HANDBOOK

Premium Dx® ColdPlex Covid & Flu & RSV

Cat. N° PREMDX005 - 100 reactions
Cat. N° PREMDX006 - 400 reactions

Detection of SARS-CoV-2, Influenza A&B and RSV A&B viruses genomes by real-time RT-PCR (qRT-PCR) with Endogenous internal positive control (IPC)

HUMAN

Sample types

- Nasal Swabs

Recommended nucleic acids (NA) extractions

- Magnetic beads extraction (*e.g.* BioSellal – BioExtract® SuperBall® Cat. N° BES384 - BioExtract® Premium Mag Cat. N° BEPM96, BEPM1K, BEPM5K.
- Silica membrane columns extraction (*e.g.* BioSellal – BioExtract® Column Cat. N° BEC050 or BEC250)

For in vitro diagnostic

CE

IVD

ADMINISTRATIVE INFORMATION

Name and address of the producer responsible for placing the product on the market and of the manufacturer:

BioSellal, Bâtiment B, 27 Chemin des Peupliers, 69570 Dardilly, France
Ph.: +33 (0)4 2678 4760 Fax +33 (0)4 7844 1068

Place of manufacture, control, and packaging:

BioSellal, Bâtiment B, 27 Chemin des Peupliers, 69570 Dardilly, France

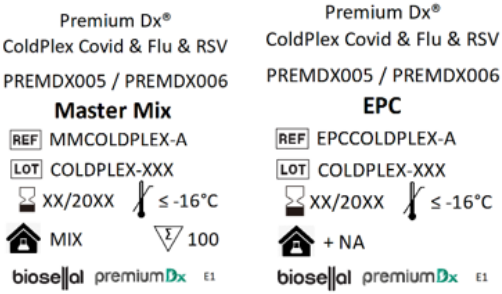
DOCUMENTS MANAGEMENT

The Premium Dx® ColdPlex Covid & Flu & RSV has two technical handbooks:

- The extraction handbook shared between all the kits related to respiratory viruses' detection belonging to the Human line, displaying BioSellal's validated or recommended extraction protocols for each type of sample.
- The Premium Dx® ColdPlex Covid & Flu & RSV qRT-PCR handbook, presenting the instruction information to perform the qRT-PCR.

The last versions in use for each handbook are indicated on the certificate of analysis (CA) provided with the Premium Dx® ColdPlex Covid & Flu & RSV.

Kit labels


Premium Dx®
ColdPlex Covid & Flu & RSV
PREMDX005 / PREMDX006
Master Mix
REF MMCOLDPLEX-A
LOT COLDPLEX-XXX
XX/20XX
≤ -16°C
MIX
100
biosellal premiumDx E1
Premium Dx®
ColdPlex Covid & Flu & RSV
PREMDX005 / PREMDX006
EPC
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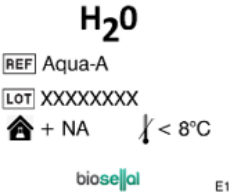
LOT COLDPLEX-XXX
XX/20XX
≤ -16°C
+ NA
biosellal premiumDx E1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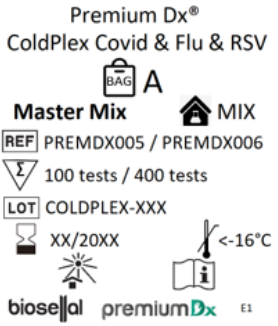
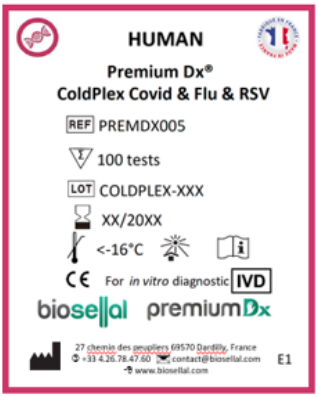
+ NA
< 8°C
biosellal
E1


Premium Dx®
ColdPlex Covid & Flu & RSV
BAG A
Master Mix
MIX
REF PREMDX005 / PREMDX006
100 tests / 400 tests
LOT COLDPLEX-XXX
XX/20XX
<-16°C
biosellal premiumDx E1
Premium Dx®
ColdPlex Covid & Flu & RSV
BAG B
H2O & EPC
+ NA
REF PREMDX005 / PREMDX006
100 tests / 400 tests
LOT COLDPLEX-XXX
XX/20XX
<-16°C
biosellal premiumDx E1


HUMAN
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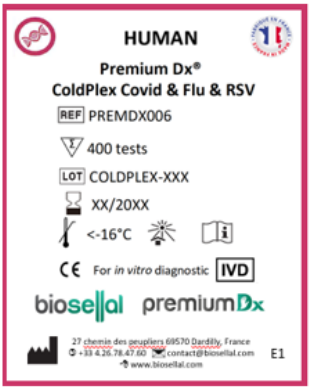
For in vitro diagnostic
IVD
biosellal premiumDx
27 chemin des peupliers 69570 Dardilly, France
+33 4.26.78.47.60 contact@biosellal.com
www.biosellal.com
E1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E1

MODIFICATIONS MANAGEMENT

BioSellal indicates modifications done to this document by highlighting them using the rules presented in the Table below:

MODIFICATIONS MANAGEMENT			
Type of modification Highlighting color	**Minor modifications**	**Type 1 Major modifications**	**Type 2 Major modifications**
Impact on revision / version	**Change of revision date** **No change of version**	**Change of revision date** **+ change of version**	**Change of revision date** **+ change of version**
Examples of modifications	Corrections: typographical, grammatical or turns of phrase	EPC reference modification	Modification of Master Mix composition
	Addition of new sample type for extraction	Exogenous IPC reference modification	Modification of validated extraction protocol
	Addition of information giving more details or alternative protocol		
	Addition/Suppression of optional information		

PRESENTATION

Recommendations for sampling, shipping and storage of samples

Real-time RT-PCR is a powerful technique allowing the detection of few amounts of pathogen genome. Genome can be rapidly degraded depending on the pathogen nature (bacteria / parasites, enveloped viruses...), the genome nature (DNA / RNA) and the sample type (presence of DNase / RNase). Thus, BioSellal recommends the following instructions to guarantee an optimal diagnosis.

Sampling

In accordance with opinion no. 2020.0020/AC/SEAP of March 6th, 2020, from the college of the Haute Autorité de Santé, nasopharyngeal samples should be taken, if necessary, at the home of the patient by an authorised health professional (in particular a doctor, medical biologist, state-certified nurse) wearing the recommended personal protective equipment.
To prevent cross-contamination between samples that could result in false positive result, it is important to use disposable sampling equipment and avoid direct contact between each sampling.

Shipping

Samples must be sent to the medical biology laboratory in triple packaging which identifies the SARS-CoV-2, Influenza or RSV risks and secures transport in accordance with the recommendations of the French Society of Microbiology.
It is mandatory to ship immediately after sampling or by default to store it at ≤ -16°C. Shipment has to be done within 12h under cover of positive cold.

Storage after reception

Analyses must be carried out in a biosafety level 2 laboratory (LSB2). Samples for analysis are processed immediately after receipt and stored at 5°C ± 3 for up to five days (for subsequent re-analysis if necessary), then frozen at ≤-16°C for a few months and at ≤-65°C after one year.

Description of the Premium Dx® ColdPlex Covid & Flu & RSV

The **Premium Dx® ColdPlex Covid & Flu & RSV** (Cat. N° PREMDX005/PREMDX006) contains a ready to use **one-step RT-PCR Master Mix** allowing the detection **in the same reaction well of**:

- ***E and N genes* of SARS-CoV-2 (SARS-CoV-2)** with a 6-FAM labelling,
- ***M gene* of influenza type A and B (Influenza A&B)** with a TEXAS RED labelling,
- **N gene of respiratory syncytial virus (RSV A&B)** with a VIC labelling,
- **An Endogenous internal positive control IPC** (RNASE P), with a Cy5 labelling, to assess the presence of sufficient amount of host cells, sample integrity, nucleic acids extraction quality.

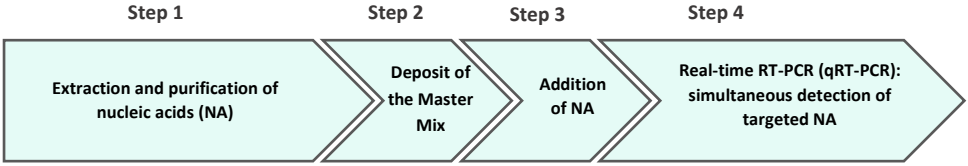
Extraction protocols recommended or validated by BioSellal are described in the extraction handbook shared between all the kits related to respiratory viruses' detection belonging to the Human line.

RISK MANAGEMENT PROCEDURES RELATING TO USE OF THE KIT AND REAGENT DISPOSAL

The implementation of the qRT-PCR protocol associated with the Premium Dx® ColdPlex Covid & Flu & RSV does not pose any hazard to the handler or the environment. However, it is recommended that all contact of reagents with the skin be avoided. In the event of contact with the skin, wash with plenty of water and contact a doctor.

It should be noted that the implementation of the qRT-PCR extraction protocols associated with the Premium Dx® ColdPlex Covid & Flu & RSV generates a chemical and biological hazard to the handler and to the environment. For more information, refer to the extraction instructions for Premium Dx® ColdPlex Covid & Flu & RSV and the safety data sheets of the products used.

Description of the whole process



Extraction handbook for respiratory viruses' detection belonging to the Humain line		qRT-PCR handbook of the Premium Dx® ColdPlex Covid & Flu & RSV		
Nasal swabs	BioExtract® SuperBall® BioExtract® Premium Mag BioExtract® Column	Ready-to-use Master Mix MMCOLDPLEX-A	Samples NC/NCS Process positive control EPC (EPCCOLDPLEX-A)	Dyes: FAM/TEXASRED/VIC/Cy5 Passive reference: None Program: COLDPLEX Standard ramping

Kit contents and storage

Table 1. Description of the kit contents

Description	Reference	Volume/tube		Presentation	Storage
		PREMDX005 100 reactions	PREMDX006 400 reactions		
Master Mix (MM) Ready to use	MMCOLDPLEX-A	1500 µl	4x1500 µl	Transparent cap tube Bag A	≤-16°C Protected from light, « MIX » Area
External Positive Control (EPC) Positive PCR control of SARS-CoV-2, Influenza A&B and RSV A&B	EPCCOLDPLEX-A	200 µl		Red cap tube Bag B	≤-16°C « Addition of Nucleic acids » Area
Water RNase/DNase free	Aqua-A	1 ml		Blue cap tube Bag B	5°C ± 3 or ≤-16°C « Addition of Nucleic acids » Area

Kit reagents are stable until the expiration date stated on the label, subject to compliance with good storage conditions.

List of consumables and reagents not included in kit

Table 2. Consumables and reagents not included in kit

Consumables/ Reagents	Description		Provider	Cat. N°
BioExtract® Column	DNA/RNA column extraction kit (50)		BioSellal	BEC050
BioExtract® Column	DNA/RNA column extraction kit (250)		BioSellal	BEC250
BioExtract® SuperBall®	DNA/RNA Magnetic beads extraction kit (4 x 96)		BioSellal	BES384
BioExtract® Premium Mag	DNA/RNA Magnetic beads extraction kit	(96)	BioSellal	BEPM96
		(1000)		BEPM1K
		(5000)		BEPM5K

For consumables related to the thermal cycler, refer to the user manual of the device.

