Respiratory Flow Chip Kit

Identification of pathogens causing acute respiratory infections by multiplex PCR and reverse hybridization

For all hybriSpot platforms

Compatible with version 2.02.00.R07 of hybriSoft HSHS
For compatibility with other versions, please contact the manufacturer / supplier.

REF	**Ref. MAD-003939M-HS12**	Σ	**24 determinations**
	Ref. MAD-003939M-HS		**24 determinations**

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

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1. INTENDED USE

Respiratory Flow Chip is an in vitro diagnostic kit for the identification of the main pathogens causing acute respiratory tract infections. The organisms causing these infections include viruses and bacteria, occurring co-infections relatively often. The methods used currently to diagnose them are expensive, laborious and do not always show 100% specificity. The *Respiratory Flow Chip* kit allows the simultaneous detection of 13 pathogens: Influenza virus[1], Adenovirus[2], Bocavirus, Coronavirus[3], Metapneumovirus, Parainfluenza virus[4], Respiratory Syncytial Virus (Subtype A and Subtype B), Rhinovirus, Enterovirus[5], *Bordetella pertussis, Bordetella parapertussis* and *Mycoplasma pneumoniae*, using viral DNA/RNA amplification by reverse transcription and multiplex PCR (RT-PCR) and subsequent reverse hybridization on a membrane containing specific probes to each species.

The *Respiratory Flow Chip* kit allows identifying these infectious agents from purified genetic material from several types of clinical samples (nasopharyngeal and oropharyngeal exudates, nasopharyngeal aspirate and bronchoalveolar lavage).

[1]Types of influenza virus identified:

- Influenza Type A: subtype H3 and subtype H1N1 (pandemic 2009)
- Influenza type B

[2]Serotypes of Adenovirus detected with the kit (not detected individually):

- Type 1
- Type 2
- Type 3
- Type 4
- Type 6
- Type 7
- Type 8
- Type 11
- Type 12
- Type 16
- Type 18
- Type 21
- Type 31
- Type 34

[3]Coronavirus identified:

- 229E
- HKU-1
- NL63
- OC43
- SARS-CoV-2: RdRP gene (specific target for SARS-CoV-2) and E gene (generic to Sarbecovirus) are included.

[4]Types of Parainfluenza virus identified:

- Type 1
- Type 2
- Type 3
- Type 4

[5] Species of Enterovirus detected with the kit (not detected individually):

- EV-A
- EV-B
- EV-D (EV-D68 is included)

Microbiological status: Product not sterile

2. PRINCIPLE OF THE METHOD

The Respiratory Flow Chip kit is based on a methodology that consists of the simultaneous amplification of viral DNA, viral RNA and bacteria by RT-multiplex PCR in only one step, followed by hybridization in membranes with specific DNA probes through the DNA-Flow technology for both automatic and manual *HybriSpot* platforms. The biotinylated amplicons generated after the RT-PCR are hybridized in membranes containing an array of specific probes for each virus as well as amplification and hybridization control probes. The DNA-Flow technology allows the fast binding of the PCR product and its specific probe in a three-dimensional porous environment, as compared to the hybridization in a conventional surface. Once the binding between the specific amplicons and their corresponding probes has occurred, the signal is visualized through an immunoenzymatic colorimetric reaction with Streptavidin–Alkaline Phosphatase and a chromogen (NBT-BCIP) generating insoluble precipitates in the membrane in those positions in which there has been hybridization. The results are analyzed automatically with the *hybriSoft* software.

3. COMPONENTS

The **Respiratory Flow Chip** kit is marketed in two formats according to the type of hybridization platform to be used for the analysis of clinical samples. Both formats provide all the necessary reagents for the amplification through RT-multiplex PCR and subsequent hybridization of 24 clinical samples. Each kit format contains the following components and references:

KIT/COMPONENTS	FORMAT	REFERENCES
Respiratory Flow Chip kit (Manual)	**24 tests**	**MAD-003939M-HS12**
1. Respiratory Flow Chip kit (PCR Reagents)	**24 tests**	**MAD-003939M-P**
Respiratory PCR Mix 1	3 strips × 8 tubes (clear)	MAD-003939M-MIX1
Respiratory PCR Mix 2	3 strips × 8 tubes (yellow)	MAD-003939M-MIX2
2. Respiratory Chips	**24 tests**	**MAD-003939M-CH-HS**
3. Flow Chip Hybridization Reagents Type I (Manual)	**24 tests**	**MAD-003925M-HS12**
Hybridization Solution (Reagent A)	40 ml	MAD-003930MA-HS12-24
Blocking Solution (Reagent B)	10 ml	MAD-003930MB-HS12-24
Streptavidin-Alkaline Phosphatase (Reagent C)	10 ml	MAD-003930MC-HS12-24
Washing Buffer I (Reagent D)	35 ml	MAD-003930MD-HS12-24
Reagent E	10 ml	MAD-003930ME
Washing Buffer II (Reagent F)	18 ml	MAD-003930MF-HS12-24

Table 1. Reagents provided in the Respiratory Flow Chip kit format (Manual).

KIT/COMPONENTS	FORMAT	REFERENCES
Respiratory Flow Chip kit (Auto)	**24 tests**	**MAD-003939M-HS**
1. Respiratory Flow Chip kit (PCR Reagents)	**24 tests**	**MAD-003939M-P**
Respiratory PCR Mix 1	3 strips × 8 tubes (clear)	MAD-003939M-MIX1
Respiratory PCR Mix 2	3 strips × 8 tubes (yellow)	MAD-003939M-MIX2
2. Respiratory Chips	**24 tests**	**MAD-003939M-CH-HS**
3. Flow Chip Hybridization Reagents Type I (Auto)	**24 tests**	**MAD-003925M-HS**
Hybridization Solution (Reagent A)	60 ml	MAD-003930MA-HS24-24
Blocking Solution (Reagent B)	10 ml	MAD-003930MB-HS24-24
Streptavidin-Alkaline Phosphatase (Reagent C)	10 ml	MAD-003930MC-HS24-24
Washing Buffer I (Reagent D)	35 ml	MAD-003930MD-HS24-24
Reagent E	10 ml	MAD-003930ME-HS24

Table 2. Reagents provided in the Respiratory Flow Chip kit format (Manual).

- Both presentations include 2 vials with 2mL of DNase/RNase-free double distilled water for the handling of clinical samples: RNASE/DNASE-FREE DISTILLED WATER; Ref: MAD-DDW.

- **Respiratory Flow Chip kit (PCR Reagents):** it is commercialized in a format of strips of 8 tubes of 0.2 ml containing the lyophilized reagents corresponding to two PCR mixes – mix 1 and mix 2.
  - The tubes corresponding to the mix 1 (clear) are disposed in a lyophilized sphere format in white whose components are: PCR buffer, dNTPs (U/T), DNase and RNase-free water, biotinylated and non-biotinylated primers, DNA of an exogenous amplification control, RNase inhibitors, *Hot Start Polymerase*, *Reverse Transcriptase* and *Uracil DNA Glycosylase*. The primers included are specific to the amplification of 9 viral species: Influenza A virus, Influenza A virus Subtype H3, Influenza A virus Subtype H1N1 (pandemic 2009), Influenza B virus, Respiratory Syncytial Virus Subtype A, Respiratory Syncytial Virus Subtype B, Rhinovirus, Enterovirus and Metapneumovirus. Furthermore, primers are included for amplification of a fragment of human genomic DNA (endogenous control) and a fragment of synthetic DNA, used as exogenous amplification control.

