HANDBOOK

Bio-T kit® TriStar Covid-19

Cat. N° BIOTK121 - 500 reactions
Cat. N° BIOTK122 - 1000 reactions

**Detection of the Coronavirus responsible of the severe acute respiratory syndrom (SRAS-CoV-2)
by real-time RT-PCR (RT-PCR)
with Exogenous internal positive control (IPC)**

HUMAN

Sample types

- Oral, nasal swabs
- Samples of the lower respiratory tract (Alveolar bronchial washing, sputum)

Recommended nucleic acids (NA) extractions

- Magnetic beads extraction (ex : BioSellal – BioExtract® SuperBall® Cat. N° BES384, BioSellal – BioExtract® Premium Mag Cat. N° BEPM96, BEPM1K, BEPM2K, BEPM5K)
- Silica membrane columns extraction (ex : BioSellal – BioExtract® Column Cat. N° BEC050 ou BEC250)

CE

For in vitro diagnostic

IVD

DOCUMENTS MANAGEMENT

The Bio-T kit® TriStar Covid-19 has two technical handbooks:

- The extraction handbook displaying BioSellal's recommended extraction protocols for each type of sample.
- The Bio-T kit® TriStar Covid-19 RT-PCR handbook, presenting the instruction information to perform the RT-PCR.

The last versions in use for each handbook are indicated on the certificate of analysis (CA) provided with the Bio-T kit® TriStar Covid-19.

Besides these two handbooks, a summary report of the validation file and a performances confirmation handbook are available on request, contact BioSellal (contact@biosellal.com).

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

The sampling must be done according to the local regulation.
In order to prevent cross-contamination between samples which could lead to a false positive result, it is important to use single-use sampling equipment and to avoid direct contact between each sampling.

Shipping

The samples must be sent to the medical biology laboratory in triple packaging which identifies the SRAS-CoV-2 risk samples and secure transport in accordance with the recommendations of your country.

It is mandatory to ship immediately after sampling or by default to store it at ≤ -16°C. Shipment has to be done within 24h under cover of positive cold.

Storage after reception

It is recommended to immediately analyze samples after receipt or freezing at ≤ -16 ° C for a few months and ≤ -65°C beyond 1 year.

Description of the Bio-T kit® TriStar Covid-19

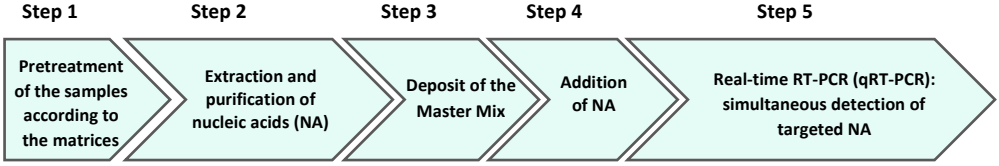
The **Bio-T kit® TriStar Covid-19** (Cat. N° BIOTK121/BIOTK122) contains a ready to use **one-step RT-PCR Master Mix** allowing the detection **in the same reaction well of**:

- ***E gene* of sarbecovirus including SRAS-CoV-2** with a 6-FAM labelling
- **ORF1ab of SRAS-CoV-2** (***RdRp***) with a VIC labelling
- An **Exogenous internal positive control IPC RNA**, with a Cy5 labelling, to add at the extraction step to assess nucleic acids extraction quality and absence of RT- PCR inhibitors.

ORF1ab detection is a product licenced from Pasteur Institute (France).

Extraction protocols recommended by BioSellal are described in the extraction handbook of the Bio-T kit® TriStar Covid-19.