Main critical points

- Wear appropriate personal protective equipment (lab coat, disposable gloves frequently changed).
- Work in dedicated and separate areas to avoid contamination: "Extraction" (unextracted samples storage, extraction equipment area), "Mix" (ready to use MM storage, qRT-PCR plates preparation), "Nucleic acids Addition" (Nucleic Acids storage and addition of extracted nucleic acids and controls in the qRT-PCR plate), "PCR" (final area containing the thermal cycler(s)).
- Use dedicated equipment for each working area (gloves, lab coat, pipettes, vortex ...).
- Use filter tips.
- Before use, thaw all components at room temperature.
- **One-step RT-PCR Master-Mix is less stable than PCR Master-Mix. To guarantee its optimal performance, it is mandatory to extemporaneously defrost the tubes just before the use, to vortex it, to keep it at 5°C ± 3 during the deposit and to refreeze it immediately afterwards.**
- Vortex and spin briefly (mini centrifuge) all reagents before use.
- Avoid the repetition of freezing-thawing cycles for samples, lysates, extracted nucleic acids.
- **Respiratory Pathogen's genome detected by the Human line's kits are RNA. Working with RNA is more demanding than working with DNA** (RNA instability and omnipresence of the RNases). For these reasons, special precautions must be taken:
    - Always wear gloves, change them frequently, especially after contact with skin or work surfaces.
    - Treat all surfaces and equipment with RNases inactivation agents (available commercially).
    - When wearing gloves and after material decontamination, minimize the contact with surfaces and equipment in order to avoid the reintroduction of RNases.
    - Use "RNase free" consumable.
    - It is recommended to store the RNA at ≤ 5°C ± 3 during the manipulation and then freeze it as soon as possible, preferably at ≤ -65°C or by default at ≤ -16°C.
    - Open and close tubes one by one in order to limit the opening times and avoid any contact with RNases present in the environment (skin, dust, working surfaces…).

DETECTION OF SARS-CoV-2, INFLUENZA A&B AND RSV A&B BY qRT-PCR WITH PREMDX005/PREMDX006

Global Procedure

1) Establish qRT-PCR plate setup defining each sample position and **including the following controls**:

- **Negative Control Sample (NCS)**: water (or PBS) replaces the sample from the first step of sample preparation. This control is mandatory for each extraction series.
- **Negative Amplification Control (NC)**: 5 µl of water RNase/DNase free (Aqua-A tube, **blue** cap) replaces sample Nucleic Acids extract on qRT-PCR plate. This control is recommended when using the kit for the first time or to verify the absence of Master Mix contamination.
- **External Positive Control (EPC)** : Synthetic DNA provided (tube **EPCCOLDPLEX-A**, **red** cap), containing specific target of SARS-CoV-2, Influenza A&B and RSV A&B.
  This control is mandatory.

⚠ CAUTION: *EPC tube handling represents nucleic acids contamination hazard, it is thus recommended to open and handle it in a restricted area, away from other PCR components and to take precautions to avoid cross-contamination with nucleic acids extracts during deposit on the qRT-PCR plate.*

- If available, **a Process Positive Control (MRI),** a weak inactivated positive sample is extracted in parallel with tested samples. After qRT-PCR, MRI Ct value will be monitored on a Shewhart control card. Obtaining conform Ct value validates the whole process. In this case, the use of the EPC, provided with the kit, is not mandatory.

2) qRT-PCR plate preparation

In the "MIX" dedicated area

1. After thawing, vortex and rapid centrifugation, **transfer 15 µl Master Mix MMCOLDPLEX-A** (transparent cap) in each well of interest (samples and controls).
   ⚠ *NOTE: One-step RT-PCR Master-Mix is less stable than PCR Master-Mix. To guarantee its optimal performance, it is mandatory to extemporaneously defrost the tubes just before the use, to vortex it, to keep it at 5°C ± 3 during the deposit and to refreeze it immediately afterwards.*

In the "Nucleic Acids addition" dedicated area

2. **Add 5 µl of extracted nucleic acids (or NCS, water, MRI or EPC**: **EPCCOLDPLEX-A red** cap tube) in each well of interest. Make sure to pipet out in the bottom of the well, in the Master Mix, and to avoid the formation of bubbles.

3. **Seal the plate with an optically clear sealer** or **close the strip caps**.

In the "PCR" amplification dedicated area

4. **Define the thermal cycler parameters** (see Table 3, Table 4,Table 5)

5. It is recommended to **spin the plate down prior to placing it in the thermal cycler**, to prevent drops in the well pit walls.

6. Start the qRT-PCR program. Approximate run time: 80 min.

3) Thermal cycler settings

This kit was developed and validated CFX96 (Bio-Rad) in standard ramping and confirmed on AriaMx™ (Agilent Technologies, Fast ramping by default) and QuantStudio®5 (Applied Biosystems) in standard ramping. It is compatible with all thermal cyclers with at least 6-FAM, VIC, TEXAS RED and Cy5 channels. For more information, contact our technical support.

Table 3. Thermal cycler configuration

	CFX96	AriaMx™	QuantStudio™ 5
Mode	All Channels	Quantitative PCR, Fluorescence Probe	Quantitation – Standard curve
Ramping	Standard Ramping	Ramping Fast by default	Ramping Standard
Passive Reference	**None**	**None**	**None**

Table 4. Thermal cycler Settings			
Target	**Detectors**		**Final Volume / well**
	Reporter	**Quencher**	
SARS-CoV-2	FAM	NFQ-MGB or None*	**20 µl** = 15 µl Master Mix + 5 µl extracted nucleic acids or controls†
Influenza A&B	TEXAS RED	NFQ-MGB or None*	
RSV A&B	VIC	NFQ-MGB or None*	
Endogenous IPC	Cy5	NFQ-MGB or None*	
To assign to samples and controls†			

* Depends on the thermal cycler model. Do not hesitate to contact the BioSellal Technical Support (tech@biosellal.com)
† Controls are NC (water), NCS (extracted water), EPC and or extracted MRI.

Table 5. ColdPlex Amplification program settings		
Standard ramping		
Cycles	**Time**	**Temperature**
1 cycle	10 min	50°C
1 cycle	5 min	95°C
40 cycles	10 sec	95°C
	45 sec + data acquisition	60°C

RESULTS INTERPRETATION

To analyze and interpret the signals obtained by qRT-PCR, the threshold must be set up. The threshold must be assigned carefully in order to obtain the most reproducible result between different manipulations. A consistent set of positives controls, usually an In-house Reference Material (MRI) or the EPC, is used to set the threshold value above the baseline and in the exponential amplification phase of the plot.
The Threshold Cycle, named « Ct » or « Cq » (depending on thermal cyclers), corresponds to the intersection between the amplification curves and the threshold line. It allows the relative measurement of the concentration of the target in the PCR reaction when a calibrated extract is analyzed in the same series.
The qRT-PCR series is validated if the controls (EPC, MRI, NCS and NC) present valid results, then the result of each sample can be interpreted.

Caution:

A negative or not detected result for a given sample does not exclude a SARS-CoV-2, Influenza A or B or RSV A or B infection and should not be used as the only basis patient care decision. A negative result should be associated with clinical observations and findings, patient history and epidemiological data.
A positive result for a tested sample indicated the presence of SARS-CoV-2, Influenza A or B or RSV A or B RNA. However, clinical correlation with the patient history and further diagnostic information are required to determine the infection status of the patient. Furthermore, a positive result does not rule out a bacterial infection or a co-infection with other viruses.

Main Scenarios

Controls Reading

Table 6. PCR Controls results interpretation

	Targets				Interpretation
	SARS-CoV-2 (FAM)	Influenza A&B (TEXAS RED)	RSV A&B (VIC)	Endogenous IPC (Cy5)	
NCS Negative Control Sample **MANDATORY**	Neg	Neg	Neg	Neg	Valid
	At least one of the four targets **Pos**				Contamination with a positive/negative sample during extraction step or during qRT-PCR plate preparation.
NC Negative PCR Control **OPTIONAL**	Neg	Neg	Neg	Neg	Valid
	At least one of the four targets **Pos**				Contamination with a positive/negative sample during qRT-PCR plate preparation or Master Mix/water contamination.
EPC SARS-CoV-2, Influenza A&B and RSV A&B PCR external positive control **MANDATORY** *IN ABSENCE OF MRI*	Pos*	Pos*	Pos*	Neg	Valid
	Neg	Neg	Neg	Neg	Problem during qRT-PCR plate preparation: Master Mix error? EPC omission?
	Pos*	Pos*	Pos*	Pos	Contamination with a sample during qRT-PCR plate preparation?
Sample process positive Control MRI **RECOMMENDED** *IF AVAILABLE*	Positive for target corresponding to the tested sample†			Pos¥	Valid
	Neg	Neg	Neg	Neg	Problem during qRT-PCR plate preparation: Master Mix error? Nucleic acids extract omission or extract not in contact with Master Mix? Process drift: extraction and/or qRT-PCR ? Degradation of the sample process positive control?

* The Ct value obtained must be conform with the value indicated on the Certificate of Analysis (CA). † The Ct value must be included within control card limits. ¥ The obtained Ct value depends on the thermal cycler and the used extraction protocol. IPC Ct values for validated extraction protocols are available upon request. BioSellal recommends you determine to your own maximal IPC Ct value depending on your own extraction method and thermal cycler.

Note:

Endogenous IPC targets a gene expressed by human cells, thus it cannot be detected in NCS, NC and EPC. However, a slight signal can be observed for IPC in the controls, the Ct value of this signal must be lower than 35.

Samples Reading

Table 7. PCR Controls results interpretation

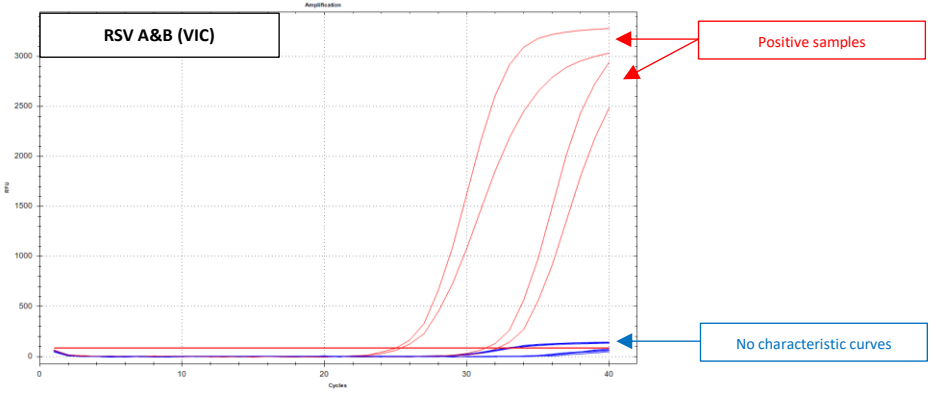
Targets				
SARS-CoV-2 (FAM)	Influenza A&B (TEXAS RED)	RSV A&B (VIC)	Endogenous IPC (Cy5)	Interpretation
Neg	Neg	Neg	Pos*	Negative or not detected
Pos	Neg	Neg		SARS-CoV-2 genome detected
Neg	Pos	Neg		Influenza A and/or B genome detected
Neg	Neg	Pos		RSV A and/or B genome detected
At least 2 positive targets[₮]				Genome detected for the positive targets[₮]
At least 1 positive target			Neg or Ct>35	**Detection of a targeted genome** Absence of sufficient cells Presence of inhibitors[ǂ]? Competition with the target?
Neg	Neg	Neg	Neg or Ct>35	**Uninterpretable = Repeat the analysis** Forgotten to add nucleic acids or not deposited them in contact of the Master Mix when preparing the plate? Presence of inhibitors[ǂ]? Degradation of nucleic acids in the sample? Sampling problem: Insufficient cells Problem during extraction?