  - The tubes corresponding to the mix 2 (yellow) are disposed in a lyophilized sphere format in white whose components are: PCR buffer, dNTPs (U/T), DNase and RNase-free water, biotinylated and non-biotinylated primers, DNA of an exogenous amplification control, RNase inhibitors, *Hot Start Polymerase*, *Reverse Transcriptase* and *Uracil DNA Glycosylase*. The primers included are specific to the amplification of 14 species of pathogenic species: Adenovirus, Bocavirus, Parainfluenza virus Type 1, Parainfluenza virus Type 2, Parainfluenza virus Type 3, Parainfluenza virus Type 4, Coronavirus 229E, Coronavirus HKU-1, Coronavirus NL63, Coronavirus OC43, Coronavirus SARS-CoV-2, *Bordetella pertussis, Bordetella parapertussis* and *Mycoplasma pneumoniae*. Furthermore, primers are included for amplification of a fragment of human genomic DNA (endogenous control) and a fragment of synthetic DNA, used as exogenous amplification control.

- **Respiratory Chips:** The kit includes 24 Chips in total (Ref: MAD-003939M-CH-HS) that contain an array of DNA probes specific to each of the pathogens included in the analysis, as well as others corresponding to the internal controls included in this kit. The position of all them on the Chip can be referred to in the section 10 of this manual (INTERPRETATION OF RESULTS).

- **Flow Chip Hybridization Reagents:** It contains all the reagents necessary for the reverse Flow-Through hybridization process.

4. ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1. Reagents and materials

A. Common reagents to manual and automatic platforms:

- Disposable gloves.
- Pipette tips with DNase/RNase-free filters.
- Extraction kits of starting genetic material (see Table 4).

B. Specific reagents (Auto, ref: MAD-003939M-HS)

- Washing Reagent (Ref: MAD-003930WSH).

4.2. Equipment

A. Common equipment for the manual and automatic platforms:

- Microcentrifuge.
- Automatic micropipettes: P1000, P200, P20 and P2.
- For the extraction of genetic material from samples of bronchoalveolar lavage, nasopharyngeal aspirates and nasopharyngeal / oropharyngeal exudates, it is recommended to use a RNA/DNA purification kit. In table 4, it is showed in detail the information on the DNA/RNA purification kits and extraction equipment with which the Respiratory Flow Chip kit has been validated.
- HybriSoft software.

B. Specific equipment:

- Respiratory Flow Chip kit (Manual) (Ref: MAD-003939M-HS12):
    - Manual equipment for hybridization hybriSpot 12 (VIT-HS12).
    - Thermocycler
    - Thermal block to heat PCR tubes (can be substituted by a thermocycler)
    - Cold plate (4°C)
    - Thermostatic bath / heater.
- Respiratory Flow Chip kit (Auto) (Ref: MAD-003939M-HS):
    - Automatic equipment for hybridization hybriSpot 24 (VIT-HS24) or hybriSpot 12 PCR AUTO (VIT-HS12a).
    - Thermocycler (not necessary for hybriSpot 12 PCR AUTO).
    - Thermal block to heat PCR tubes (not necessary for hybriSpot 12 PCR AUTO).
    - Cold plate (4°C).

5. STORAGE AND STABILITY CONDITIONS

The *Respiratory Flow Chip* kit consists of two components that are supplied in separate boxes:

- **Respiratory Flow Chip kit (PCR Reagents):** Shipped and stored at 2-8°C.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. Once the package containing the tube strip with the lyophilized PCR mix is opened, store the remaining tubes up to a maximum of one week at 2-8°C in the original package.
- **Respiratory Chips:** Shipped and stored between 2-8°C*. **Do not freeze**. The Chips are stable until their indicated expiration date.
- **Hybridization reagents:** Shipped and stored between 2-8°C. **Do not freeze**. The hybridization reagents are stable until the expiration date indicated. Previous recommendations on the hybridization reagents:
  - The hybridization reagent A must be preheated at 41°C in a thermostatic bath or heater (only before using in manual equipment) before its use.
  - The rest of the hybridization reagents must be used at room temperature (15-25°C).

Previous chip considerations:

- Once the packaging containing the chips has been opened, keep the cylindrical foam and sorbent packet inside until end of use to ensure the adequate preservation of the membranes.

*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 of use before using this product.**
- **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.
- **Respiratory Flow Chip** kit uses as starting material, nucleic acids previously extracted and purified. 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 carrier.
  - 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 greatest contamination source is normally the same amplified PCR product. Therefore, it is recommended to carry out the handling of the amplified products in a different area than the one the PCR reaction is performed. It is recommended to work on different pre- and post-PCR areas where the handling of the test DNA and preparation of the PCR tubes (pre-PCR) and the handling and hybridization 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 diagnosis. 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 dUTP, is included in the kit.

  It is recommended to include negative amplification controls containing all the reagents handled in the kit, from the extraction to the amplification, except for the DNA/RNA sample, in order to detect and control any possible contamination of the reagents with test samples or amplified products. The hybridization in membrane of this control must be negative, marking only the hybridization control and the amplification exogenous control. This way, it is verified that there is no contamination of DNA of patients and/or amplified DNA in the pre-PCR area.

- **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/CE 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. Rubbish/Waste generated from hybridization reagents 2. Disposal of Liquid Wastes ("Wastes" in the manual and automatic platforms)	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"
3. Chips used 4. Perishable material (tubes, tips, aluminum foil, etc.) 5. 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"
6. 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: English acronym for *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 *Respiratory Flow Chip* kit has been validated for its use with purified genetic material from bronchoalveolar lavage, nasopharyngeal aspirate and nasopharyngeal / 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 nasopharyngeal aspirates, a probe is introduced through the nasal cavities to the posterior wall of the pharynx. Then, 1 mL of sterile saline solution is introduced in one of the nostrils using a syringe attached to the probe and a light suction with a vacuum pump or a syringe is applied, aspirating as much nasopharyngeal secretion as possible. 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 throat (oropharynx). The tip of the nasopharyngeal / oropharyngeal swab to be used 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 with transport medium. The samples are collected in a sterile recipient and kept at 2-8 °C for a maximum of 48 hours. Once the samples are classified, they are stored at -80 °C in order to preserve the viral viability.

7.2. Extraction of nucleic acids from bronchoalveolar lavages, nasopharyngeal aspirates and nasopharyngeal / oropharyngeal exudates

The *Respiratory Flow Chip* kit has been tested with purified genetic material from human bronchoalveolar lavages, nasopharyngeal aspirates or nasopharyngeal / 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 INSTRUMENTS
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)
NucliSENS EasyMAG (Biomerieux)	NucliSENS EasyMAG (Biomerieux)
PureLink Viral RNA/DNA extraction mini kit (Invitrogen)	Manual system

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. ANALYSIS PROCEDURE FOR HS12 AND HS24 PLATFORMS

8.1. Multiplex DNA/RNA amplification reaction

The following thermal cyclers have been validated with *Respiratory Flow Chip:*

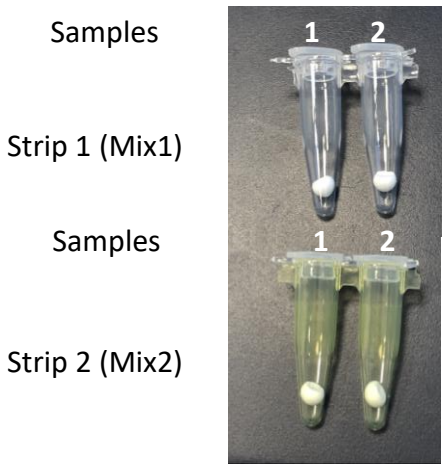
- *Veriti 96 Well Thermal Cycler* (Applied Biosystems)
- *SimpliAmp Thermal Cycler* (Applied Biosystems)
- *LifeEco (BioER) thermocycler*
- MyCycler™ Thermal Cycler System (BioRad)
- GeneAmp PCR system 9700 (Applied BioSystems)
- *TProfessional ThermoCycler* (Biometra)

The PCR reaction is carried out in a final volume of 30 µl for each PCR mix. The lyophilized spheres correspond to the mix 1 and mix 2 are supplied in separated strips. Two PCR tubes must be used for each sample, one for each strip.