Description of the whole process



Extraction handbook of the Bio-T kit® TriStar Covid-19		RT-PCR handbook of the Bio-T kit® TriStar Covid-19		
Nasal and oral swabs Alveolar bronchial washing* Sputum*	BioExtract® SuperBall® BioExtract® Premium Mag BioExtract® Column	Ready-to-use Master Mix MMTRISTARCOV-B	Samples NC/NCS Process positive control EPC (EPCTRISTARCOV-A)	Dyes: FAM/VIC/Cy5 Passive reference: ROX Program: TRISTAR Standard ramping

* pretreatment mandatory

Procedures for managing the risk related to the kit use

RT-PCR protocol associated with the Bio-T Kit® TriStar Covid-19 does not generate any risk for the handler and the environment. However, it is recommended to avoid contact between reagents and skin. In case of contact, wash with plenty of water and contact a doctor.

It should be noted that the implementation of the extraction protocols associated with the Bio-T® kit TriStar Covid-19 generates a chemical and biological risk for the handler and the environment. Please refer to the Bio-T® TriStar Covid-19 extraction instructions and the safety data sheets of the products used for further information.

Kit contents and storage

Table 1. Description of the kit contents					
Description	**Reference**	**Volume/tube**		**Presentation**	**Storage**
		BIOTK121 500 reactions	BIOTK122 1000 reactions		
Master Mix (MM) Ready to use	MMTRISTARCOV-B	7500 µl	2x7500 µl	Grey cap tube Bag A	≤-16°C Protected from light, « MIX » Area
Exogenous Internal Positive Control (IPC)	IPCTRISTAR-B	2500 µl	2x2500 µl	Grey cap tube Bag B	≤-16°C « Extraction » Area
External Positive Control (EPC) Positive PCR control of SRAS-CoV-2	EPCTRISTARCOV-A	200 µl		Red cap tube Bag C	≤-16°C « Addition of Nucleic acids » Area
Water RNase/DNase free	Aqua-A	1 ml		Blue cap tube Bag C	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°**
ATL Buffer	Lysis Buffer		BioSellal	ATL19076
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
		2000		BEPM2K
		5 000		BEPM5K

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

List of reagents to confirm laboratory performances

Synthetic RNA of SRAS-CoV-2 (titrated in number of copies/RT-PCR) used by BioSellal for the validation of the kit can be used to confirm the performance of your thermal cycler(s). For monitoring of process performance over time, an Internal Reference Material (IRM) is also available.
BioSellal sells these reagents under the following references:

Table 3. Optional reagents*

Reagent	Description	Provider	Cat. N°
***E gene* of SRAS-CoV-2 RNA**	Quantified RNA of *E gene* SRAS-CoV-2 ($6x10^4$ copies/qRT-PCR)	BioSellal	cARN-ESRAS-001
ORF1ab SRAS-CoV-2 RNA	Quantified RNA of ORF1ab SRAS-CoV-2 ($3x10^4$ copies/qRT-PCR)	BioSellal	cARN-IPSRAS-001
MRI SRAS-CoV-2	Inactivated SRAS-CoV-2 low positive sample	BioSellal	MRI-COVID-001

* These reagents are available only on demand, please contact BioSellal (contact@biosellal.com).

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, RT-PCR plates preparation), "Nucleic acids Addition" (Nucleic Acids storage and addition of extracted nucleic acids and controls in the RT-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.
- **Genome of SRAS-CoV-2 is 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 SRAS-CoV-2 BY RT-PCR WITH BIOTK121/BIOTK122 KIT

Global Procedure

1) **Establish RT-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 RT-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 of SRAS-CoV-2 (EPC)** : Synthetic DNA(tube **EPCTRISTARCOV-A**, **red**), containing specific targets of SRAS-CoV-2
  This control is mandatory.

⚠ CAUTION: *Due to the high sensitivity of the real-time RT-PCR process, good laboratory practice is essential for the proper performance of this test. Therefore, care must be taken to ensure that reagents are not contaminated. This includes opening and handling the Positive Control (EPC) in a delimited area away from other components and taking precautions to avoid cross-contamination with other sample during plate deposition. The NC and NCS Negative Controls ensure respectively that the Master Mix and the overall process including extraction are free of contamination. If these controls are positive, refer to the table on page 13.*

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

BioSellal also offers a ready-to-use MRI (Cat. N°MRI-COVID-001), for more information contact BioSellal.