* The Ct value obtained depends on the thermal cycler, the matrix analysed, and the extraction methods used. IPC values, obtained from the various matrices with the methods validated by BioSellal, are available on request. BioSellal recommends that the laboratory determine its own maximum tolerated IPC value on the basis of its extraction method and thermal cycler.

ǂ If inhibition is suspected, 1) Repeat the qRT-PCR by prediluting the nucleic acids extracted to one part in 10 (or even 1 part in 100) in free DNase/RNase water or 2) Resume analysis after extraction.

₮ For the RSV A & B valence (VIC), a positive signal may be observed when the SARS-CoV-2 valence (FAM) is found positive. This signal is not specific and may create curves with a very low fluorescence level and of uncharacteristic aspects. BioSellal recommends considering such curves on the VIC canal to be negative when there's a relatively strong FAM signal in the same reaction well.

As an example, this type of curves is presented in the figure on the next page, compared with actual positive samples.



PERFORMANCES OF
PREMIUM DX® ColdPlex Covid & Flu & RSV
Characterisation de la qRT-PCR

1) Verification of analytical specificity

a. Experimental inclusivity

Premium Dx® ColdPlex Covid & Flu & RSV can be used to detect the presence of the SARS-CoV-2 genome, influenza type A & B genomes and RSV type A & B genomes. The analytical specificity of the primer and probe systems was verified on whole inactivated virus strains supplied by the National Reference Center (NRC) for respiratory infection viruses including influenza (Lyon, France) and viral RNA provided by the American Type Culture Collection (ATCC).

Data obtained from the various SARS-CoV-2 strains:

Strains	Origin	Premium Dx® ColdPlex Covid & Flu & RSV		
		SARS-CoV-2	Influenza A/B	RSV A/B
SARS-CoV-2 Clade 20C	NRC	Detected	Not Detected	Not Detected
SARS-CoV-2 lineage 20I.501Y.V1		Detected	Not Detected	Not Detected
SARS-CoV-2 lineage 20H/501Y.V2		Detected	Not Detected	Not Detected
SARS-CoV-2 lineage 20I/484Q		Detected	Not Detected	Not Detected
SARS-CoV-2 lineage 20A.452R B.1.617.2		Detected	Not Detected	Not Detected
SARS-CoV-2 lineage AY.4.2		Detected	Not Detected	Not Detected
SARS-CoV (2003)		Detected	Not Detected	Not Detected

Data obtained from the various Influenza A strains:

Strains	Origin	Premium Dx® ColdPlex Covid & Flu & RSV		
		SARS-CoV-2	Influenza A/B	RSV A/B
Influenza A H3N2 A/Singapore/INIFMIH-16-0019/2016	NRC	Not Detected	Detected	Not Detected
Influenza A H3N2 A/Hong Kong/8/68		Not Detected	Detected	Not Detected
Influenza A H3N2 A/Port Chalmers/1/73		Not Detected	Detected	Not Detected
Influenza A H3N2 A/Panama/2007/1999		Not Detected	Detected	Not Detected
Influenza A H3N2 A/Wisconsin/67/05		Not Detected	Detected	Not Detected
Influenza A H3N2 A/Brisbane/10/2007		Not Detected	Detected	Not Detected
Influenza A H3N2 A/Wisconsin/12/2010		Not Detected	Detected	Not Detected
Influenza A H3N2 A/Indiana/8/2010		Not Detected	Detected	Not Detected
Influenza A H3N2 A/Texas/50/2012		Not Detected	Detected	Not Detected
Influenza A H1N1 A/Brisbane/2/2018		Not Detected	Detected	Not Detected
Influenza A H1N1 A/PR/8/34		Not Detected	Detected	Not Detected
Influenza A H1N1 A/New Jersey/8/76		Not Detected	Detected	Not Detected
Influenza A H1N1 A/New Caledonia/20/1999		Not Detected	Detected	Not Detected
Influenza A H1N1 A/Solomon Island/3/2006		Not Detected	Detected	Not Detected
Influenza A H1N1 A/Brisbane/59/2007		Not Detected	Detected	Not Detected
Influenza A H1N1 A/California/7/2009		Not Detected	Detected	Not Detected
Influenza A H1N1 A/Solomon Island/3/2006		Not Detected	Detected	Not Detected
Influenza A H1N1 A/Brisbane/59/2007		Not Detected	Detected	Not Detected
Influenza A H1N1 A/California/7/2009		Not Detected	Detected	Not Detected
Influenza A virus strain A/HongKong/8/68	ATCC	Not Detected	Detected	Not Detected
Influenza A virus (H3N2) A/Aichi/2/68		Not Detected	Detected	Not Detected
Influenza A virus (H1N1pdm) A/California/07/2009 NTMC X-179A		Not Detected	Detected	Not Detected
Influenza A virus (H3N2) strain A/Virginia/ATCC6/2012		Not Detected	Detected	Not Detected
Influenza A virus (H1N1) strain A/Virginia/ATCC1/2009		Not Detected	Detected	Not Detected

Data obtained from the various Influenza B strains:

Strains	Origin	Premium Dx® ColdPlex Covid & Flu & RSV		
		SARS-CoV-2	Influenza A/B	RSV A/B
Influenza B/Colorado/6/2017	NRC	Not Detected	Detected	Not Detected
Influenza B/Phuket/3073/2013		Not Detected	Detected	Not Detected
Influenza B/Lee/40		Not Detected	Detected	Not Detected
Influenza B/Panama/45/90		Not Detected	Detected	Not Detected
Influenza B/Florida/07/2004		Not Detected	Detected	Not Detected
Influenza B/Hong Kong/5/72		Not Detected	Detected	Not Detected
Influenza B/Hong Kong/5/72		Not Detected	Detected	Not Detected
Influenza B/Wisconsin/01/2010		Not Detected	Detected	Not Detected
Influenza B/Malaysia/2506/04		Not Detected	Detected	Not Detected
Influenza B/Brisbane/60/2008		Not Detected	Detected	Not Detected
Influenza B virus strain B/Taiwan/2/62	ATCC	Not Detected	Detected	Not Detected
Influenza B virus strain B/Lee/40		Not Detected	Detected	Not Detected

Data obtained from the various RSV strains:

Strains	Origin	Premium Dx® ColdPlex Covid & Flu & RSV		
		SARS-CoV-2	Influenza A/B	RSV A/B
RSV A 28-589A P14	NRC	Not Detected	Not Detected	Detected
RSV B 28-591B P10		Not Detected	Not Detected	Detected
RSV B RSVB R18-126-79 P3		Not Detected	Not Detected	Detected
RSV A RSVA R22-106-22 P4		Not Detected	Not Detected	Detected
RSV B RSVB R22-30-76 P3		Not Detected	Not Detected	Detected
Respiratory Syncytial Virus (Subtype B) (MBC083)	ATCC	Not Detected	Not Detected	Detected
Human Respiratory Syncytial Virus long strain (ATCC RSV A VR-26DQ)		Not Detected	Not Detected	Detected
Human Respiratory Syncytial Virus strain 9320 (ATCC RSV B VR-955D)		Not Detected	Not Detected	Detected
Human Respiratory Syncytial Virus B strain 18537 (ATCC RSV VR-1580DQ)		Not Detected	Not Detected	Detected
Human Respiratory Syncytial Virus strain ATCC-2012-11		Not Detected	Not Detected	Detected

b. Experimental exclusivity

Experimental exclusiveness was verified on viruses, bacteria, and parasites present in the same ecological niches, or which leads to pathologies or clinical symptoms similar to those associated with the presence of SARS-CoV-2, influenza A&B or RSV A&B available in the BioSellal sample library.

Data obtained for the various viruses, bacteria and parasites:

Strain	Origin	Premium Dx® ColdPlex Covid & Flu & RSV		
		SARS-CoV-2	Influenza A/B	RSV A/B
Rhinovirus (MBC091)	ATCC	Not Detected	Not Detected	Not Detected
Bordetella holmesii (MBC092)		Not Detected	Not Detected	Not Detected
Bordetella parapertussis (MBC007)		Not Detected	Not Detected	Not Detected
Chlamydophila pneumoniae strain CM-1		Not Detected	Not Detected	Not Detected
Haemophilus influenzae		Not Detected	Not Detected	Not Detected
Legionella pneumophilia (MBC031)		Not Detected	Not Detected	Not Detected
Moraxella catarrhalis (MBC117)		Not Detected	Not Detected	Not Detected
Mycoplasma pneumoniae		Not Detected	Not Detected	Not Detected
Salmonella enterica subsp. Enterica serovar Enteritidis strain MZ1486		Not Detected	Not Detected	Not Detected
Human parainfluenza virus 2 strain Greer		Not Detected	Not Detected	Not Detected
Human parainfluenza virus 3		Not Detected	Not Detected	Not Detected
Human parainfluenza virus 4		Not Detected	Not Detected	Not Detected
Human coronavirus 229E		Not Detected	Not Detected	Not Detected
Human coronavirus NL63		Not Detected	Not Detected	Not Detected
MERS Coronavirus		Not Detected	Not Detected	Not Detected
Human coronavirus HKU1		Not Detected	Not Detected	Not Detected
Betacoronavirus 1 strain OC43		Not Detected	Not Detected	Not Detected
Human enterovirus 71 strain BrCr		Not Detected	Not Detected	Not Detected
Enterovirus 68 strain Fermon		Not Detected	Not Detected	Not Detected
Human adenovirus 1 strain Adenoid 71		Not Detected	Not Detected	Not Detected
Human adenovirus 2 strain Adenoid 6		Not Detected	Not Detected	Not Detected
Human adenovirus 3 strain G.B.		Not Detected	Not Detected	Not Detected
Human adenovirus 4 strain RI-67		Not Detected	Not Detected	Not Detected
Human adenovirus 5 strain Adenoid 75		Not Detected	Not Detected	Not Detected
Human adenovirus 6 strain Tonsil99		Not Detected	Not Detected	Not Detected
Human adenovirus 7 strain Gomen		Not Detected	Not Detected	Not Detected

⇨ **The analytical specificity of Premium Dx® ColdPlex Covid & Flu & RSV was confirmed *in silico* and in experiments on all sequences present in the databases and on all isolates tested.**

2) Determination of analytical sensitivity: $LD_{RT\text{-}PCR}$

The limit of detection of qRT-PCR ($LD_{RT\text{-}PCR}$) corresponds to the minimum number of copies of target nucleic acids detected by the system in 95% of cases.

It was determined experimentally for each target gene using the RNAs transcribed from the *E gene* and *N gene* of the SARS-CoV-2, the *M genes* of influenza A & B and the *N genes* of RSV A & B, quantified by fluorimetry as a number of copies of the target sequence by qRT-PCR (number of copies in 5 µl).