If the number of samples to be analyzed is lower or higher than 8, the necessary tubes can be separated from each strip with no need for using complete strips.

Once the strip is open, **the rest of the lyophilized tubes that are not going to be used at that moment must be stored for maximum of 1 week at 4°C in their original package.**

The diagram below shows an example of the distribution of samples/strips in case 2 test samples are used:



Procedure:

- Take a tube of mix 1 and a tube of mix 2 containing the lyophilized PCR mixes for each sample to be analyzed.
- Add up to 30 µl of genetic material from each sample previously purified (the extraction eluate) to each of the corresponding tubes to both PCR mixes.

If such quantity of starting sample is not available, a lower volume can be used (up to a minimum of 3 µl/PCR reaction) and complete the rest up to 30 µl with DNase/RNase-free water, although the kit specifications in terms of clinical sensitivity and specificity are based on the use of 30 µl of starting sample.

- Homogenize the mix by pipetting and centrifuge for a few seconds.
- Place the tubes in the thermocycler and set the following amplification conditions:

PCR PROGRAM		
25 °C	5 min	1 cycle
50 °C	20 min	1 cycle
95 °C	5 min	1 cycle
95 °C	30 sec	45 cycles
60 °C	1 min	
8 °C	∞	

Table 5. PCR program.

Keep the tubes refrigerated at 8-10 °C when the reaction is finished. If the samples are not going to be processed at that moment, they can be stored in the post-PCR zone at 8-10°C for 1-2 days. In order to store them for a longer period of time, it is recommended to do it at -20**°**C up to a maximum of a week.

8.2. Flow-through reverse hybridization

All the reagents are provided in a "ready-to-use" format.
The Chips are single-use. They must be handled with gloves and away from any contamination source.
Depending on the type of kit with which we are working, we will proceed as follows:

A. Respiratory Flow Chip kit for HS12 (Manual, ref: MAD-003939M-HS12):

The full hybridization process is performed semi-automatically in hybriSpot (HS12) following the instructions provided by the wizard of the system. The sample management, the capture of images and the analysis and report of the results are performed by the *hybriSoft software*.

Note: Configure the instrument by following the instructions of the user manual (provided with the instrument).

Before starting the hybridization process:

- Pre-heat **Reagent A at 41°C for at least 20 min** in a thermostatically controlled bath.
- **Mix the PCR products obtained with the mix 1 and mix 2** and aliquot 50 µl of the mix in a new tube, being this the material used in the following steps.
- **Denature the PCR products** by heating them at 95 °C for 10 min (in a thermal cycler or a heating block) and cool down quickly by keeping the samples at 4 °C for at least 2 min.
- Place every **Respiratory Chip** in the position indicated in the platform (HS12).

Manual hybridization protocol:

a) Set the temperature of the equipment at 41 °C. Add **300 µl** of **Reagent A (Hybridization Solution)** preheated for at least 20 minutes at 41 °C for every Chip and incubate for at least **2 min at 41 °C**.
b) Remove the **reagent A (Hybridization Solution)** by activating the vacuum pump.

c) Mix **50 µl** of each PCR sample from the combination of the two PCR products obtained with the mix 1 and mix 2 (previously denatured and kept in ice) with **230 µl** of **Reagent A (Hybridization Solution)** (41 °C) and dispense the mix on the corresponding **Respiratory Chip**.
d) Incubate at **41 °C** for **8 min**.
e) Activate the pump for at least 30 s to remove the PCR products.
f) Wash **3** times with **300 µl** with **Reagent A (Hybridization Solution)** (41 ºC).
g) Set the temperature at **29 °C**.
h) Add **300 µl** of **Reagent B (Blocking Solution)** and incubate for 5 min.
i) Activate the pump to remove the reagent B.
j) When the temperature reaches **29 °C**, add **300 µL** of **Reagent C (Streptavidin-Alkaline Phosphatase)** to each Chip.
k) Incubate for **5 min at 29 ºC**.
l) Activate the pump to remove the reagent.
m) Set the temperature at **36 °C**.
n) Wash the membranes **4** times with **300 µl** with **reagent D (Washing buffer I).**
o) When the temperature has reached **36 °C**, add **300 µl** of **Reagent E (developer solution)** to every Chip. Incubate for **10 min** at **36 ºC**.
p) Activate the pump to remove the reagent.
q) Wash the membranes **2** times with **300 µl** with **reagent F (Washing buffer II).**
r) Activate the pump to remove the reagent.
s) Perform the image capture, analysis and result report following the instructions of the equipment's user manual.

B. For Respiratory Flow Chip kit for HS24 (Auto, ref: MAD-003939M-HS):

The whole hybridization process is performed automatically on hybriSpot 24 (HS24). The sample management, the capture of images and the analysis and report of the results are performed through the hybriSoft software.

Note: Configure the instrument by following the instructions of the user manual (provided with the instrument).

Before starting the hybridization process:

1. Denature the PCR products by heating them at **95 °C for 10 min** in a thermocycler or a heating block and cool quickly in ice for at least **2 min**.
2. Follow the instructions in the manual to place the PCR tubes of the Mix 1 and 2, the Respiratory Chips and the reagents in their corresponding positions of the automatic platform HS24.
3. Select the corresponding protocol in the equipment to start the automatic process.

9. ANALYSIS PROCEDURE FOR HS12a

The amplification through PCR and hybridization processes are performed automatically in the Hs12a platform.

The processing of the sample, the capture of images and the results analysis are performed with the *hybriSoft software*.

Before starting the process, it is recommended to read carefully the user manual (included in the Hs12a platform). Follow the instructions in the manual to place the PCR tube strips, CHIPs and hybridization reagents in the instrument.

Protocol:

- Take a tube containing each of the lyophilized PCR mixes per sample to be analyzed.
- Add up to 30 µl of sample in each tube following the recommended protocol in section 8.
- Homogenize the mix by pipetting and centrifuge for a few seconds.
- If the number of samples to be analyzed is lower or higher than 8, the necessary tubes can be separated from the strip with no need for using complete strips. The rest of the lyophilized tube strip that is not going to be used at that moment must be stored for maximum of 1 week at 4 °C in its original package.
- Follow the instructions described in the manual of the Hs12a instrument to place the PCR tube strips, chips and hybridization reagents in the instrument and start the process.

10. QUALITY CONTROL PROCEDURE

The *Respiratory Flow Chip* kit contains several internal controls to control the quality of the results.

SPOTS	CONTROL	POSITION (see Figure 1)
B	Hybridization control	1A-1B-2I-5E-8A
CI-1	Exogenous amplification control mix 1	1C-5F
CI-2	Exogenous amplification control mix 2	1D-5G
RNaseP	Endogenous amplification control mix 1	1E-6A
BG	Endogenous amplification control mix 2	1F-6B

Table 6. Control probes included in *Respiratory Chip.*

Hybridization control: After the development of the membranes, an intense signal must appear in all five hybridization control positions (B), which serve as a quality control. This signal indicates that the hybridization reagents and developing have worked properly. If the signal does not appear, it indicates that an error has occurred during the hybridization process or that a reagent has not been used properly. Furthermore, this signal allows the software to orientate correctly the probe panel to perform the subsequent analysis.