2) RT-PCR plate preparation

In the "MIX" dedicated area

1. After thawing, vortex and rapid centrifugation, **transfer 15 µl Master Mix MMTRISTARCOV-B (grey 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**: **EPCTRISTARCOV-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.

Note: if the exogenous IPC was not added during sample extraction, it can be added directly in the RT-PCR plate:
- Add 1 µl of IPC (grey cap) with the extracted nucleic acids
- Or add directly the IPC (1 µl per reaction) in an aliquot of Master Mix before the deposits of 16 µl of this mix into each well of interest. Then add 5 µl of extracted nucleic acids.
The reaction volume will be increased to 21 µl, without impacting the performances of the RT-PCR.

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 4, Table 5, Table 6,)

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

6. Start the RT-PCR program. Approximate run time: 90 min.

3) Thermal cycler settings

This kit was developed and validated on AriaMx™ (Agilent Technologies, Fast ramping by default) and confirmed on ABI PRISM® 7500 Fast (Applied Biosystems) in standard ramping. It is compatible with all thermal cyclers with at least 6-FAM, VIC and Cy5 channels. For more information, contact our technical support.

Table 4. Thermal cycler configuration

	ABI PRISM® 7500 Fast	AriaMx™
Mode	Quantitation – Standard curve	Quantitative PCR, Fluorescence Probe
Ramping	Standard Ramping	Fast ramping by default
Passive Reference	**ROX**	**ROX**

Table 5. Thermal cycler Settings

Target	Detectors		Final Volume / well
	Reporter	Quencher	
E gene	FAM	NFQ-MGB or None*	**20 µl** = 15 µl Master Mix + 5 µl extracted nucleic acids or controls†
ORF1ab	VIC	NFQ-MGB or None*	
Exogenous 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 6. TRISTAR Amplification program settings

Standard ramping		
Cycles	**Time**	**Temperature**
1 cycle	20 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 RT-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 RT-PCR series is validated if the controls (EPC, MRI, NCS and NC) present valid results, then the result of each sample can be interpreted.

Warning: A negative result for the tested sample does not exclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. A negative result must be combined with clinical observations, patient history and epidemiological information.
Positive results are indicative of the presence of SARS-CoV-2 RNA, however, clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Moreover, positive results do not rule out bacterial infection or co-infection with other viruses.

Main Scenarios

Controls Reading

Table 7. PCR Controls results interpretation

	Targets *E gene* (FAM)	Targets ORF1ab (VIC)	Exogenous IPC (Cy5)	Interpretation
NCS Negative Control Sample **MANDATORY**	Neg	Neg	Pos	Valid
	At least one of the two targets Pos		Pos	Contamination with a positive sample during extraction step or during qPCR plate preparation. In this case, it is recommended to repeat the samples extraction and add NCS in larger numbers, then repeat the RT-PCR assay. If the contamination persists, it may come either from environmental contamination of your lab or from the Master Mix. The latter possibility can be assessed by depositing 24 to 48 NC without any samples or control. If this test is positive, discard the tube of Master Mix.
	Neg	Neg	Neg	Omission of exogenous IPC addition? Defective extraction ?
NC Negative PCR Control **OPTIONAL**	Neg	Neg	Neg	Valid
	At least one of the three targets Pos			Contamination with a negative or a positive sample during PCR plate preparation? or Master Mix / Water contamination? To test this hypothesis, it is recommended to deposit 24 to 48 NC without any samples or control. If this test is positive, discard the tube of Master Mix.
EPC SRAS-CoV-2 PCR external positive control **MANDATORY** *IN ABSENCE OF MRI*	Pos*	Pos*	Neg	Valid
	Neg	Neg	Neg	Problem during RT-PCR plate preparation: Master Mix error? EPC omission?
	Pos*	Pos*	Pos	Contamination with a sample during RT-PCR plate preparation?
Sample process positive Control MRI **RECOMMENDED** *IF AVAILABLE*	Pos†	Pos†	Pos¥	Valid
	Neg	Neg	Neg	Problem during RT-PCR plate preparation: Master Mix error? Nucleic acids extract omission or extract not in contact with Master Mix? Process drift: extraction and/or RT-PCR ?
	Neg	Neg	Pos¥	Process drift: extraction (in case of exogenous IPC addition directly into RT-PCR plate and not during extraction) Problem with MRI preparation? 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, the sample type and the used extraction protocol. IPC Ct values for recommended extraction protocols are available upon request. BioSellal recommends you determine your own maximal IPC Ct value depending on your own extraction method and thermal cycler.