A first approach by serial dilutions of 10 by 10 has made it possible to estimate the $LD_{RT\text{-}PCR}$ between 1 and 10 copies of RNA by RT-PCR for the SARS-CoV-2, between 10 and 100 copies for influenza A&B and RSV A&B. Ranges of reason 2 are produced from 50 to 1,6 copy by qRT-PCR for SARS-CoV-2, from 300 to 2.35 copy by qRT-PCR for Influenza A, from 150 to 4.99 copy by qRT-PCR for Influenza B and RSV B and from 123 to 3.84 copy by qRT-PCR for RSV A in order to frame the estimated value of the $LD_{RT\text{-}PCR}$ over six to eight dilutions.

Experimental design:

Number of dilutions	Number of replicas per dilution and per series	Number of independent series
6 - 8	**8**	**3**

Data obtained for the target SARS-CoV-2:

Number of copies /RT-PCR*	Number of replicas detected			Number of replicas detected	Frequency of detection
	Série 1	Série 2	Série 3		
50	8/8	8/8	8/8	24/24	100 %
25	8/8	8/8	8/8	24/24	100 %
12.5	8/8	8/8	8/8	24/24	100 %
6.25	**8/8**	**8/8**	**8/8**	**24/24**	**100 %**
3.13	6/8	6/8	8/8	20/24	83%
1.6	4/8	3/8	6/8	13/24	54%

⇨ **The experimental approach indicates that the 95% $LD_{RT\text{-}PCR}$ for the SARS-CoV-2 valence (the last dilution giving a minimum of 23 positive results out of 24) is 6.25 copies/RT-PCR.**

Data obtained for the target influenza type A:

Number of copies /RT-PCR*	Number of replicas detected			Number of replicas detected	Frequency of detection
	Série 1	Série 2	Série 3		
300	8/8	8/8	8/8	24/24	100%
150	8/8	8/8	8/8	24/24	100%
75	**8/8**	**8/8**	**8/8**	**24/24**	**100%**
37.5	7/8	6/8	8/8	21/24	88%
18.75	7/8	6/8	7/8	20/24	83%
9.38	2/8	3/8	6/8	11/24	46%
4.69	4/8	2/8	5/8	11/24	46%
2.35	0/8	0/8	3/8	3/24	13%

⇨ **The experimental approach indicates that the 95% $LD_{RT\text{-}PCR}$ for the influenza type A valence (the last dilution giving a minimum of 23 positive results out of 24) is 75 copies/RT-PCR.**

Data obtained for the target influenza type B:

Number of copies /RT-PCR*	Number of replicas detected			Number of replicas detected	Frequency of detection
	Série 1	Série 2	Série 3		
150	8/8	8/8	8/8	24/24	100%
75	8/8	8/8	8/8	24/24	100%
37.5	8/8	8/8	8/8	24/24	100%
18.75	**8/8**	**8/8**	**8/8**	**24/24**	**100%**
9.38	5/8	7/8	8/8	20/24	83%
4.99	5/8	5/8	5/8	15/24	63%

⇨ **The experimental approach indicates that the 95% LD_{RT-PCR} for the influenza type B valence (the last dilution giving a minimum of 23 positive results out of 24) is 18.75 copies/RT-PCR.**

Data obtained for the target RSV type A:

Number of copies /RT-PCR*	Number of replicas detected			Number of replicas detected	Frequency of detection
	Série 1	Série 2	Série 3		
150	8/8	8/8	8/8	24/24	100%
75	8/8	8/8	8/8	24/24	100%
37.5	**7/8**	**8/8**	**8/8**	**23/24**	**96%**
18.75	7/8	8/8	6/8	21/24	88%
9.38	4/8	6/8	6/8	16/24	67%
4.99	1/8	1/8	2/8	4/24	17%

⇨ **The experimental approach indicates that the 95% LD_{RT-PCR} for the RSV type A valence (the last dilution giving a minimum of 23 positive results out of 24) is 37.50 copies/RT-PCR.**

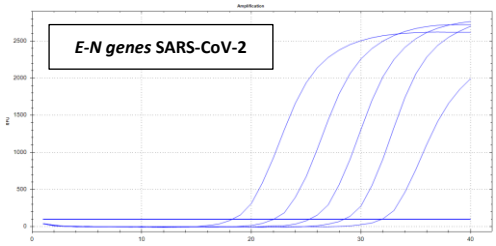
Data obtained for the target RSV type B:

Number of copies /RT-PCR*	Number of replicas detected			Number of replicas detected	Frequency of detection
	Série 1	Série 2	Série 3		
123	8/8	8/8	8/8	24/24	100%
61.5	8/8	8/8	8/8	24/24	100%
30.75	8/8	8/8	8/8	24/24	100%
15.38	8/8	8/8	8/8	24/24	100%
7.69	**8/8**	**8/8**	**7/8**	**23/24**	**96%**
3.84	8/8	6/8	7/8	21/24	88%

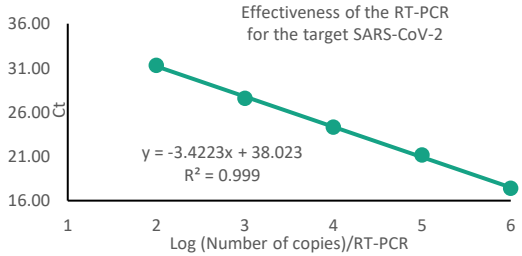
⇨ **The experimental approach indicates that the 95% LD_{RT-PCR} for RSV type B valence (the last dilution giving a minimum of 23 positive results out of 24) is 7.69 copies/RT-PCR.**

3) Efficiency

The efficiencies of each qRT-PCR for the different targets were determined from 10 by 10 serial dilutions of the transcribed RNAs quantified by fluorimetry as a number of copies of the target sequence by qRT-PCR (copies in 5 µl).

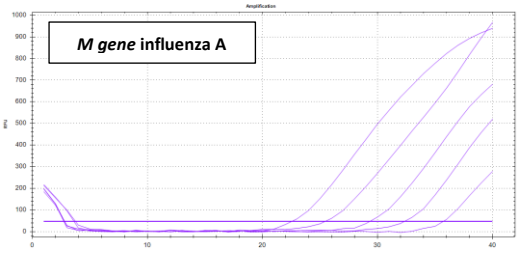


Copies/RT-PCR	Ct
1 x 10 ^{6}	17.38
1 x 10 ^{5}	21.14
1 x 10 ^{4}	24.31
1 x 10 ^{3}	27.55
1 x 10 ^{2}	31.29

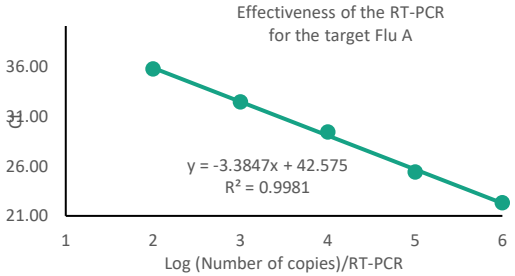


The effectiveness deduced from the slope of the line is: $E = (10^{-1/gradient} - 1) \times 100 = 96\%$

⇨ **The effectiveness of Premium Dx® ColdPlex Covid & Flu & RSV is 96% for the valence SARS-CoV-2.**

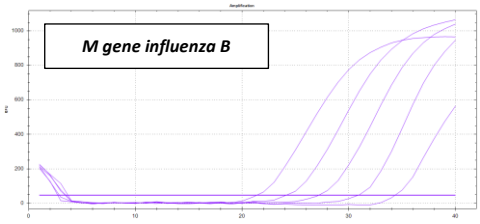


Copies/RT-PCR	Ct
1 x 10 ^{6}	22.30
1 x 10 ^{5}	25.38
1 x 10 ^{4}	29.40
1 x 10 ^{3}	32.39
1 x 10 ^{2}	35.72

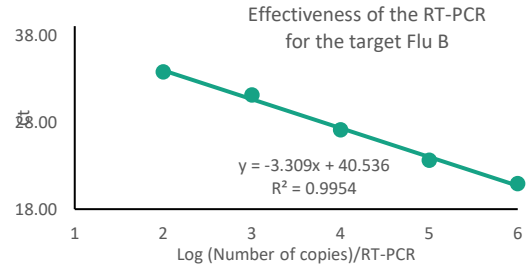


The effectiveness deduced from the slope of the line is: E = ($10^{-1/gradient}$ -1) x 100 = 97.4%

⇨ **The effectiveness of Premium Dx® ColdPlex Covid & Flu & RSV is 97.4% for the valence influenza A.**

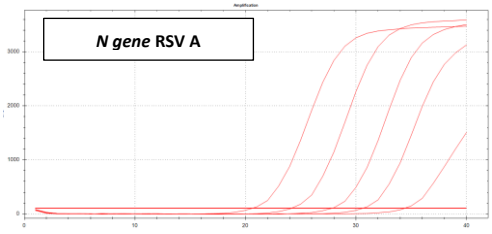


Copies/RT-PCR	Ct
1 x 10 ^{6}	20.93
1 x 10 ^{5}	23.63
1 x 10 ^{4}	27.10
1 x 10 ^{3}	31.10
1 x 10 ^{2}	33.74

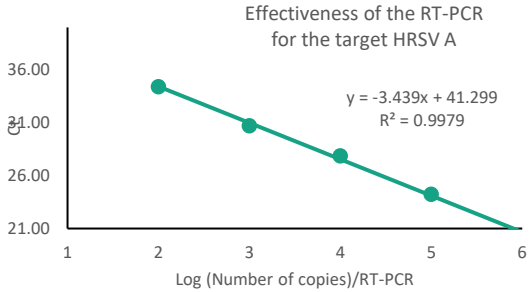


The effectiveness deduced from the slope of the line is: E = ($10^{-1/gradient}$ -1) x 100 = 100.5%

⇨ **The effectiveness of Premium Dx® ColdPlex Covid & Flu & RSV is 100.5% for the valence influenza B.**

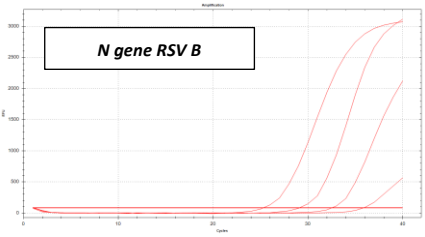


Copies/RT-PCR	Ct
1 x 10 ^{6}	20.46
1 x 10 ^{5}	24.23
1 x 10 ^{4}	27.89
1 x 10 ^{3}	30.73
1 x 10 ^{2}	34.40

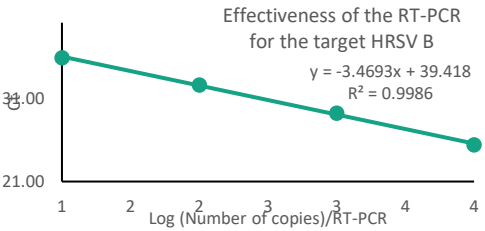


The effectiveness deduced from the slope of the line is: E = ($10^{-1/gradient}$ -1) x 100 = 95.3%

⇨ **The effectiveness of Premium Dx® ColdPlex Covid & Flu & RSV is 99% for the valence RSV A.**



Copies/RT-PCR	Ct
1 x 10 ^{4}	25.38
1 x 10 ^{3}	29.20
1 x 10 ^{2}	32.58
1 x 10 ^{1}	35.82



The effectiveness deduced from the slope of the line is: E = ($10^{-1/gradient}$ -1) x 100 = 94.2%

⇨ **The effectiveness of Premium Dx® ColdPlex Covid & Flu & RSV is 94.2% for the valence RSV B.**

4) Repeatability of the RT-PCR

The repeatability of the qRT-PCR was determined using dilutions of the transcribed RNAs of the targets in order to obtain three levels of positivity. An independent series of qRT-PCR was carried out by depositing the three dilutions in duplicate following the Premium Dx® ColdPlex Covid & Flu & RSV protocol described on page 11 (ColdPlex program). The repeatability coefficient of variation (CV) of the Ct values was then determined by dividing the standard deviations by the mean according to the formula:

$$CV_{repeatability} = \frac{Sr}{M} * 100$$

Where Sr corresponds to the standard deviations of repeatability, and M to the general mean of the values of the series.