Exogenous amplification control (CI-1): probe to detect a synthetic DNA included in the mix 1 of the PCR. This DNA will be co-amplified along with the genetic material of the sample. Two positive signals in the Exogenous amplification control 1(CI-1) will indicate that the PCR reaction in the mix 1 has worked correctly. A negative result in this control does not invalidate the result if the endogenous control 1 has correctly amplified and/or the sample has been positive for any of the organisms included in the mix.

Exogenous amplification control (CI-2): probe to detect a synthetic DNA included in the mix 2 of the PCR. This DNA will be co-amplified along with the genetic material of the sample. Two positive signals in the Exogenous amplification control 2(CI-2) will indicate that the PCR reaction in the mix 2 has worked correctly. A negative result in this control does not invalidate the result if the endogenous control 2 has correctly amplified and/or the sample has been positive for any of the organisms included in the mix.

Endogenous amplification control mix 1 (RNaseP): probe to detect the DNA of the gene of the human RNaseP which is co-amplified during the PCR when the mix 1 is used. All the samples where the test DNA has been amplified correctly will have a positive signal in the Endogenous amplification Control (RNaseP).

This signal shows the quality/quantity of the DNA used in the amplification. A positive signal indicates that the amplification has worked correctly and that the quality and quantity of the DNA used for it have been optimal. The lack of signal for this control indicates errors during the amplification, due to low quality/quantity of the DNA used in the amplification or lack of human DNA in the amplification. A negative result in this control does not invalidate the result if the exogenous control 1 has correctly amplified and/or the sample has been positive for any of the organisms included in the mix. The latter case is likely to occur with clinical specimen types containing a lower number of human cells.

Endogenous amplification control mix 2 (BG): probe to detect the DNA of the gene of the human beta-globulin, which is co-amplified during the PCR when the mix 2 is used. All the samples where the test DNA has been amplified correctly will have a positive signal in the Endogenous amplification control (BG). This signal shows the quality/quantity of the DNA used in the amplification. A positive signal indicates that the amplification has worked correctly and that the quality and quantity of the DNA used for it have been optimal. The lack of signal for this control indicates errors during the amplification, due to low quality/quantity of the DNA used in the amplification or lack of human DNA in the amplification. A negative result in this control does not invalidate the result if the exogenous control 2 has correctly amplified and/or the sample has been positive for any of the organisms included in this mix. The latter case is likely to occur with clinical specimen types containing a lower number of human cells.

When a sample is positive for any of the pathogens included in the kit, with a negative result for the exogenous and endogenous amplification controls, the report for the automatic analysis of the results with *HybriSoft software* shows a warning of *"no exogenous control / no human DNA control"* for the user to perform the appropriate verifications before validating the result.

The user is responsible for determining the appropriate quality control procedures for their laboratory and comply with the applicable legislation.

11. INTERPRETATION OF RESULTS

The interpretation of results is done automatically using *HybriSoft*'s analysis software. The following scheme shows the arrangement of the probes on the *Respiratory Chip*:

	1	2	3	4	5	6	7	8	9
A	B	FluA	PIV-1	CoV-OC43		RNase P	RSV-A	B	
B	B	FluA-H1N1	PIV-2	BP		BG	RSV-B	CoV-229E	
C	CI-1	FluA-H3	PIV-3	BPP			RhV	CoV-HKU1	
D	CI-2	FluB	PIV-4	MP			PIV-1	CoV-NL63	
E	RNase P	MPV	Adv	EV	B	FluA	PIV-2	BPP	
F	BG	RSV-A	BoV	CoV-2	CI-1	Flua-H1N1	PIV-3	MP	
G		RSV-B	CoV-229E	SARS	CI-2	FluA-H3	PIV-4	EV	
H		RhV	CoV-HKU1		CoV-OC43	FluB	Adv	CoV-2	
I		B	CoV-NL63		BP	MPV	BoV	SARS	

Figure 1: Scheme of the arrangement of the probes on the array.

"B": Hybridization control
"CI-1": Exogenous amplification control mix 1
"CI-2": Exogenous amplification control mix 2
"RNaseP": Endogenous amplification control mix 1 (fragment human RNaseP)
"BG": Endogenous amplification control mix 2 (fragment human ß-Globin)
"X": Specific probes for each pathogen

All the probes are duplicated to guarantee the reliability in the automatic analysis of the results. The hybridization control (B) is repeated in 5 positions and allows the software to orientate correctly the probe panel for its analysis afterwards.

The distribution of the different probes included in the **Respiratory Chip** as well as the possible expected results and their interpretation are shown below:

EXPECTED RESULTS (DETECTED PATHOGENS)	PROBE ID	PROBE/POSITION (column-row)					
		PROBE	B	CI-1	CI-2	RNaseP	BG
Influenza A	**FluA**	2A-6E	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Influenza A, subtype H1N1 (pandemic 2009)*	**FluA-H1N1**	2B-6F	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Influenza A, subtype H3**	**FluA-H3**	2C-6G	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Influenza B	**FluB**	2D-6H	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Metapneumovirus	**MPV**	2E-6I	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Respiratory Syncytial Virus Subtype A	**RSV-A**	2F-7A	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B

Respiratory Syncytial Virus Subtype B	**RSV-B**	2G-7B	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Rhinovirus	**RhV**	2H-7C	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Enterovirus	**EV**	4E-8G	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Parainfluenza Type 1	**PIV-1**	3A-7D	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Parainfluenza Type 2	**PIV-2**	3B-7E	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Parainfluenza Type 3	**PIV-3**	3C-7F	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Parainfluenza Type 4	**PIV-4**	3D-7G	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Adenovirus	**AdV**	3E-7H	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Bocavirus	**BoV**	3F-7I	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Coronavirus 229E	**CoV-229E**	3G-8B	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Coronavirus HKU-1	**CoV-HKU1**	3H-8C	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Coronavirus NL63	**CoV-NL63**	3I-8D	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Coronavirus OC43	**CoV-OC43**	4A-5H	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Coronavirus SARS-like	**SARS**	4G-8I	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Coronavirus SARS-CoV2***	**SARS + CoV-2**	4G-8I - 4F-8H	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Bordetella pertussis	**BP**	4B-5I	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Bordetella parapertussis	**BPP**	4C-8E	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Mycoplasma pneumoniae	**MP**	4D-8F	1A-1B-2I-5E-8A	--/1C-5F	--/1D-5G	-- /1E-6A	-- /1F-6B
Negative sample	--	--	1A-1B-2I-5E-8A	1C-5F	1D-5G	1E-6A	1F-6B
Blank	--	--	1A-1B-2I-5E-8A	1C-5F	1D-5G	--	--
Invalid results	--	--	1A-1B-2I-5E-8A	--	1D-5G	--	--
Invalid results	--	--	1A-1B-2I-5E-8A	1C-5F	--	--	--
Invalid results	--	--	1A-1B-2I-5E-8A	--/1C-5F	--	--/1E-6A	--
Invalid results	--	--	1A-1B-2I-5E-8A	--	--/1D-5G	--	-- /1F-6B
Invalid results	--	--	1A-1B-2I-5E-8A	1C-5F	1D-5G	1E-6A	--
Invalid results	--	--	1A-1B-2I-5E-8A	1C-5F	1D-5G	--	1F-6B
Negative sample	--	--	1A-1B-2I-5E-8A	--	--/1D-5G	1E-6A	1F-6B
Negative sample	--	--	1A-1B-2I-5E-8A	--/1C-5F	--	1E-6A	1F-6B
Invalid results	--	--	1A-1B-2I-5E-8A	--	--	--	--
Hybridization error	--	--	--	--	--	--	--

Table 7. Position of the probes on the *Respiratory Chip* and interpretation of results.

