tested:

Cross-reactivity test			
Adenovirus	-	Coronavirus humano NL63	-
Virus Parainfluenza humano 1	-	Coronavirus humano HKU1	-
Virus Parainfluenza humano 2	-	Bocavirus humano	-
Virus Parainfluenza humano 3	-	Virus Respiratorio Sincitial (VRS) A	-
Virus Parainfluenza humano 4	-	Virus Respiratorio Sincitial (VRS) B	-

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Virus rhinovirus humano	-	Enterovirus	-
Virus metapneumovirus humano	-	*Bordetella pertusis*	-
Coronavirus humano OC43	-	*Bordetella parapertussis*	-
Coronavirus humano 229E	-	*Mycoplasma pneumoniae*	-

10.3 Clinical sensitivity and specificity

The FLU-COVID RT-PCR kit was evaluated in a retrospective study with 42 respiratory samples that had been previously characterized by a molecular reference method. This panel includes 8 positive samples for SARS-CoV-2, 14 positive samples for Inluenza A, 10 positive samples for Influenza B (two of them presented a coinfection with FluA), and 10 negative samples.

The agreement obtained for FLU-COVID RT-PCR kit with the gold standard method was 100%, showing an 100% of sensitivity and specificity.

11 LIMITATIONS OF THE TEST

1. The results of the test must be evaluated by a healthcare professional in the context of medical history, clinical symptoms, and other diagnostic tests.

2. This test can be used with different types of samples, although it has only been validated with RNA extracted from respiratory samples (nasopharyngeal and oropharyngeal smear and bronchoalveolar lavages).

3. The correct functioning of the test depends on the quality of the sample; the nucleic acid must be properly extracted from the clinical samples. Improper collection, storage and/or transport of samples can result in false negatives.

4. A low number of target copies below the detection limit can be detected, but the results may not be reproducible.

5. A positive test for FluA, FluB and/or SARS-CoV-2 does not exclude the possibility that other pathogens are present in the clinical sample.

6. The test works within the genomic regions in which the probes have been designed. Due to the high variability of RNA, certain subtypes may not be detected. However, at the time of design, no mutations were observed in the target regions.

7. A negative result of the test does not exclude that there is an infection with FluA, FluB and/or SARS-CoV-2, and it should not be used as the sole diagnostic method to establish a treatment or patient management regime.

8. A negative result of the test must be analyzed in the context of medical history of the patient and

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13 LABEL AND BOX SYMBOLS

IVD	Health product for in vitro diagnosis.		Expiration date
REF	Catalog number		Temperature limit
LOT	Lot code		Manufacturer
	Refer to the instructions of use		Sufficient content for <n> assays
	Material safety data sheet		Keep away from sunlight

14 CHANGELOG

Date	Description
2022-01-05	• Inclusion of the section changelog • Inclusion of the explanation of the pictogram of the Safety Sheet • Storage temperature is modified in Section 5.
2022-03-08	• Inclusion of the explanation of the pictogram “Keep away from sunlight”.
2023-02-03	• In section 1 "Intended Use" it is included that fluorescent primers and probes are used for the N target genes (SARS-CoV-2), with independent regions for N1 and N2.

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