Data obtained for the target SARS-CoV-2:

	Repeatability (Ct)			Repeatability	
				Mean (Ct)	CV%
Strong positive	20.72	20.78	20.90	20.80	**0.44**
Average positive	27.40	27.38	27.42	27.40	**0.07**
Weak positive	30.52	31.09	30.60	30.74	**1.00**

⇨ **The repeatability variation coefficient according to level varies from 0.07% to 1.00% for the SARS-CoV-2 valence.**

Data obtained for the target influenza type A:

	Repeatability (Ct)			Repeatability	
				Mean (Ct)	CV%
Strong positive	20.63	20.62	20.87	20.71	**0.68**
Average positive	28.02	28.14	28.02	28.06	**0.25**
Weak positive	30.78	30.95	31.32	31.02	**0.89**

⇨ **The repeatability variation coefficient according to level varies from 0.25% to 0.89% for the influenza type A valence.**

Data obtained for the target influenza type B:

	Repeatability (Ct)			Repeatability	
				Mean (Ct)	CV%
Strong positive	22.46	21.73	21.85	22.01	**1.78**
Average positive	28.86	29.25	28.55	28.89	**1.21**
Weak positive	31.22	31.95	31.41	31.53	**1.20**

⇨ **The repeatability variation coefficient according to level varies from 1.20% to 1.78% for the influenza type B valence.**

Data obtained for the target RSV type A:

	Repeatability (Ct)			Repeatability	
				Mean (Ct)	CV%
Strong positive	21.00	20.97	21.39	21.12	**1.11**
Average positive	27.54	27.52	27.54	27.53	**0.04**
Weak positive	30.97	31.12	30.80	30.96	**0.52**

⇨ **The repeatability variation coefficient according to level varies from 0.04% to 1.11% for the RSV type A valence.**

Data obtained for the target RSV type B:

	Repeatability (Ct)			Repeatability	
				Mean (Ct)	CV%
Strong positive	20.20	20.22	20.19	20.20	**0.08**
Average positive	27.20	27.14	27.11	27.15	**0.17**
Weak positive	30.30	30.74	30.58	30.54	**0.73**

⇨ **The repeatability variation coefficient according to level varies from 0.08% to 0.73% for the RSV type B valence.**

5) Intermediate fidelity of the RT-PCR

The intermediate precision of the qRT-PCR was determined using the transcribed RNAs of the targets in order to obtain three levels of positivity. Three independent series were carried out on two thermal cyclers, using two manipulators, by depositing the three dilutions in triplicate for each series as per the protocol of Premium Dx® ColdPlex Covid & Flu & RSV described on page 11 (ColdPlex program). At the end of these three series, the intermediate coefficient of variation (CV) of the Ct values can be determined, provided that the threshold is positioned according to common criteria. The calculation formula used is:

$$CV_{\text{intermediate precision}} = \frac{Sr}{M} * 100$$

Where Sr corresponds to the standard deviations of intermediate precision, and M to the general mean of the values of the three series.

Data obtained for the target SARS-CoV-2:

	Intermediate precision (Ct)			CV%
	Ct series 1	Ct series 2	Ct series 3	Intermediate precision
Strong positive	20.72	21.65	20.86	**2.38**
Average positive	27.4	28.02	27.66	**1.12**
Weak positive	30.52	31.16	32.08	**2.51**

⇨ **The intermediate precision variation coefficient according to level varies from 1.12% to 2.51% for the SARS-CoV-2 valence.**

Data obtained for the target influenza type A:

	Intermediate precision (Ct)			CV% Intermediate precision
	Ct series 1	Ct series 1	Ct series 1	
Strong positive	20.63	21.21	20.56	**1.72**
Average positive	28.02	28.36	27.75	**1.09**
Weak positive	30.78	31.28	30.40	**1.43**

⇨ **The repeatability variation coefficient according to level varies from 1.09% to 1.72% for the influenza type A valence.**

Data obtained for the target influenza type B:

	Intermediate precision (Ct)			CV% Intermediate precision
	Ct series 1	Ct series 1	Ct series 1	
Strong positive	22.46	22.73	22.07	**1.48**
Average positive	28.86	29.26	28.01	**2.22**
Weak positive	31.22	32.50	31.01	**2.55**

⇨ **The repeatability variation coefficient according to level varies from 1.48% to 2.55% for the influenza type B valence.**

Data obtained for the target RSV type A:

	Intermediate precision (Ct)			CV% Intermediate precision
	Ct series 1	Ct series 1	Ct series 1	
Strong positive	21.00	21.48	20.30	**2.84**
Average positive	27.54	28.19	26.69	**2.74**
Weak positive	30.97	31.06	30.22	**1.50**

⇨ **The repeatability variation coefficient according to level varies from 1.50% to 2.84% for the RSV type A valence.**

Data obtained for the target RSV type B:

	Intermediate precision (Ct)			CV% Intermediate precision
	Ct series 1	Ct series 1	Ct series 1	
Strong positive	20.20	21.02	20.15	**2.39**
Average positive	27.20	28.07	26.27	**3.31**
Weak positive	30.30	31.91	29.65	**3.80**

⇨ **The repeatability variation coefficient according to level varies from 2.39% to 3.80% for the RSV type B valence.**

6) Confirmation of qRT-PCR performance on other thermal cyclers

We have confirmed the characteristics of Premium Dx® ColdPlex Covid & Flu & RSV on three thermal cyclers:

- QuantStudio™ 5 (Applied Biosystems, ramping standard)
- AriaMx™ (Agilent Technologies, ramping Fast)
- CFX96 (Bio-Rad, ramping standard)

These three thermal cyclers were chosen because they represent three brands that are particularly present in analysis laboratories equipped with so-called open systems. They have a different distribution of Peltier blocks, as well as different fluorescence reading methods (CFX96, 1 Peltier block, AriaMx™ and QuantStudio™ 5, 6 Peltier blocks, reading per line). Premium Dx® ColdPlex Covid & Flu & RSV can be used on any other thermal cycler that, as a minimum, contains the 6-FAM, VIC, TEXAS RED and Cy5 playback channels. In all cases, BioSellal recommends the user laboratory verify the performance of the test on their devices by verifying the detectability of a level equivalent to $3xLD_{RT\text{-}PCR}$ in order to qualify their real-time thermal cycler, not only on the thermal part (coverage of the set of Peltier blocks which operate independently) but also on the optical part. For more information, contact BioSellal.

For this test, the amplification program used is the one described on page 11 (ColdPlex program). To this end, the RNAs of each target were diluted to reach an amount corresponding to 3x the $LD_{RT\text{-}PCR}$ per reaction, *i.e.* 3 x $LD_{RT\text{-}PCR}$ in 5 µl. After a prior deposit of 15 µl of Master Mix, this quantity is deposited in at least three replicates and at least one replica per Peltier block. The wells used for these performance confirmations are those that correspond to the positions of the thermal block verified during the metrological connection of the temperatures.

Performance criteria to be met for the adoption of the qRT-PCR:

Stage	Experimental design				Expected results
	Number of levels of dilutions	Number of replicas	Number of series / thermal cycler	Number of thermal cyclers	
Limit of detection $LD_{RT\text{-}PCR}$	1 =3 x $LD_{RT\text{-}PCR}$ *i.e.* 20 copies/RT-PCR for the target **SARS-CoV-2** 1 =3 x $LD_{RT\text{-}PCR}$ *i.e.* 225 copies/RT-PCR for the target **influenza type A** 1 =3 x $LD_{RT\text{-}PCR}$ *i.e.* 60 copies/RT-PCR for the target **influenza type B** 1 =3 x $LD_{RT\text{-}PCR}$ *i.e.* 115 copies/RT-PCR for the target **RSV type A** 1 =3 x $LD_{RT\text{-}PCR}$ *i.e.* 25 copies/RT-PCR for the target **RSV type B**	4 for the CFX96 6 for the AriaMx™ 6 for the QuantStudio™ 5	1	3	**100% of positive results**

Data obtained: Frequency of detection

Measured criterion	Target	Number of copies / RT-PCR	CFX96 Standard ramping		AriaMx™ (Fast ramping)		QuantStudio 5 Standard ramping	
			Number of replicas detected	Frequency of detection	Number of replicas detected	Frequency of detection	Number of replicas detected	Frequency of detection
Limit of detection $3xLD_{RT-PCR}$	SARS-CoV-2	20	4/4	**100%**	6/6	**100%**	6/6	**100%**
	Influenza type A	225	4/4	**100%**	6/6	**100%**	6/6	**100%**
	Influenza type B	60	4/4	**100%**	6/6	**100%**	6/6	**100%**
	RSV type A	115	4/4	**100%**	6/6	**100%**	6/6	**100%**
	RSV type B	25	4/4	**100%**	6/6	**100%**	6/6	**100%**

⇨ **The values of the 3 x LD_{RT-PCR} (= 20 copies/RT-PCR for SARS-CoV-2; 225 copies/RT-PCR for influenza type A ; 60 copies/RT-PCR for influenza type B ; 115 copies/RT-PCR for RSV type A ; 25 copies/RT-PCR for RSV type B) have been confirmed for the CFX96, AriaMx™ and QuantStudio™ 5 thermal cyclers.**

7) Robustness of the qRT-PCR

The robustness of the qRT-PCR was evaluated by analysing the 3 x LD_{RT-PCR} level on six replicates per series and by varying the critical parameters of the qRT-PCR used compared to the reference conditions described on page 11 (ColdPlex program):

Variation of critical parameters	
Reference conditions	5 µl of nucleic acids 15 µl of Master Mix Hybridization-Elongation of the primers at 63°C for 45 seconds
For a volume of 15 µl of Master Mix, variation of ± 10% of the volume of RNA	4.5 µl and 5.5 µl
For a volume of 15 µl of Master Mix, variation of ± 1°C in the hybridization and elongation temperature of the primers	59°C and 61°C
For a volume of 15 µl of Master Mix, variation of ± 5 sec. in the duration of the hybridization and primer extension stage	40 seconds and 50 seconds

Raw data from robustness tests:

		CFX96 (Bio-Rad)						
		Reference condition	15 µl MM 4.5 µl AN	15 µl MM 5.5 µl AN	15 µl MM 59°C	15 µl MM 61°C	15 µl MM 40 sec.	15 µl MM 50 sec.
SARS-CoV-2	Replica 1	34.46	33.67	33.25	33.44	33.00	33.06	33.67
	Replica 2	33.59	32.89	33.69	34.97	33.85	33.12	32.79
	Replica 3	34.04	33.23	33.78	33.45	34.22	33.56	34.05
	Replica 4	33.58	33.06	33.81	33.31	33.05	33.32	33.93
Influenza type A	Replica 1	35.64	36.02	35.61	34.52	34.43	31.37	34.01
	Replica 2	35.23	33.95	34.97	34.77	32.06	33.30	34.89
	Replica 3	33.91	35.4	35.52	34.98	34.45	34.06	35.44
	Replica 4	35.51	35.86	34.45	34.25	34.45	34.31	34.23
Influenza type B	Replica 1	34.07	34.19	33.82	34.15	33.82	33.45	33.25
	Replica 2	33.63	34.52	34.14	34.07	35.00	33.71	34.18
	Replica 3	33.24	33.59	33.47	32.58	33.81	33.02	33.33
	Replica 4	33.58	33.84	33.74	33.06	33.89	33.91	33.47
RSV type A	Replica 1	33.10	33.79	33.33	33.53	34.02	33.36	33.03
	Replica 2	33.15	33.35	33.11	32.61	33.37	33.00	32.81
	Replica 3	33.21	33.52	33.70	33.33	33.20	32.86	33.17
	Replica 4	33.26	33.19	33.49	33.48	32.95	33.04	33.16
RSV type B	Replica 1	34.69	35.15	35.52	35.35	34.22	34.34	34.41
	Replica 2	34.81	36.90	35.21	35.43	34.52	35.27	34.77
	Replica 3	35.21	34.41	35.43	34.64	33.78	34.41	34.15
	Replica 4	36.33	36.45	35.51	34.18	34.14	34.46	34.05

The variation of ± 10% of the volume of nucleic acid, of ± 5 seconds of the duration of the primers' elongation and of ± 1°C of the temperature of the primers' elongation did not affect the analytical sensitivity of the qRT-PCR, since the four replicas of each valence at the 3 x $LD_{RT\text{-}PCR}$ level provided a signal detected in 100% of cases.