***Note: The positive identification of the Influenza A virus subtype H1N1 is obtained through the positivity of the FluA-H1N1 probe along with the positivity for the FluA probe (although a negative result for FluA does not invalidate the result).**

****Note: The positive identification of the Influenza A subtype H3 is obtained through the positivity of the FluA-H3 probe along with the positivity for the FluA probe (although a negative result for FluA does not invalidate the result).**

*****Note: The positive identification of the Coronavirus SARS-CoV-2 is obtained through the double positivity of the CoV-2 probe (specific for SARS-CoV-2, designed to target the RdRP gene) along with the positivity for the SARS probe (generic to Sarbecovirus, designed to target the E gene).**

- **A positive result for CoV-2 probe and a negative result for SARS probe should be considered as uncertain positiveness for SARS-CoV-2. It is recommended to repeat the test or start from a new RNA extraction. If**

the repeat result remains uncertain, additional confirmation testing (to sequence the sample) should be conducted if clinically indicated.

- **A positive result for SARS probe and a negative result for CoV-2 probe should be considered as presumed positive for SARS-CoV-2 in pandemic status. It is recommended to repeat the test or start from a new RNA extraction. If the repeat result remains unclear, additional confirmation testing (to sequence the sample) should be conducted if clinically indicated.**

An example of a report in which the analyzed sample has been positive for human Coronavirus SARS-CoV-2 is shown below:

Respiratory Flow Chip Kit

LOTS

PCR:	RES008L	30/09/2022
Chips:	RES004.2HS	30/10/2022
Reagent:	H079LF	30/08/2021

SAMPLE DETAILS

ID SAMPLE: Sample-01 **SAMPLE TYPE:**

ID PATIENT: **PATIENT:**

SEX: - **BIRTHDATE:** **AGE:**

REPORT

RES POSITIVE

Positive sample for:

Human Coronavirus SARS-CoV-2

The sample is negative for the rest of bacteria and virus included in the RES flow chip test.

PROTOCOL

Detection of a panel of viruses and bacteria that causing acute respiratory infections by multiplex-RT-PCR and Automatic Reverse Dot Blot that includes:

- Virus: Influenza Virus A/Influenza Virus A H1N1 2009/Influenza Virus A H3/Influenza Virus B/human Metapneumovirus/Respiratory syncytial virus A/Respiratory syncytial virus B/human Rhinovirus/Enterovirus/human Parainfluenza virus type 1/ human Parainfluenza virus type 2/ human Parainfluenza virus type 3/ human Parainfluenza virus type 4/Adenovirus/human Bocavirus/human Coronavirus 229E/human Coronavirus HKU/human Coronavirus NL63/human Coronavirus OC43/human Coronavirus SARS-CoV-2
- Bacteria: Bordetella pertussis/Bordetella parapertussis/Mycoplasma pneumoniae

Add cell suspension/purified DNA for PCR amplification:

PCR Protocol: 1x [25° 5 min]; 1x [50° 20 min]; 1x [95° 5 min]; 45x [95° 30 s-60° 1min]; 1x [8°C, ∞].

REVERSE-DOT BLOT protocol:

- Hybridization of the biotinilated PCR products to the Respiratory Flow CHIP.
- Post-hybridization washes.
- Streptavidin-Alkaline Phosphatase incubation.
- NBT-BCIP development.

Automatic analysis of results

NOTES

FACULTATIVE: Default Doctor, doctor **Validated:** 21/05/2020

Performed by: Default Tech, tech **Processed:** 21/05/2020

Instr. : Mock **Serial Nº:** Mock **hybriSoft:** HSHS 2.2.0.R07 (HS24) / HSHS IPL 1.0.1.R0000

Respiratory Flow Chip Kit

LOTS

PCR:	RES008L	30/09/2022
Chips:	RES004.2HS	30/10/2022
Reagent:	H079LF	30/08/2021

SAMPLE DETAILS

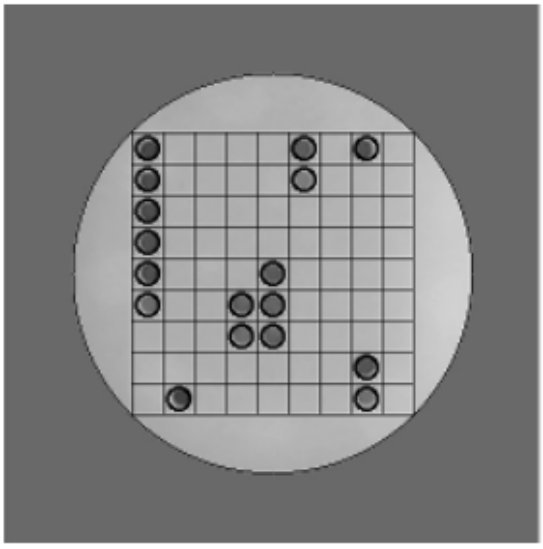
ID SAMPLE: Sample-01 **SAMPLE TYPE:**

ID PATIENT: **PATIENT:**

SEX: - **BIRTHDATE:** **AGE:**

REPORT

B	FluA	PIV-1	CoV-OC43		RNaseP	RSV-A	B	
B	FluA-H1N1	PIV-2	BP		BG	RSV-B	CoV-229E	
CI-1	FluA-H3	PIV-3	BPP			RhV	CoV-HKU1	
CI-2	FluB	PIV-4	MP			PIV-1	CoV-NL63	
RNaseP	MPV	AdV	EV	B	FluA	PIV-2	BPP	
BG	RSV-A	BoV	CoV-2	CI-1	FluA-H1N1	PIV-3	MP	
	RSV-B	CoV-229E	SARS	CI-2	FluA-H3	PIV-4	EV	
	RhV	CoV-HKU1		CoV-OC43	FluB	AdV	CoV-2	
	B	CoV-NL63		BP	MPV	BoV	SARS	



- Spot"B": Hybridization control (5 signals to orientate the CHIP)
- Spot "CI-1": Amplification control for reaction mixture Mix-1. - Spot "CI-2": Amplification control for reaction mixture Mix-2.
- Spot "RNaseP": DNA Control for reaction mixture Mix-1. - Spot "BG": DNA Control for reaction mixture Mix-2.
- Spot "#":Pathogen specific probes

All the spots are printed in duplicate.

ANALYSIS INFORMATION

Threshold: 4

FACULTATIVE: Default Doctor, doctor **Validated:** 21/05/2020

Performed by: Default Tech, tech **Processed:** 21/05/2020

Instr. : Mock **Serial Nº:** Mock **hybriSoft:** HSHS 2.2.0.R07 (HS24) / HSHS IPL 1.0.1.R0000

Figure 2. Example of a report obtained for an only positive case for Coronavirus Type 229E (CoV-229E).