Samples Reading

Table 8. Different types of results obtained for the samples			
Targets			
E gene (FAM)	ORF1ab (VIC)	Exogenous IPC (Cy5)	Interpretation
Neg	**Neg**	**Pos***	**Negative or Undetected**
Pos	**Pos**		**Positive or Detected** SRAS-CoV-2 genome detection
Pos	**Neg¥**		**Positive or Detected** *E gene* of sarbecovirus including detection
Neg	**Pos¥**		**Positive or Detected** ORF1ab of SRAS-CoV-2 detection
Pos	**Pos**	**Neg or Ct>35**	**Positive or Detected** Problem during the IPC addition? Presence of inhibitors ǂ? Competition with the main target?
One of the targets is **Neg**		**Neg or Ct>35**	**Positive or Detected for the positive target** **Uninterpretable for the negative target = Repeat the analysis** IPC exogenous omission during the extraction and/or RT-PCR Presence of inhibitors ǂ? Nucleic acids degradation in the sample? Extraction problem? Competition with the main target?
Neg	**Neg**	**Neg or Ct>35**	**Uninterpretable = Repeat the analysis** Nucleic acids extract omission or extract not in contact with Master Mix ? Presence of inhibitors ǂ? Nucleic acids degradation in the sample? Problem during the IPC addition? Extraction problem?

* The obtained Ct value depends on the thermal cycler, the sample type and the used extraction protocol. This value must be, at least, included within the specified range in the certificate of analysis (CA). IPC Ct values for recommended extraction protocols are available upon request. BioSellal recommends you determine your own maximal IPC Ct value depending on your own extraction method and thermal cycler.

¥ Samples positive for SARS-CoV-2 must be positive for both the *E gene* and ORF1ab. As the *E gene* is not specific for SARS-CoV-2, it is possible that the positive result in *gene E* and negative in ORF1ab may be consistent with the presence of another coronavirus of the genus sarbecovirus. In the case of a sample at the limit of detection (Ct>35), the result may be inconsistent between the two targets. In the event of a conflicting result BioSellal recommend to repeat the analysis and/or sequence the strain to detect any mutations.

ǂ In case of inhibition suspicion, 1) Repeat the RT-PCR with the dilution of extracted nucleic acids at 1/10 or 1/100 in the DNase/RNase free water. 2)Restart the analysis from the extraction step.

BIO-T KIT® TRISTAR COVID-19 PERFORMANCES

Validation data for the Bio-T kit® Tristar Covid-19 are detailed in the kit validation file. In order to comply with Directive 98/79/CE, analytical sensitivity and specificity, repeatability and intermediate precision, and diagnostic sensitivity and specificity are described below.

Characterization of RT-PCR

1) Analytical specificity

In silico

In silico analytical specificity was verified by analysing all SARS-CoV-2 sequences available in public databases (GenBank, NCBI) and performing alignments using MegAlign Pro software (DNAstar® Lasergene®).
The *in silico* specificity was confirmed by alignment using the online tool BLAST.

Experimental inclusivity

The Bio-T kit® TriStar Covid-19 can detect the presence of two SARS-CoV-2 target genes. The primers and probes system allowing the detection of *the E gene* (6-FAM channel) chosen by BioSellal is based on the protocol published by the World Health Organization (WHO) dated January 17, 2020. The primers and probes system for the detection of the ORF1ab of SARS-CoV-2 (VIC channel) chosen by BioSellal is a product under license from Pasteur Institute (France). Also, the analytical specificity is guaranteed by the initial publications for these two detection systems. For more information, please do not hesitate to contact BioSellal technical support.