⇨ **The robustness of Premium Dx® ColdPlex Covid & Flu & RSV was verified for all five valences when using 15 µL of Master Mix for variations in nucleic acid volume and duration and temperature of the primers' elongation and hybridization.**

8) Interferences

The presence of interferences has been evaluated by preparing samples containing RNA of one target at the $3xLD_{RT\text{-}PCR}$ level with the RNA of another target at a strong level of positivity. RNA for each target have been diluted to reach the desired level then analyzed by qRT-PCR using the Premium Dx® ColdPlex Covid & Flu & RSV according to the amplification program described on page 11.

$3xLD_{RT\text{-}PCR}$ level target	Strong positive target	Ct SARS-CoV-2	Ct Influenza type A	Ct Influenza type B	Ct RSV type A	Ct RSV type B
SARS-CoV-2	-	**33.42**	-	-	-	-
Influenza type A	-	-	**33.94**	-	-	-
Influenza type B	-	-	-	**34.21**	-	-
RSV type A	-	-	-	-	**33.83**	-
RSV type B	-	-	-	-	-	**34.62**
SARS-CoV-2	Influenza type A	**33.39**	*23.93*	-	-	-
SARS-CoV-2	Influenza type B	**33.72**	-	*25.27*	-	-
SARS-CoV-2	RSV type A	**33.61**	-	-	*24.31*	-
SARS-CoV-2	RSV type B	**34.47**	-	-	-	*23.82*
Influenza type A	SARS-CoV-2	*24.37*	**35.60**	-	-	-
Influenza type A	RSV type A	-	**32.68**	-	*24.30*	-
Influenza type A	RSV type B	-	**34.24**	-	-	*23.97*
Influenza type B	SARS-CoV-2	*24.10*	-	**34.12**	-	-
Influenza type B	RSV type A	-	-	**32.34**	24.28	-
Influenza type B	RSV type B	-	-	**32.83**	-	*24.23*
RSV type A	SARS-CoV-2	*24.18*	-	-	**30.13***	-
RSV type A	Influenza type A	-	*23.25*	-	**33.17**	-
RSV type A	Influenza type B	-	-	*25.78*	**33.35**	-
RSV type B	SARS-CoV-2	*24.17*	-	-	-	**31.24***
RSV type B	Influenza type A	-	*23.75*	-	-	**34.04**
RSV type B	Influenza type B	-	-	*25.88*	-	**33.65**

* For the RSV A & B valence (VIC), a positive signal may be observed when the SARS-CoV-2 valence (FAM) is found positive. This signal is not specific and may create curves with a very low fluorescence level and of uncharacteristic aspects. Here, the presence of SARS-CoV-2 in competition with RSV A&B lowers the value of the Ct for weak positives. However, the signal is still characteristic of the presence of the RSV in a small quantity.

⇨ **The presence of the SARS-CoV-2 at a strong level of positivity may alter the aspect of the curves for influenza B signal when the latter is at a low level of positivity. However, the signal for influenza B is still detected.**

⇨ **No other interference was observed in this study.**

Characterization of the complete method for deep nasopharyngeal swab samples

1) Limit of detection of the complete method

Premium Dx® ColdPlex Covid & Flu & RSV (Cat. PREMDX005 / PREMDX006) allows the detection of the SARS-CoV-2, influenza A&B and RSV A&B in samples using multiple detection systems. The LD_{METHOD} was determined for each of these targets on serial dilutions of whole inactivated viruses titrated in negative swab eluate. Strains were provided by National Reference Center (NRC) for respiratory infection viruses including influenza (Lyon, France):

- **SARS-CoV-2 lineage 20H/501Y.V2**
- **Influenza type A strain H1N1 19/4/2019 A/Brisbane/2/2018**
- **Influenza type B strain 21/06/2018 B/Colorado/6/2017**
- **RSV type A strain 28Long 28-589A P14**
- **RSV type B strain 28B/60 MERIEUX 28-591B P10**

The nucleic acid extraction system tested was the BioExtract® Premium Mag (BioSellal). The LD_{METHOD} approach was performed on different concentration levels with 5 replicates per concentration. The lowest concentration with a detection rate of 100% was selected to confirm the LD_{METHOD} on 20 replicates. The amplifications have been performed using the Premium Dx® ColdPlex Covid & Flu & RSV (program detailed on page 11) using the CFX96 (Bio-Rad) thermal cycler.

Result of the LD_{METHOD} approach for the detection of the SARS-CoV-2 genome

Experimental design:

Number of dilutions	Number of replicas per series	Number of independent series
3	5	1

Raw data (Ct values) of the LD_{METHOD} approach to test for the presence of SARS-CoV-2:

Positivity level		BioExtract® Premium Mag						
		Replica	Ct SARS-CoV-2	Ct Influenza type A	Ct Influenza type B	Ct RSV type A	Ct RSV type B	Ct IPC
$7.2.10^4$ copies/ml of swab eluate	**720 copies/qRT-PCR**	1	29.62	N/A*	N/A*	N/A*	N/A*	29.99
		2	30.49					29.74
		3	30.84					29.97
		4	30.59					30.08
		5	30.65					29.65
$7.2.10^3$ copies/ml of swab eluate	**72 copies/qRT-PCR**	1	33.47	N/A*	N/A*	N/A*	N/A*	31.12
		2	33.35					31.19
		3	33.25					30.76
		4	32.97					30.51
		5	33.32					30.70
$7.2.10^2$ copies/ml of swab eluate	**7.2 copies/qRT-PCR**	1	36.86	N/A*	N/A*	N/A*	N/A*	30.74
		2	35.18					31.12
		3	35.13					30.97
		4	35.82					31.13
		5	35.88					31.25

N/A*: not analyzed

⇨ **For the SARS-CoV-2 valence, the approached LD_{METHOD} would be $7.2.10^2$ copies/ml of swab eluate (7.2 copies/qRT-PCR). However, considering the shape of the curves and the late Ct, the concentration $7.2.10^3$ copies/ml of swab eluate (72 copies/qRT-PCR) was selected for the $LD_{METHODE}$ confirmation.**

Results of the LD_{METHOD} confirmation for the detection of the SARS-CoV-2 genome

Experimental design:

Number of dilutions	Number of replicas per series	Number of operators	Number of independent series
1	20	1	1

Raw data (Ct values) of the LD_{METHOD} confirmation to test for the presence of SARS-CoV-2:

Positivity level		BioExtract® Premium Mag						
		Replica	Ct SARS-CoV-2	Ct Influenza type A	Ct Influenza type B	Ct RSV type A	Ct RSV type B	Ct IPC
$7.2.10^3$ copies/ml of swab eluate	72 copies/qRT-PCR	1	34.21	N/A*	N/A*	N/A*	N/A*	32.39
		2	33.42	N/A*	N/A*	N/A*	N/A*	32.83
		3	32.16	N/A*	N/A*	N/A*	N/A*	32.41
		4	33.42	N/A*	N/A*	N/A*	N/A*	32.81
		5	32.96	N/A*	N/A*	N/A*	N/A*	31.77
		6	33.01	N/A*	N/A*	N/A*	N/A*	31.34
		7	33.86	N/A*	N/A*	N/A*	N/A*	32.88
		8	33.62	N/A*	N/A*	N/A*	N/A*	32.04
		9	33.33	N/A*	N/A*	N/A*	N/A*	32.46
		10	33.36	N/A*	N/A*	N/A*	N/A*	32.16
		11	32.89	N/A*	N/A*	N/A*	N/A*	32.03
		12	33.18	N/A*	N/A*	N/A*	N/A*	31.69
		13	33.49	N/A*	N/A*	N/A*	N/A*	32.07
		14	32.94	N/A*	N/A*	N/A*	N/A*	32.09
		15	33.33	N/A*	N/A*	N/A*	N/A*	32.30
		16	33.43	N/A*	N/A*	N/A*	N/A*	32.00
		17	33.63	N/A*	N/A*	N/A*	N/A*	31.80
		18	33.34	N/A*	N/A*	N/A*	N/A*	32.29
		19	32.96	N/A*	N/A*	N/A*	N/A*	32.37
		20	33.14	N/A*	N/A*	N/A*	N/A*	31.59

N/A*: not analyzed

⇨ **For the SARS-CoV-2 valence, the LD_{METHOD} is $7.2.10^3$ copies/mL of swab eluate (72 copies/qRT-PCR).**

Result of the LD_{METHOD} approach for the detection of the influenza type A genome

Experimental design:

Number of dilutions	Number of replicas per series	Number of independent series
4	5	1

Raw data (Ct values) of the LD_{METHOD} approach to test for the presence of influenza type A:

Positivity level		BioExtract® Premium Mag						
		Replica	Ct SARS-CoV-2	Ct Influenza type A	Ct Influenza type B	Ct RSV type A	Ct RSV type B	Ct IPC
$1.10^{6.1}$ TCID50/ml of swab eluate	$1.10^{4.1}$ TCID50/qRT-PCR	1	N/A*	30.48	N/A*	N/A*	N/A*	30.25
		2		30.28				30.03
		3		30.72				30.04
		4		30.72				29.60
		5		30.47				30.36
$5.10^{5.1}$ TCID50/ml of swab eluate	$5.10^{3.1}$ TCID50/qRT-PCR	1	N/A*	32.52	N/A*	N/A*	N/A*	30.45
		2		33.02				30.31
		3		32.97				30.72
		4		32.97				30.51
		5		32.98				29.91
$1.10^{5.1}$ TCID50/ml of swab eluate	$1.10^{3.1}$ TCID50/qRT-PCR	1	N/A*	32.79	N/A*	N/A*	N/A*	25.63
		2		33.29				25.35
		3		32.95				25.52
		4		32.94				25.47
		5		32.55				25.52
$1.10^{4.1}$ TCID50/ml of swab eluate	$1.10^{2.1}$ TCID50/qRT-PCR	1	N/A*	N/A	N/A*	N/A*	N/A*	25.44
		2		N/A				25.41
		3		N/A				25.35
		4		N/A				25.23
		5		N/A				25.42