12. PERFORMANCE CHARACTERISTICS

12.1. Analytical functioning on a manual platform

12.1.1. Repeatability

The repeatability of the method was analyzed by testing the method 6 times for each pathogen included in the panel, using a known concentration of genomic material of the different viruses/bacteria from the company Vircell (Influenza A, subtype H1N1 (pandemic 2009), Influenza A, subtype H3, Respiratory Syncytial Virus Subtype A, Respiratory Syncytial Virus Subtype B, Rhinovirus, Parainfluenza Type 1, Parainfluenza Type 2, Parainfluenza Type 3, Parainfluenza Type 4, Coronavirus 229E and *Mycoplasma pneumoniae*), except for those whose material is not available, for which synthetic DNA/RNA of the target sequence is used (Influenza A, Influenza B, Metapneumovirus, Adenovirus, Bocavirus, Enterovirus, Coronavirus HKU-1, Coronavirus NL63, Coronavirus OC43, Coronavirus SARS-CoV-2, *Bordetella pertussis* and *Bordetella parapertussis*). The test was performed by the same operator, in a single location and using the same reagent lot and the same hybriSpot equipment. The results obtained were analyzed with *hybriSoft HSHS v.2.2.0.R04*. For Enterovirus and SARS-CoV-2, the results obtained were analyzed with *hybriSoft v.2.2.0.R05.*

TARGET	PROBES	No. COPIES / REACTION	POSITIVE / TESTED	% POSITIVE
Influenza A*	**FluA**	100	6/6	100%
Influenza A, subtype H1N1 (pandemic 2009)*	**FluA-H1N1**	10	6/6	100%
Influenza A, subtype H3	**FluA-H3**	100	6/6	100%
Influenza B	**FluB**	500	6/6	100%
Metapneumovirus	**MPV**	500	6/6	100%
Respiratory Syncytial Virus Subtype A	**RSV-A**	100	6/6	100%
Respiratory Syncytial Virus Subtype B	**RSV-B**	100	6/6	100%
Rhinovirus	**RhV**	100	6/6	100%
Enterovirus**	**EV**	100	6/6	100%
Parainfluenza Type 1	**PIV-1**	100	6/6	100%
Parainfluenza Type 2	**PIV-2**	100	6/6	100%
Parainfluenza Type 3	**PIV-3**	100	6/6	100%
Parainfluenza Type 4	**PIV-4**	250	6/6	100%
Adenovirus	**AdV**	100	6/6	100%
Bocavirus	**BoV**	100	6/6	100%
Coronavirus 229E	**CoV-229E**	250	6/6	100%
Coronavirus HKU-1	**CoV-HKU1**	100	6/6	100%
Coronavirus NL63	**CoV-NL63**	500	6/6	100%
Coronavirus OC43	**CoV-OC43**	100	6/6	100%
SARS-CoV-2	**CoV-2**	100	6/6	100%
	SARS	100	6/6	100%
Bordetella pertussis	**BP**	250	6/6	100%

Bordetella parapertussis	**BPP**	50	6/6	100%
Mycoplasma pneumoniae	**MP**	100	6/6	100%

Table 8. Repeatability test for each of the pathogens included in the panel *Respiratory Flow Chip* kit. *The target Influenza A contains two different probes, each of them with different sensitivity. ** The target Enterovirus contains three different probes, each of them with different sensitivity.

12.1.2. Reproducibility

The reproducibility of the method was analyzed by simulating the inter-laboratory variability, changing the operator, the equipment used in the process and the lots of PCR mix. 47 samples of RNA from strains of influenza virus (*Threshold cycle* between 18 and 40 obtained with Simplexa™ Flu A/B & RSV Kit, Focus Diagnostics) and 24 negative samples were tested. After the incorporation of SARS-CoV-2 in the detection panel of the kit, a total of 21 clinical samples of nasopharyngeal origin were tested for inter-laboratory reproducibility under the previously described conditions, of which 15 were positive for SARS-CoV-2 and 6 were negative. The concordance was calculated, obtaining a kappa index of 0.908, a standard error of 0.052 and a 95% CI of 0.807-1.010, demonstrating a statistical significance of the reproducibility tests with *Respiratory Flow Chip* kit.

12.1.3. Analytical specificity

Experiments to determine potential cases of cross non-specificity between members of the panel were performed using a specific number of copies of each of the synthetic oligos ($1x10^6$ copies) representing each pathogen, with no cross non-specificity observed between members of the panel:

ORGANISM	SPECIFICITY
Influenza A	100%
Influenza A, subtype H1N1 (pandemic 2009)*	100%
Influenza A, subtype H3	100%
Influenza B	100%
Metapneumovirus	100%
Respiratory Syncytial Virus Subtype A	100%
Respiratory Syncytial Virus Subtype B	100%
Rhinovirus	100%
Enterovirus	100%
Parainfluenza Type 1	100%
Parainfluenza Type 2	100%
Parainfluenza Type 3	100%
Parainfluenza Type 4	100%
Adenovirus	100%
Bocavirus	100%
Coronavirus 229E	100%
Coronavirus HKU-1	100%
Coronavirus NL63	100%
Coronavirus OC43	100%
SARS-CoV-2	100%
Bordetella pertussis	100%

Bordetella parapertussis	100%
Mycoplasma pneumoniae	100%

Table 9. *Respiratory Flow Chip* kit intra-panel specificity.

Non-specificity with other viruses, bacteria or fungi that might be related phylogenetically with the members of the panel or present in the characteristic flora of the respiratory tract was not observed:

TESTED MICROORGANISMS ($1x10^4$ TOTAL COPIES)		
Bacteria	Virus	Fungi
Staphylococcus aureus	CMV	*Candida albicans*
Haemophilus influenzae	VHS-1	
Serratia marscescens	MUV	
M. tuberculosis	VHS-2	
N. gonorrhoeae	VEB	
C. trachomatis	MEV	
M. hominis		
S. pneumoniae		
S. agalactiae		
N. meningitidis		
M. genitalium		
B. burgdorferi		
C. neoformans		
L. monocytogenes		
E. coli		

Table 10. List of pathogens included in the "inter-panel" Specificity tests analyzed with the *Respiratory Flow Chip* kit.

12.1.4. Analytical sensitivity

In order to analytically verify the correct performance of the system designed, we worked with genomic material of the different viruses/bacteria from the company Vircell (Influenza A, subtype H1N1 (pandemic 2009), and subtype H3, Respiratory Syncytial Virus Subtype A, Respiratory Syncytial Virus Subtype B, Rhinovirus, Parainfluenza Type 1, Parainfluenza Type 2, Parainfluenza Type 3, Parainfluenza Type 4, Coronavirus 229E and *Mycoplasma pneumoniae*), was used, except for those whose material is not available, for which synthetic DNA/RNA that mimes the different target regions of the pathogens included in the target sequence panel (Influenza A, Influenza B, Metapneumovirus, Adenovirus, Bocavirus, Enterovirus, Coronavirus HKU-1, Coronavirus NL63, Coronavirus OC43, Coronavirus SARS-CoV-2, *Bordetella pertussis* and *Bordetella parapertussis*). The kit's limit of detection (LoD) was calculated for each one of the analyzed genes. The determination of the minimum number of copies detected was performed through serial dilutions of gene material or, failing this, the synthetic DNA/RNA of each one of the pathogens included in the panel with 20 ng of human genomic DNA. In order to calculate sensitivity, each case was repeated 12 times. All PCRs were hybridized by using the manual platform. The results were analyzed with *hybriSoft v.2.2.0.R02* and v.2.2.R05, and the value established for a positive signal was 4 (gray intensity).

