Respiplex (RSV-MPV-RhV) RT-PCR

Diagnostic kit for the detection of RNA from the Respiratory Syncytial Virus (RSV), Metapneumovirus (MPV) and Rhinovirus (RhV) by One-step Real-Time RT-PCR

REF **Ref. MAD-003943M-W** **100 determinations**

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

Rev.: 2022/03/08

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IVD

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1 INTENDED USE

The **Respiplex (RSV-MPV-RhV) RT-PCR** kit is an in vitro diagnostic kit for the simultaneous qualitative detection and differentiation of RNA of the Respiratory Syncytial Virus[1], Metapneumovirus (MPV) and/or Rhinovirus (RHV) from RNA extracted from human clinical samples of different origin such as nasopharyngeal and oropharyngeal exudates and bronchoalveolar lavages (BAL). It can be used simultaneously with the FLU-COVID RT-PCR kit for the detection of Influenza A (FluA), Influenza B (FluB) and/or SARS-CoV-2 in a complementary manner.
It is based on the multiplex One-Step RT-PCR technique, using primers and fluorescent probes for the target genes: matrix protein gene (M) of RSV, nucleoprotein gene (N) of MPV and 5' region UTR of RhV.

[1]Types of Respiratory Syncytial Viruses detected: A and B.

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

Target	Fluorophore
Respiratory Syncytial Virus	ROX
Metapneumovirus	JOE
Rhinovirus	FAM
RNaseP	Cy5

Table 1. Detection channels for the different targets of the Respiplex (RSV-MPV-RhV) RT-PCR kit.

Microbiological status: Non-sterile product.

2 PRINCIPLE OF THE METHOD

The **Respiplex (RSV-MPV-RhV) RT-PCR** kit is a multiplex test based on the reverse transcription and real-time polymerase chain reaction. The Master mix contains three sets of primers and probes for the detection of RNA of the Metapneumovirus, Rhinovirus and Respiratory Syncytial Virus. It also includes primers and probes for the detection of the human gene of the RNaseP in clinical or control samples. The oligonucleotides used as primers and probes were selected in evolutionary conserved regions. In a single assay, the test allows differentiating between the Respiratory Syncytial Virus, Metapneumovirus and Rhinovirus. This Mix can be prepared simultaneously with the FLU-COVID RT-PCR mix to differentiate at the same time, and in a complementary way, between Influenza A, Influenza B and SARS-CoV-2.

In a first step of the reverse transcription, the RNA regions complementary to the primers are transformed to cDNA, which is subsequently amplified by polymerase chain reaction (PCR). The detection of the amplicons obtained is based on the TaqMan probe technology.
If the target nucleic acids are present, these are amplified and, during the PCR process, the probes will bind

specifically in the complementary regions located between the forward and reverse primers. While the extension phase of the PCR occurs, the 5' nuclease activity of the polymerase DNA degrades the probes bound specifically to their targets, causing the split between the reporter and the quencher, and a fluorescent signal will generate. The specific probes for each virus will generate a fluorescent signal at different longitudes of waves, allowing the Real Time PCR instrument to differentiate between the different signals. In each denaturation-extension cycle, the split of new molecules of reporter occurs, and, consequently, the intensity of the fluorescent signal increases. The intensity of the fluorescence is monitored on the real-time PCR instruments in each of the cycles and the data are analyzed with a specific analysis software for each platform.

The detection of viral RNA, besides helping in the diagnosis of the disease, provides valuable information in terms of epidemiology and monitoring.

3 COMPONENTS

The Respiplex (RSV-MPV-RhV) RT-PCR kit is commercialized as a ready-to-use 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 DNase/RNase-free DEPC-treated water to include in the negative controls (NTC) are supplied along with the Mix of RT-PCR.

Components of the kit for 100 tests:

REFERENCE (DESCRIPTION)		CONTENT	AMOUNT
MAD-003943M-100-W [Respiplex (RSV-MPV-RhV) MMIX]	**MAD-003943-MIX-W [Respiplex (RSV-MPV-RhV) 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**
	MAD-DDW-DEPC (RNAse/DNAse free water)	---	**1 vial (200 µl)**
MAD-Respiplex (RSV-MPV-RhV) [Respiplex (RSV-MPV-RhV) PC]		Synthetic non-infectious DNA/RNA containing part of the genome of MPV, RhV, RSV and human DNA	**1 vial (100 µl)**

Table 2. Reagents supplied in the Respiplex (RSV-MPV-RhV) RT-PCR kit.