N/A*: not analyzed

⇨ **For the influenza type A valence, the approached LD_{METHOD} is $1.10^{5.1}$ TCID50/ml of swab eluate ($1.10^{3.1}$ TCID50/qRT-PCR).**

Results of the LD_{METHOD} confirmation for the detection of the influenza type A genome

Experimental design:

Number of dilutions	Number of replicas per series	Number of operators	Number of independent series
1	20	1	1

Raw data (Ct values) of the LD_{METHOD} confirmation to test for the presence of influenza type A:

Positivity level		BioExtract® Premium Mag						
		Replica	Ct SARS-CoV-2	Ct Influenza type A	Ct Influenza type B	Ct RSV type A	Ct RSV type B	Ct IPC
$1.10^{5.1}$ TCID50/ml of swab eluate	$1.10^{3.1}$ TCID50/qRT-PCR	1	N/A*	33.45	N/A*	N/A*	N/A*	29.79
		2	N/A*	34.01	N/A*	N/A*	N/A*	30.08
		3	N/A*	33.97	N/A*	N/A*	N/A*	30.29
		4	N/A*	34.02	N/A*	N/A*	N/A*	29.90
		5	N/A*	34.17	N/A*	N/A*	N/A*	30.16
		6	N/A*	33.70	N/A*	N/A*	N/A*	30.00
		7	N/A*	33.69	N/A*	N/A*	N/A*	30.11
		8	N/A*	33.79	N/A*	N/A*	N/A*	30.09
		9	N/A*	33.54	N/A*	N/A*	N/A*	29.77
		10	N/A*	34.03	N/A*	N/A*	N/A*	30.94
		11	N/A*	33.79	N/A*	N/A*	N/A*	29.94
		12	N/A*	33.69	N/A*	N/A*	N/A*	30.41
		13	N/A*	34.02	N/A*	N/A*	N/A*	30.25
		14	N/A*	34.26	N/A*	N/A*	N/A*	29.74
		15	N/A*	33.96	N/A*	N/A*	N/A*	30.74
		16	N/A*	33.46	N/A*	N/A*	N/A*	29.57
		17	N/A*	33.69	N/A*	N/A*	N/A*	30.30
		18	N/A*	33.60	N/A*	N/A*	N/A*	30.03
		19	N/A*	34.10	N/A*	N/A*	N/A*	30.30
		20	N/A*	34.12	N/A*	N/A*	N/A*	30.55

N/A*: not analyzed

⇨ **For the influenza type A valence, the LD_{METHOD} is $1.10^{5.1}$ TCID50/ml of swab eluate ($1.10^{3.1}$ TCID50).**

Result of the LD_{METHOD} approach for the detection of the influenza type B genome

Experimental design:

Number of dilutions	Number of replicas per series	Number of independent series
4	5	1

Raw data (Ct values) of the LD_{METHOD} approach to test for the presence of influenza type B:

Positivity level		BioExtract® Premium Mag						
		Replica	Ct SARS-CoV-2	Ct Influenza type A	Ct Influenza type B	Ct RSV type A	Ct RSV type B	Ct IPC
$1.10^{5.2}$ TCID50/ml of swab eluate	$1.10^{3.2}$ TCID50/qRT-PCR	1	N/A*	N/A*	26.38	N/A*	N/A*	29.78
		2			26.91			30.28
		3			27.01			30.29
		4			27.44			30.54
		5			27.81			30.61
$5.10^{4.2}$ TCID50/ml of swab eluate	$5.10^{2.2}$ TCID50/qRT-PCR	1	N/A*	N/A*	29.12	N/A*	N/A*	30.43
		2			29.10			30.50
		3			29.31			30.37
		4			29.46			30.43
		5			29.41			30.95
$1.10^{4.2}$ TCID50/ml of swab eluate	158 TCID50/qRT-PCR	1	N/A*	N/A*	31.03	N/A*	N/A*	25.43
		2			31.22			25.23
		3			31.16			25.25
		4			31.24			25.18
		5			31.93			25.36
$1.10^{3.2}$ TCID50/ml of swab eluate	15.8 TCID50/qRT-PCR	1	N/A*	N/A*	34.99	N/A*	N/A*	25.35
		2			36.41			25.10
		3			N/A			25.39
		4			N/A			25.31
		5			38.61			25.17

N/A*: not analyzed

⇨ **For the influenza type B valence, the approached LD_{METHOD} is $1.10^{4.2}$ TCID50/ml of swab eluate (158 TCID50/qRT-PCR).**

Results of the LD_{METHOD} confirmation for the detection of the influenza type B genome

Experimental design:

Number of dilutions	Number of replicas per series	Number of operators	Number of independent series
1	20	1	1

Raw data (Ct values) of the LD_{METHOD} confirmation to test for the presence of influenza type B:

Positivity level		BioExtract® Premium Mag						
		Replica	Ct SARS-CoV-2	Ct Influenza type A	Ct Influenza type B	Ct RSV type A	Ct RSV type B	Ct IPC
$1.10^{4.2}$ TCID50/ml of swab eluate	158 TCID50/qRT-PCR	1	N/A*	N/A*	30.03	N/A*	N/A*	30.25
		2	N/A*	N/A*	30.03	N/A*	N/A*	30.36
		3	N/A*	N/A*	30.21	N/A*	N/A*	30.06
		4	N/A*	N/A*	30.37	N/A*	N/A*	30.39
		5	N/A*	N/A*	30.51	N/A*	N/A*	30.33
		6	N/A*	N/A*	30.23	N/A*	N/A*	30.22
		7	N/A*	N/A*	30.39	N/A*	N/A*	30.27
		8	N/A*	N/A*	30.21	N/A*	N/A*	29.99
		9	N/A*	N/A*	29.71	N/A*	N/A*	30.23
		10	N/A*	N/A*	30.02	N/A*	N/A*	30.02
		11	N/A*	N/A*	30.18	N/A*	N/A*	30.35
		12	N/A*	N/A*	29.68	N/A*	N/A*	29.67
		13	N/A*	N/A*	30.17	N/A*	N/A*	30.21
		14	N/A*	N/A*	30.04	N/A*	N/A*	30.04
		15	N/A*	N/A*	29.92	N/A*	N/A*	29.98
		16	N/A*	N/A*	29.61	N/A*	N/A*	29.93
		17	N/A*	N/A*	29.88	N/A*	N/A*	30.28
		18	N/A*	N/A*	30.04	N/A*	N/A*	30.03
		19	N/A*	N/A*	29.99	N/A*	N/A*	30.09
		20	N/A*	N/A*	29.86	N/A*	N/A*	30.02

N/A*: not analyzed

⇨ **For the influenza type B valence, the LD_{METHOD} is $1.10^{4.2}$ TCID50/ml of swab eluate (158 TCID50/qRT-PCR).**

Result of the LD_{METHOD} approach for the detection of the RSV type A genome

Experimental design:

Number of dilutions	Number of replicas per series	Number of independent series
4	5	1

Raw data (Ct values) of the LD_{METHOD} approach to test for the presence of RSV type A:

Positivity level		BioExtract® Premium Mag						
		Replica	Ct SARS-CoV-2	Ct Influenza type A	Ct Influenza type B	Ct RSV type A	Ct RSV type B	Ct IPC
$1.10^{3.62}$ TCID50/ml of swab eluate	$1.10^{1.62}$ TCID50/qRT-PCR	1	N/A*	N/A*	N/A*	28.17	N/A*	25.49
		2				28.01		25.49
		3				28.08		25.37
		4				27.86		25.35
		5				27.85		25.26
$1.10^{2.62}$ TCID50/ml of swab eluate	4.2 TCID50/qRT-PCR	1	N/A*	N/A*	N/A*	31.00	N/A*	25.33
		2				30.83		25.33
		3				31.27		25.49
		4				30.96		25.47
		5				30.56		25.29
$1.10^{1.62}$ TCID50/ml of swab eluate	0.42 TCID50/qRT-PCR	1	N/A*	N/A*	N/A*	35.95	N/A*	25.38
		2				34.34		25.19
		3				34.18		25.23
		4				35.19		25.33
		5				33.54		25.26
$1.10^{0.62}$ TCID50/ml of swab eluate	0.042 TCID50/qRT-PCR	1	N/A*	N/A*	N/A*	N/A	N/A*	25.46
		2				N/A		25.49
		3				N/A		25.30
		4				N/A		25.32
		5				N/A		25.22

N/A*: not analyzed

⇨ **For the RSV type A valence, the approached LD_{METHOD} is $1.10^{1.62}$ TCID50/ml of swab eluate (0.42 TCID50/qRT-PCR).**

Results of the LD_{METHOD} confirmation for the detection of the RSV type A genome

Experimental design:

Number of dilutions	Number of replicas per series	Number of operators	Number of independent series
1	20	1	1

Raw data (Ct values) of the LD_{METHOD} confirmation to test for the presence of RSV type A:

Positivity level		BioExtract® Premium Mag						
		Replica	Ct SARS-CoV-2	Ct Influenza type A	Ct Influenza type B	Ct RSV type A	Ct RSV type B	Ct IPC
$1.10^{1.62}$ TCID50/ml of swab eluate	0.42 TCID50/qRT-PCR	1	N/A*	N/A*	N/A*	34.58	N/A*	25.16
		2	N/A*	N/A*	N/A*	33.91	N/A*	25.19
		3	N/A*	N/A*	N/A*	33.60	N/A*	25.30
		4	N/A*	N/A*	N/A*	33.32	N/A*	25.13
		5	N/A*	N/A*	N/A*	33.55	N/A*	25.07
		6	N/A*	N/A*	N/A*	33.56	N/A*	25.32
		7	N/A*	N/A*	N/A*	33.34	N/A*	25.11
		8	N/A*	N/A*	N/A*	34.19	N/A*	25.06
		9	N/A*	N/A*	N/A*	33.45	N/A*	25.12
		10	N/A*	N/A*	N/A*	33.77	N/A*	25.05
		11	N/A*	N/A*	N/A*	33.51	N/A*	25.12
		12	N/A*	N/A*	N/A*	34.48	N/A*	25.33
		13	N/A*	N/A*	N/A*	33.78	N/A*	24.97
		14	N/A*	N/A*	N/A*	34.10	N/A*	25.10
		15	N/A*	N/A*	N/A*	33.38	N/A*	25.41
		16	N/A*	N/A*	N/A*	33.26	N/A*	25.17
		17	N/A*	N/A*	N/A*	33.69	N/A*	25.28
		18	N/A*	N/A*	N/A*	33.30	N/A*	25.18
		19	N/A*	N/A*	N/A*	33.54	N/A*	25.04
		20	N/A*	N/A*	N/A*	34.89	N/A*	25.51

N/A*: not analyzed

⇨ **For the RSV type A valence, the LD_{METHOD} is $1.10^{1.62}$ TCID50/mL of swab eluate (0.42 TCID50/qRT-PCR).**

Result of the LD_{METHOD} approach for the detection of the RSV type B genome

Experimental design:

Number of dilutions	Number of replicas per series	Number of independent series
4	5	1

Raw data (Ct values) of the LD_{METHOD} approach to test for the presence of RSV type B:

Positivity level		BioExtract® Premium Mag						
		Replica	Ct SARS-CoV-2	Ct Influenza type A	Ct Influenza type B	Ct RSV type A	Ct RSV type B	Ct IPC
$1.10^{3.62}$ TCID50/ml of swab eluate	$1.10^{1.62}$ TCID50/qRT-PCR	1	N/A*	N/A*	N/A*	N/A*	28.99	25.78
		2					28.79	25.36
		3					28.54	25.38
		4					28.46	25.22
		5					28.50	25.24
$1.10^{2.62}$ TCID50/ml of swab eluate	4.2 TCID50/qRT-PCR	1	N/A*	N/A*	N/A*	N/A*	32.02	25.70
		2					31.83	25.54
		3					31.61	25.38
		4					32.07	25.31
		5					30.39	25.42
$1.10^{1.62}$ TCID50/ml of swab eluate	0.42 TCID50/qRT-PCR	1	N/A*	N/A*	N/A*	N/A*	35.16	25.34
		2					36.59	25.50
		3					34.37	25.31
		4					34.47	25.47
		5					35.40	25.46
$1.10^{0.62}$ TCID50/ml of swab eluate	0.042 TCID50/qRT-PCR	1	N/A*	N/A*	N/A*	N/A*	N/A	25.30
		2					N/A	25.51
		3					N/A	25.54
		4					N/A	25.49
		5					N/A	25.60

N/A*: not analyzed

⇨ **For the RSV type B valence, the approached LD_{METHOD} is $1.10^{1.62}$ TCID50/ml of swab eluate (0.42 TCID50/qRT-PCR).**

Results of the LD_{METHOD} confirmation for the detection of the RSV type B genome

Experimental design:

Number of dilutions	Number of replicas per series	Number of operators	Number of independent series
1	20	1	1

Raw data (Ct values) of the LD_{METHOD} confirmation to test for the presence of RSV type B:

Positivity level		BioExtract® Premium Mag						
		Replica	Ct SARS-CoV-2	Ct Influenza type A	Ct Influenza type B	Ct RSV type A	Ct RSV type B	Ct IPC
$1.10^{1.62}$ TCID50/ml of swab eluate	0.42 TCID50/qRT-PCR	1	N/A*	N/A*	N/A*	N/A*	35.64	25.27
		2	N/A*	N/A*	N/A*	N/A*	34.17	25.34
		3	N/A*	N/A*	N/A*	N/A*	35.32	25.31
		4	N/A*	N/A*	N/A*	N/A*	35.42	25.29
		5	N/A*	N/A*	N/A*	N/A*	35.63	25.18
		6	N/A*	N/A*	N/A*	N/A*	34.97	25.06
		7	N/A*	N/A*	N/A*	N/A*	35.50	25.07
		8	N/A*	N/A*	N/A*	N/A*	36.05	25.13
		9	N/A*	N/A*	N/A*	N/A*	36.56	25.15
		10	N/A*	N/A*	N/A*	N/A*	34.76	25.15
		11	N/A*	N/A*	N/A*	N/A*	35.28	25.34
		12	N/A*	N/A*	N/A*	N/A*	34.12	25.07
		13	N/A*	N/A*	N/A*	N/A*	33.69	24.99
		14	N/A*	N/A*	N/A*	N/A*	36.08	25.22
		15	N/A*	N/A*	N/A*	N/A*	35.23	24.95
		16	N/A*	N/A*	N/A*	N/A*	36.49	24.88
		17	N/A*	N/A*	N/A*	N/A*	35.69	25.02
		18	N/A*	N/A*	N/A*	N/A*	35.89	25.08
		19	N/A*	N/A*	N/A*	N/A*	35.73	25.11
		20	N/A*	N/A*	N/A*	N/A*	36.16	25.19

N/A*: not analyzed

⇨ **For the RSV type B valence, the LD_{METHOD} is $1.10^{1.62}$ TCID50/ml of swab eluate (0.42 TCID50/qRT-PCR).**

2) Diagnostic specificity and sensitivity on samples with known status

The diagnostic specificity and sensitivity of the complete method combining the Premium Dx® ColdPlex Covid & Flu & RSV with the BioExtract® Premium Mag extraction kit was determined for each valence on a deep nasopharyngeal swab eluate matrix.

c. Specificity and sensitivity for the detection of the SARS-CoV-2 genome

For the detection of the SARS-CoV-2 (presence/absence result), the diagnostic sensibility and sensitivity were determined *in vitro* on 94 samples from patients previously determined to be positive, and on 94 samples from patients previously determined to be negative for this virus. The positive or negative status for each sample was obtained using the Bio-T kit® TriStar Covid-19 CE-IVD. These results were obtained on CFX96.

The results are analysed and expressed as follows:
For diagnostic specificity (Sp): As a percentage of negatives found among expected negatives.
For diagnostic sensitivity (Se): As a percentage of positives found among expected positives.

Results for the SARS-CoV-2 valence:

		Bio-T Kit® TriStar Covid-19 status		
		Presence of the SARS-CoV-2 genome	Absence of the SARS-CoV-2 genome	Total
Premium Dx® ColdPlex Covid & Flu & RSV results	Presence of the SARS-CoV-2 genome	94	0	94
	Absence of the SARS-CoV-2 genome	0	94	94
	Total	94	94	188

⇨ **On the panel of samples analysed, the diagnostic sensibility is Se = 100 %.**
The lower limit of the confidence interval for sensibility is 90 %.

⇨ **On the panel of samples analysed, the diagnostic specifity is Sp = 100 %.**

d. Specificity and sensitivity for the detection of influenza A&B genomes

For the detection of influenza A & B (presence/absence result), the diagnostic sensibility and sensitivity were determined *in vitro* on 65 samples from patients previously determined to be positive, and on 96 samples from patients previously determined to be negative for this virus. The positive or negative status for each sample was obtained by NGS sequencing done by the National Reference Center (NRC) for respiratory infection viruses including influenza (Lyon, France). These results were obtained on CFX96.
The results are analysed and expressed as follows:
For diagnostic specificity (Sp): As a percentage of negatives found among expected negatives.
For diagnostic sensitivity (Se): As a percentage of positives found among expected positives.

Results for the Influenza A&B valence:

		Sequencing status		
		Presence of the Influenza A&B genome	Absence of the Influenza A&B genome	Total
Premium Dx® ColdPlex Covid & Flu & RSV results	Presence of the Influenza A&B genome	65	0	65
	Absence of the Influenza A&B genome	0	96	96
	Total	65	96	161

⇨ **On the panel of samples analysed, the diagnostic sensibility is Se = 100 %.**
The lower limit of the confidence interval for sensibility is 88 %.

⇨ **On the panel of samples analysed, the diagnostic specifity is Sp = 100 %.**

Note: the sample status given by the sequencing specifies the type of the Influenza virus (A or B). The results of sensibility and specificity are equivalent between the two types.

e. Specificity and sensitivity for the detection of RSV A&B genomes

For the detection of RSV A & B (presence/absence result), the diagnostic sensibility and sensitivity were determined *in vitro* on 49 samples from patients previously determined to be positive, and on 96 samples from patients previously determined to be negative for this virus. The positive or negative status for each sample was obtained by NGS sequencing done by the National Reference Center (NRC) for respiratory infection viruses including influenza (Lyon, France). These results were obtained on CFX96.
The results are analysed and expressed as follows:
For diagnostic specificity (Sp): As a percentage of negatives found among expected negatives.
For diagnostic sensitivity (Se): As a percentage of positives found among expected positives.

Results for the RSV A&B valence:

		Sequencing status		
		Presence of the RSV A genome	Absence of the RSV A genome	Total
Premium Dx® ColdPlex Covid & Flu & RSV results	Presence of the RSV A&B genome	47	0	47
	Absence of the RSV A&B genome	2	96	98
	Total	49	96	145

⇨ **On the panel of samples analysed, the diagnostic sensibility is Se = 96 %.**
The lower limit of the confidence interval for sensibility is 82 %.

⇨ **On the panel of samples analysed, the diagnostic specifity is Sp = 100 %.**

Conclusions

1) CHARACTERISATION OF THE RT-PCR

Experimental inclusivity	Verified *in silico* on all strains present in the bank and experimentally on whole inactivated viruses provided by the National Reference Center.
Experimental exclusivity	Verified on viruses, bacteria present in the same ecological niches and/or niches that are genetically related
$LD_{RT\text{-}PCR}$ at 95%	*E-N genes* of SARS-CoV-2: 6.25 copies of RNA transcribed per RT-PCR *M gene* of influenza type A: 75 copies of RNA transcribed per RT-PCR *M gene* of influenza type B: 18.75 copies of RNA transcribed per RT-PCR *N gene* of RSV type A: 37.5 copies of RNA transcribed per RT-PCR *N gene* of RSV type B: 7.7 copies of RNA transcribed per RT-PCR
Efficiency of the qRT-PCR	*E-N genes* of SARS-CoV-2: 96.0 % *M gene* of influenza type A: 97.4 % *M gene* of influenza type B: 100.5 % *N gene* of RSV type A: 95.3 % *N gene* of RSV type B: 94.2 %
Repeatability variation coefficient	Varies between 0.07 % to 1.00 % for the *E-N gene* of the SARS-CoV-2 Varies between 0.25 % to 0.89 % for the *M gene* of influenza A Varies between 1.20 % to 1.78 % for the *M gene* of influenza B Varies between 0.04 % to 1.11 % for the *N gene* of RSV A Varies between 0.08 % to 0.73 % for the *N gene* of RSV B
Intermediate precision variation coefficient	Varies between 1.12 % to 2.51 % for the *E-N gene* of the SARS-CoV-2 Varies between 1.09 % to 1.72 % for the *M gene* of influenza A Varies between 1.48 % to 2.55 % for the *M gene* of influenza B Varies between 1.50 % to 2.84 % for the *N gene* of RSV A Varies between 2.39 % to 3.80 % for the *N gene* of RSV B
Confirmation of performance of the qRT- PCR on other parameters or thermal cyclers.	<u>Usable program:</u> - ColdPlex program <u>Validated thermal cyclers:</u> - CFX96 standard ramping - AriaMx™ default fast ramping - QuantStudio™ 5 standard ramping
Robustness	Verified on the 3xLD_{PCR} level by allowing a variation in: The volume of nucleic acids (4.5-5.5µl) of +/- 10% The hybridization/elongation temperature (59-61°C) of +/- 1°C The hybridization/elongation time (40-50 sec) of +/- 5 seconds

3) CHARACTERISATION OF THE COMPLETE METHOD

Matrix: deep nasopharyngeal swab		
$LD_{METHODE}$		**BioExtract® Premium Mag**
	SARS-CoV-2	$7.2.10^{3}$ copies/mL of swab eluate
	Influenza type A	$1.10^{5.1}$ TCID50/mL of swab eluate
	Influenza type B	$1.10^{4.2}$ TCID50/mL of swab eluate
	RSV type A	$1.10^{1.62}$ TCID50/mL of swab eluate
	RSV type B	$1.10^{1.62}$ TCID50/mL of swab eluate
Diagnostic sensitivity	**SARS-CoV-2**	100 %
	Influenza type A&B	100 %
	RSV type A&B	96 %
Diagnostic specificity	**SARS-CoV-2**	100 %
	Influenza type A&B	100 %
	RSV type A&B	100 %

biosellal

www.biosellal.com

Technical Support

tech@biosellal.com +33 (0) 4 26 78 47 62

Information and orders

contact@biosellal.com +33 (0) 4 26 78 47 60