ORGANISM	PROBE	No. COPIES/ REACTION	POSITIVE/ TESTED	SENSITIVITY	95% CONFIDENCE INTERVAL	SPECIFICITY	95% CONFIDENCE INTERVAL
Influenza A	**FluA**	100	12/12	100%	80.5%-100%	99%	97.8%-99.7%
	FluA	50	2/3	67%	20.8%-93.9%	99%	97.8%-99.7%
Influenza A, subtype H1N1 (pandemic 2009)*	**FluA-H1N1**	100	3/3	100%	43.8%-100%	100%	99.2%-100%
	FluA-H1N1	10	10/10	100%	77.2%-100%	100%	99.2%-100%
Influenza A, subtype H3	**FluA-H3**	250	10/10	100%	77.2%-100%	100%	98.5%-100%
	FluA-H3	100	17/21	81%	60%-92.3%	100%	98.5%-100%
Influenza B	**FluB**	500	12/12	100%	69%-100%	100%	98.5%-100%
	FluB	250	2/6	33.3%	9.7%-70%	100%	98.5%-100%
Metapneumovirus	**MPV**	500	10/10	100%	61%-100%	100%	98.5%-100%
	MPV	250	3/6	50%	18.8%-81.2%	100%	98.5%-100%
Respiratory Syncytial Virus Subtype A	**RSV-A**	100	10/10	100%	61%-100%	100%	98.5%-100%
	RSV-A	50	2/6	33.3%	9.7%-70%	100%	98.5%-100%
Respiratory Syncytial Virus Subtype B	**RSV-B**	50	8/8	100%	61%-100%	100%	98.5%-100%
	RSV-B	10	0/3	0%	0%-69%	100%	98.5%-100%
Rhinovirus	**RhV**	100	10/10	100%	77.2%-100%	100%	98.5%-100%
	RhV	50	11/13	66.7%	30%-90.3%	100%	98.5%-100%
Enterovirus	**EV-A**	50	9/9	100%	62.8%-100%	100%	98.5%-100%
		10	6/9	66.6%	31%-91%	100%	98.5%-100%
	EV-B	1000	9/9	100%	62.8%-100%	100%	98.5%-100%
		500	3/9	33.3%	9.04%-70%	100%	98.5%-100%
	EV-D	10	9/9	100%	62.8%-100%	100%	98.5%-100%
Parainfluenza Type 1	**PIV-1**	100	13/13	100%	61%-100%	100%	98.5%-100%
	PIV-1	50	0/6	0%	0%-48%	100%	98.5%-100%
Parainfluenza Type 2	**PIV-2**	50	10/10	100%	77.2%-100%	100%	98.5%-100%
	PIV-2	10	2/3	67%	20.8%-93.9%	100%	98.5%-100%
Parainfluenza Type 3	**PIV-3**	250	13/13	100%	61%-100%	100%	98.5%-100%
	PIV-3	100	11/15	73.3%	48%-89.1%	100%	98.5%-100%
Parainfluenza Type 4	**PIV-4**	250	7/7	100%	61%-100%	100%	98.5%-100%
	PIV-4	100	2/3	66.7%	20.8%-93.9%	100%	98.5%-100%
Adenovirus	**AdV**	100	10/10	100%	77.2%-100%	100%	98.5%-100%
	AdV	50	2/6	33.3%	9.7%-70%	100%	98.5%-100%

Bocavirus	**BoV**	100	15/15	100%	61%-100%	99%	97.6%-99.82%
	BoV	50	5/6	83.3%	43.6%-97%	99%	97.6%-99.82%
Coronavirus 229E	**CoV-229E**	250	15/15	100%	61%-100%	100%	98.5%-100%
	CoV-229E	100	3/5	66.7%	20.8%-93.9%	100%	98.5%-100%
Coronavirus HKU-1	**CoV-HKU1**	250	7/7	100%	61%-100%	100%	98.5%-100%
	CoV-HKU1	100	11/15	73.3%	48%-89.1%	100%	98.5%-100%
Coronavirus NL63	**CoV-NL63**	250	10/10	100%	77.2%-100%	100%	98.5%-100%
	CoV-NL63	100	2/6	33.3%	9.7%-70%	100%	98.5%-100%
Coronavirus OC43	**CoV-OC43**	100	10/10	100%	77.2%-100%	100%	98.5%-100%
	CoV-OC43	50	5/6	83.3%	43.6%-97%	100%	98.5%-100%
SARS-CoV-2	**CoV-2**	100	12/12	100%	69%-100%	100%	98.5%-100%
	CoV-2	50	9/12	75%	42.8%-93.3%	100%	98.5%-100%
	SARS	50	12/12	100%	69%-100%	100%	98.5%-100%
	SARS	10	3/12	25%	6.7%-57.1%	100%	98.5%-100%
Bordetella pertussis	**BP**	250	10/10	100%	77.2%-100%	100%	98.5%-100%
	BP	100	0/3	0%	0%-69%	100%	98.5%-100%
Bordetella parapertussis	**BPP**	100	10/10	100%	77.2%-100%	100%	98.5%-100%
	BPP	50	4/6	66.7%	30%-90.3%	100%	98.5%-100%
Mycoplasma pneumoniae	**MP**	100	10/10	100%	77.2%-100%	100%	98.5%-100%
	MP	50	2/3	66.7%	20.8%-93.9%	100%	98.5%-100%

Table 11. Analytical sensitivity and specificity test using different number of copies of genomic material or, failing this, synthetic DNA/RNA corresponding to each of the pathogens included in the panel, establishing a positivity cut-off value of 4.

12.2. Analytical functioning on the automatic platform Hybrispot 24

The performance and robustness of the *Respiratory Flow Chip* kit on the automatic platform was validated by analyzing a number of limit copies of gene material of four pathogens included in the panel. The reproducibility of the results obtained with the automatic platform HS24 was evaluated by comparing the results obtained in the manual platform. Two types of test were performed:

12.2.1. Reproducibility of results in programs for a different number of samples

Replicas of a positive sample containing a number of limit copies of RNA of the Influenza Virus A Type H3 and Parainfluenza Virus Type 2 (100 copies of each pathogen) or synthetic DNA fragments for both the specific and the generic genes of SARS-CoV-2 (100 copies of each synthetic) were made. In order to evaluate the reproducibility of the automatic platform HS24, the replicates were placed in different positions of the reaction chamber in the equipment, and four different protocols were evaluated:

- Protocol for 2 samples (2 replicas)
- Protocol for 12 samples (3 replicas)

- Protocol for 15 samples (4 replicas)
- Protocol for 24 samples (6 replicas)

The results were analyzed automatically with *hybriSoft* v.*2.2.0.R00 – v.2.2.0.R05* and no differences between the different positions of the reaction chamber nor the protocols used were detected.

12.2.2. Reproducibility of results in different hybridization positions in the automatic platform

Eight replicates for three pathogens from the panel were prepared and located in different positions of the two reaction chambers of the equipment, using the protocol for 24 samples. The results were analyzed automatically with *hybriSoft* v.*2.2.0.R00*- v.*2.2.0.R05* (to analyze SARS-CoV-2), showing a high rate of reproducibility for all the pathogens analyzed in different positions.

ORGANISM	No. COPIES/REACTION	POSITIVE/TESTED	DIFFERENCES BETWEEN POSITIONS
Influenza A-H3	100	4/4	No
Adenovirus	100	4/4	No
Mycoplasma pneumoniae	50	4/4	No
Coronavirus SARS-CoV2	100	4/4	No

Table 12. Reproducibility results obtained with the automatic platform and the *Respiratory Flow Chip* kit. The results were analyzed automatically with *hybriSoft* v.*2.2.0.R00 or hybriSoft v.2.2.R05*, establishing a cut-off value of 4.

This validation proves the reproducibility of the results between the positions 1 and 24 of the equipment and the reproducibility of the results with different programs for a different number of samples.

12.3. Analytical functioning in the automatic platform Hybrispot 12 PCR AUTO

The functioning and the robustness of the Respiratory Flow Chip was validated in the automatic equipment HS12a by analyzing limit concentrations of synthetic fragments of all the pathogens included in the panel. This validation also proves the reproducibility of the results with different programs for different number of samples.

12.3.1. Reproducibility of results in programs for a different number of samples

Replicas of a positive sample containing a number of limit copies of gene material of two pathogens included in the panel, RNA of the Influenza A virus type H3 and Parainfluenza Virus Type 2, or synthetic DNA fragments for both the specific and the generic genes of SARS-CoV-2 (100 copies of each synthetic) were made. These replicas were placed in different positions of the reaction chamber of the HS12a system and different protocols were evaluated:

- Protocol for 2 samples (2 replicas)
- Protocol for 12 samples (3 replicas)

The results were automatically analyzed with hybriSoft v.*2.2.0.R00*- v.*2.2.0.R05* (to analyze SARS-CoV-2) and no differences between the different positions of the reaction chamber nor the used protocol were detected.