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 Respiplex (RSV-MPV-RhV) RT-PCR kit must be transported and stored at -10 °C to -30°C *. Nonetheless, besides the recommended transport at -10 °C to -30°C, it is also possible to transport it at refrigeration temperature (2 °C - 8 °C), as long as the transit period does not exceed a maximum of ten days. In any case, the kit must be stored at a temperature of -10 °C to -30°C upon receipt.

The reaction mix Respiplex (RSV-MPV-RhV) MMix is sensitive to matter state changes and it has been proven that it supports up to five freeze-thaw 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 DNA or PCR products contamination.

*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.

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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 Respiplex (RSV-MPV-RhV) MMix must be thawed before use and handled on 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 Respiplex (RSV-MPV-RhV) 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, which act quickly degrading RNA. 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 waste 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 WASTE 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, this waste must be considered as "waste 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	"Waste 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 containing waste 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 Respiplex (RSV-MPV-RhV) RT-PCR kit has been validated for its use 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.

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 Respiplex (RSV-MPV-RhV) 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 60-100 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)
RNA/DNA Viral Extraction kit (Vitro S.A.)	OT-2 (Opentrons)
VIRAL EXTRACTION VN143 (Genolution)	Nextractor® NX-48 (Genolution)

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.

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8 PCR PROTOCOL

8.1 Preparation of the Reaction mix

The RT-PCR reaction is carried out in a final volume of 20 µl. Prepare the Master Mix as indicated below:

1. Thaw and homogenize Respiplex (RSV-MPV-RhV) MMix(do not use vortex). Once it is thawed, centrifuge briefly.
2. Mix in each PCR tube the following volumes for each sample:

Reagent	V/test
Respiplex (RSV-MPV-RhV) MMix	12 µl
Sample	8 µl

3. Include a negative control by adding 8 µl of the water included in the kit.
4. Include a positive control by adding 8 µl of the positive Respiplex (RSV-MPV-RhV) PC included in the kit.
5. Centrifuge briefly to make sure there are no air bubbles in the wells.

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

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 PCR targets (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 Respiplex (RSV-MPV-RhV) RT-PCR kit.

*The fluorescence data must be collected during the extension phase (*) by means of the FAM (Rhinovirus), HEX, JOE or VIC (Metapneumovirus), ROX (Syncytial Respiratory Virus) and Cy5 (internal control).

This kit has been validated with the platforms:

- QuantStudio™ 5 Real-Time PCR System (Applied Biosystems)
- 7500 Real-Time PCR System (Applied Biosystems)

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- 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 QuantStudio™ 5 Real-Time PCR System and Stratagene Mx3005P™ Real Time PCR System, the option of the 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 Respiplex (RSV-MPV-RhV)	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).

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

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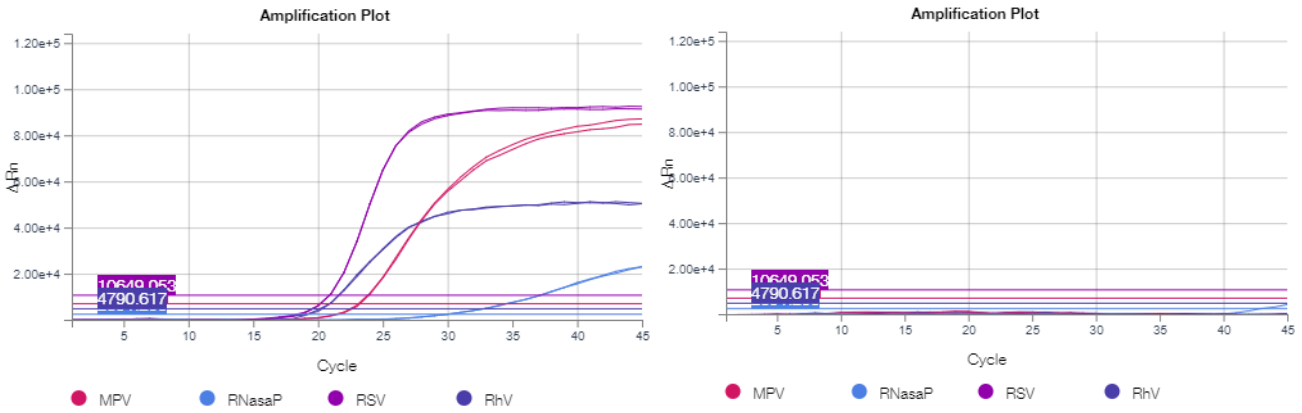

Figure 1: Graphs of amplification of the positive control PC (A) and of a negative control with water NTC (B). (Expected Cts values for PC: MPV (JOE) 23±2, RhV (FAM) 20±2; RSV A-B (ROX) 19 ±2; RNaseP (Cy5) 28±2. Experiment performed in Applied Biosystems QuantStudio™ 5 Real-Time PCR System.