12.3.2. Verification of sensitivity limit

The performance and the robustness of the Respiratory Flow Chip was validated in the automatic equipment HS12a by analyzing concentrations at limit of detection of synthetic fragments of DNA/RNA and/or gene material of all the pathogens included in the panel.
3 replicas of each positive sample were made. The whole process was performed automatically using two different HS12a platforms, and the results were analyzed with hybriSoft v.*2.2.0.R00*- v.*2.2.0.R05* (to analyze Enterovirus and SARS-CoV-2).

TARGET	No. COPIES / REACTION	POSITIVE/ TESTED
Influenza A	100	3/3
Influenza A, subtype H1N1 (pandemic 2009)	10	3/3
Influenza A, subtype H3	100	3/3
Influenza B	500	3/3
Metapneumovirus	500	3/3
Respiratory Syncytial Virus Subtype A	100	3/3
Respiratory Syncytial Virus Subtype B	100	3/3
Rhinovirus	100	3/3
Enterovirus A	50	3/3
Enterovirus D	10	3/3
Enterovirus B	1000	3/3
Parainfluenza Type 1	100	3/3
Parainfluenza Type 2	100	3/3
Parainfluenza Type 3	100	3/3
Parainfluenza Type 4	250	3/3
Adenovirus	100	3/3
Bocavirus	100	3/3
Coronavirus 229E	250	3/3
Coronavirus HKU-1	100	3/3
Coronavirus NL63	500	3/3
Coronavirus OC43	100	3/3
Coronavirus SARS-Cov2	100	3/3
Bordetella pertussis	250	3/3
Bordetella parapertussis	50	3/3
Mycoplasma pneumoniae	100	3/3

Table 13: Verification of sensitivity limit of Respiratory Flow Chip kit in HS12a. The positivity was analyzed with the hybriSoft software by establishing as a cut-off point a value of 4.

12.4. Clinical performance

The clinical performance of the Respiratory Flow Chip kit was validated from purified gene material with the extraction methods mentioned above in section 7 of this manual. The diagnostic capacity of the *Respiratory Flow Chip* kit was evaluated by testing its diagnostic sensitivity and specificity. These two parameters are defined and calculated as follows:

- 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).

- 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 values (FP), or 100 × TN/ (TN + FP).

12.4.1. Diagnostic Sensitivity and Specificity

The purified DNA/RNA from 386 clinical samples was analyzed retrospectively with the *Respiratory Flow Chip* kit. This study was performed retrospectively and in parallel with the method considered as *GOLD standard*: NxTAG® Respiratory Pathogen panel (Luminex) and VIASURE SARS-CoV-2 Real time PCR (Certest) for SARS-CoV-2 detection. The analysis of discordant results was performed with the kit FTD Respiratory Pathogens 21 (fast-track DIAGNOSTICS) and, failing this, simplex PCR using specific primers of each pathogen.

ORGANISM	TP	TN	FP	FN	DIAGNOSTIC SENSITIVITY	DIAGNOSTIC SPECIFICITY
Influenza A	86	315	1	4	95,6%	99,7%
Influenza Type A, subtype H1N1 (pandemic 2009)	18	386	2	0	100,0%	99,5%
Influenza A, subtype H3	69	330	2	5	93,2%	99,4%
Influenza B	5	400	1	0	100,0%	99,7%
Metapneumovirus	18	381	0	7	72,0%	100,0%
Respiratory Syncytial Virus Subtype A	17	382	0	7	70,8%	100,0%
Respiratory Syncytial Virus Subtype B	28	377	1	0	100,0%	99,7%
Rhinovirus	55	333	5	13	80,9%	98,5%
Enterovirus	6	61	0	0	100,0%	100,0%
Parainfluenza Type 1	0	406	0	0	NT	100,0%
Parainfluenza Type 2	0	404	1	1	NT	99,7%
Parainfluenza Type 3	15	389	0	2	88,2%	100,0%
Parainfluenza Type 4	0	405	0	1	NT	100,0%
Adenovirus	35	364	3	4	89,7%	99,2%

Bocavirus	17	387	2	0	100,0%	99,5%
Coronavirus 229E	13	386	5	2	86,7%	98,7%
Coronavirus HKU-1	4	402	0	0	100,0%	100,0%
Coronavirus NL63	20	384	0	2	90,9%	100,0%
Coronavirus OC43	14	385	3	4	77,8%	99,2%
SARS-CoV-2	17	46	0	0	100,0%	100,0%
Bordetella pertussis	1	403	2	0	NT	99,5%
Bordetella parapertussis	1	403	2	0	NT	99,5%
Mycoplasma pneumoniae	31	373	1	1	96,9%	99,7%

Table 14. Diagnostic sensitivity and specificity results obtained with the *Respiratory Flow Chip* kit from the analysis of purified gene material. NT: not tested due to an insufficient number of samples.

13. LIMITATIONS

Use of unsuitable samples: the method has been validated with purified gene material from bronchoalveolar lavages, nasopharyngeal aspirates and nasopharyngeal / oropharyngeal exudates. The analysis of any other type of sample not indicated in this manual can lead to wrong or inconclusive results due to RT-PCR reaction inhibition by inhibiting chemical agents.

14. TROUBLESHOOTING

Problem	Causes	Solutions
No signal is observed/ there is no hybridization signal	Failure in the hybridization protocol.	Check that all the reagents have been correctly added during the hybridization process.
		Check the correct functioning of hybriSpot 12/12a/24. Repeat the test.
	PCR reagents and/or expired or not stored properly.	Check the expiration date and the storage conditions of the reagents and the Chips. Repeat the test.
	Chip probes destroyed by rests of decontamination reagents (e.g. Bleach) in the wells.	Clean with plenty of distilled water and repeat the experiment.
No signals in the endogenous amplification control.	Insufficient amount of DNA/RNA in the clinical sample or error during the extraction of nucleic acids.	Repeat the PCR by increasing the amount of starting sample. Repeat the test.

Weak hybridization signals.	Failure in the functioning of the thermal cycler (external or HS12a)	Verify the correct functioning of the thermal cycler with control positive samples. In case of error, contact the technical support of the supplier
	PCR reagents and/or expired or stored improperly.	Check the expiration date of all the reagents and the storage conditions. Repeat the test.
	Sample volume used to re-suspend the erroneous lyophilized product.	Repeat the test by using the correct sample volume
	Failure in the hybridization protocol.	Check the correct functioning of hybriSpot HS12/12a/24 and the hybridization protocol. Repeat the test.
	Low quality/quantity of DNA/RNA in the sample.	Repeat the extraction by eluting the RNA/DNA in less volume. Repeat the test

Table 15. Possibles incidents, causes and solutions against the problems that can arise during the analysis.

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16. 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	Σ	Sufficient content for <n> assays
	Material safety data sheet		

17. GLOSSARY

DNA: Deoxyribonucleic acid.
RNA: Ribonucleic acid
Cod UNG: Cod Uracil-DNA Glycosylase.
DNase: Deoxyribonuclease.
dUTP: Deoxyuridine Triphosphate.
FN: False negative results.
FP: False positive results.
HS12: *HybriSpot 12*.
HS24: *HybriSpot 24*.
HS12a: *HybriSpot 12 PCR AUTO*.
NBT-BCIP: Nitroblue Tetrazolium Chloride- 5-Bromo-4-Chloro-3-Indolyl phosphate.
PCR: Polymerase Chain Reaction.
RNase: Ribonuclease.
TN: True negative results.
TP: True positive results.

18. CHANGELOG

Date	Description
2022-01-25	• Inclusion of the section changelog • Inclusion of the explanation of the pictogram of the Safety Sheet • Room temperature is modified in Section 5