The result is considered as invalid if a graphic of amplification in the negative control can be observed, or if there is lack of amplification in the well of the positive control. 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:

Respiplex (RSV-MPV-RhV) RT-PCR				**INTERPRETATION**
MPV (JOE)	**RSV (ROX)**	**RHV (FAM)**	**RNaseP (Cy5)**	
Signal	No signal	No signal	Signal	Positive sample for MPV
			No signal	
No signal	Signal	No signal	Signal	Positive sample for RSV
			No signal	
No signal	No signal	Signal	Signal	Positive sample for RhV
			No signal	
No signal	Signal	Signal	Signal	Positive sample for RSV and RhV
			No signal	
Signal	Signal	No signal	Signal	Positive sample for MPV and RSV
			No signal	
Signal	No signal	Signal	Signal	Positive sample for MPV and RhV
			No signal	
Signal	Signal	Signal	Signal	Positive sample for MPV, RSV and RhV
			No signal	

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No signal	No signal	No signal	Signal	Negative result [1]
			No signal	Invalid [2]: Problems in the extraction or amplification

[1] Negative or below the limit of detection of the kit.
[2] It is recommended to repeat the extraction of RNA and/or repeat the RT-PCR or from a new 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.

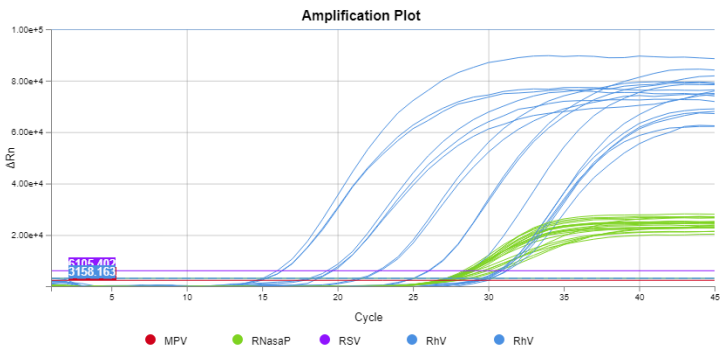
10 PERFORMANCE CHARACTERISTICS

10.1 Analytical sensitivity

The analytical sensitivity of the Respiplex (RSV-MPV-RhV) RT-PCR kit was determined by making six replicas of serial dilutions of synthetic fragments of each of the targets from 10^7 copies/rxn to 10^1 copies/rxn.

It has been established that the Respiplex (RSV-MPV-RhV) RT-PCR kit has a limit of detection of 10 copies/reaction for the Respiratory Syncytial Virus, Metapneumovirus and Rhinovirus (Figure 2).

A



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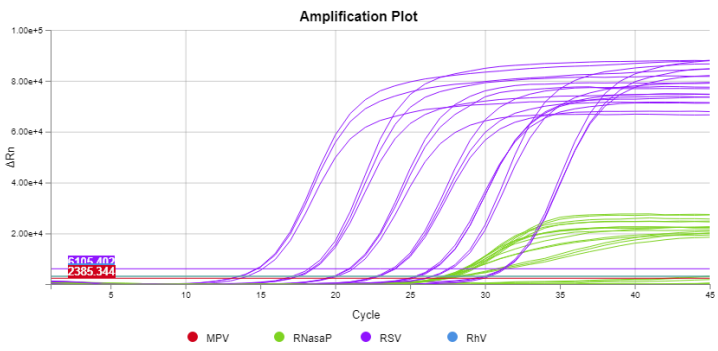

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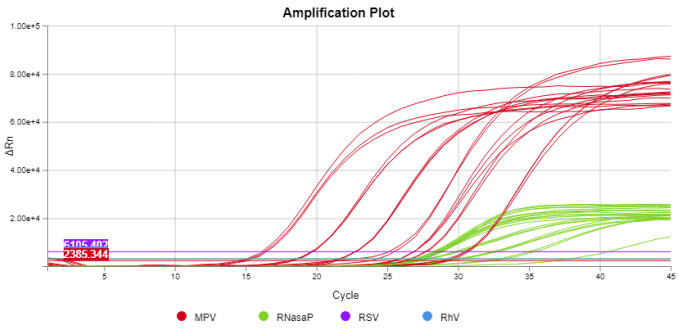
C



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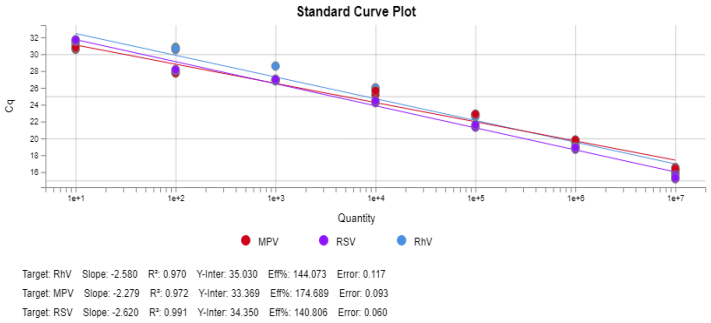

Figure 2: Serial dilutions from 10^7 copies/rxn to 10^1 copies/rxn of synthetic fragments of RhV in the FAM channel (A), RSV in ROX (B), and MPV in the JOE channel (C). Calibration lines obtained for the three targets (D).

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

The amplification of the 5' region UTR of RhV showed an efficiency of 144.07%, an R^2 of 0.97. The M gene of RSV showed an efficiency of 140.81%, an R^2 of 0.991. The N gene of MPV showed an efficiency of 174.69%, an R^2 of 0.972.

10.2 Analytical specificity

The specificity of the assay Respiplex (RSV-MPV-RhV) RT-PCR 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	-	Human coronavirus HKU1	-
Human parainfluenza virus type 1	-	Human bocavirus	-
Human parainfluenza virus type 2	-	Influenza A	-
Human parainfluenza virus type 3	-	Influenza B	-
Human parainfluenza virus type 4	-	Enterovirus	-
SARS-CoV-2	-	*Bordetella pertusis*	-
Human coronavirus OC43	-	*Bordetella parapertussis*	-
Human coronavirus 229E	-	*Mycoplasma pneumoniae*	-
Human coronavirus NL63	-		

10.3 Clinical sensitivity and specificity

In a retrospective study, a total of 63 respiratory samples were evaluated, which were previously analyzed with a reference molecular method. Of these, 48 were positive for any of the three viruses, with cases of co-infection, and 15 samples were negative.
In order to verify the diagnostic capability, the sensitivity and clinical specificity were calculated as follows:

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- The **diagnostic specificity** is expressed as a percentage (numerical fraction multiplied by 100), calculated as 100 x the number of true negative values (TN) divided by the sum of true negative values (TN) plus the number of false positive (FP) values, or 100 × TN/ (TN + FP).

- The **diagnostic sensitivity** is expressed as a percentage (numerical fraction multiplied by 100), calculated as 100 × the number of true positive values (TP) divided by the sum of true positive values (TP) plus the number of false negative values (FN), or 100 × TP/ (TP + FN).

ORGANISM	TN	FP	TP	FN	DIAGNOSTIC SPECIFICITY	DIAGNOSTIC SENSITIVITY
Respiratory Syncytial Virus	45	0	18	0	100%	100%
Metapneumovirus	55	0	8	0	100%	100%
Rhinovirus	41	0	22	0	100%	100%

Table 6. Diagnostic sensitivity and specificity of the Respiplex (RSV-MPV-RhV) RT-PCR kit.

The agreement obtained compared with the reference method was 100% for the three viruses. The results show a 100% of clinical sensitivity and specificity for Respiplex (RSV-MPV-RhV) RT-PCR kit.

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 MPV, RHV and/or RSV 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 MPV, RHV and/or RSV, 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 epidemiology.

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13 LABEL AND BOX SYMBOLS

Explanation of the symbols of the product label and box:

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
2020-11-23	• Creation of the document.
2020-12-03	• More samples in the clinical validation are included.
2022-01-05	• Temperature is modified in Section 5.
2022-03-08	• Inclusion of the explanation of the pictogram “Keep away from sunlight”.

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