Experimental inclusivity data were provided by a partner laboratory performing SARS-CoV-2 testing with the Bio-T kit® Tristar Covid-19. Thus, 13 samples from patients who were tested positive for SARS-CoV-2 with a reference technique (internal technique) were extracted using BioExtract® Premium Mag according to the protocol detailed in the Bio-T kit® Tristar Covid-19 extraction manual and then analyzed with the Bio-T kit® Tristar Covid-19 according to the protocol detailed on page 10. The comparative data detailed in the paragraph "Diagnostic Sensitivity and Specificity" demonstrate that the 13 samples expected to be positive with the reference technique are indeed positive for both targets of the Bio-T kit® Tristar Covid-19.

Experimental Exclusivity

Experimental exclusivity has been verified on viruses, bacteria or parasites present in the same ecological niches or leading to pathologies or clinical signs similar to those related to the presence of SARS-CoV-2 immediately available in BioSellal's sample library.

Raw data obtained on tested viruses, bacteria and parasites

Strain	Result
Streptococcus pneumoniae	Non- Detected
K. pneumoniae	Non-Detected
Streptococcus pyrogenes	Non-Detected
E. coli/579	Non-Detected
Leptospires pathogènes	Non- Detected
Mycobacterium bovis	Non-Detected
Mycobacterium tuberculosis	Non-Detected
E. cloacae	Non-Detected
S. marcescens	Non- Detected
S. aureus	Non-Detected
P. aeruginosa	Non-Detected
C. Burnetii	Non-Detected
Influenza A H3N2	Non- Detected
Influenza A H1N1	Non-Detected

⇨ **Analytical specificity of Bio-T kit® TriStar Covid-19 was confirmed *in silico* and experimentally on all the isolates and strains tested.**

2) Analytical sensitivity: $LD_{RT\text{-}PCR}$

The detection limit of qRT-PCR ($LD_{RT\text{-}PCR}$) corresponds to the minimum number of copies of targeted nucleic acid detected in 95% of cases.
It was determined using synthetic RNA of *E gene* and/or ORF1ab quantified by fluorimetry in target sequence copy number by RT-PCR (copies number in 5 µl).

The Bio-T kit® TriStar Covid-19 is a triplex for the simultaneous detection of two virus genes. These two genes are present at a rate of one copy each per viral genome. Also, BioSellal determined $LD_{RT\text{-}PCR}$ when each target is present with an equimolar ratio (1 copy of *E gene* + 1 copy of ORF1ab for example).

A first approach with serial 10-fold dilutions allowed to estimate the $LD_{RT\text{-}PCR}$ between 10 and 100 copies of *E gene* RNA by RT-PCR and between 1 and 10 copies of *ORF1ab* RNA by RT-PCR. Ranges dilutions by 2 were made from 80 to 2.5 copies by RT-PCR for *E gene* and 40 to 1.25 copies by RT-PCR for ORF1ab in order to frame the estimated value of $LD_{RT\text{-}PCR}$ on at least 6 dilutions.

Experimental design

Number of dilutions	Number of replicates by dilution and analytical series	Number of independent series
6	**8**	**3**

Data obtained for *E gene* of SARS-CoV-2:

Copies number/RT-PCR	Number of detected replicates			Total number of detected replicates	Frequency of detection
	Series 1	Series 2	Series 3		
80	8/8	8/8	8/8	24/24	100 %
40	8/8	8/8	8/8	24/24	100 %
20	**8/8**	**8/8**	**8/8**	**24/24**	**100 %**
10	8/8	8/8	6/8	22/24	92 %
5	5/8	3/8	6/8	14/24	58 %
2.5	4/8	3/8	3/8	10/24	42 %

The statistical value is less than 20 copies by RT-PCR (last dilution with at least 23 replicates detected out of 24). ORF1ab transcribed RNA is also present in equimolar ratio (e.g. 10 copies of gene E + 10 copies of ORF1ab).

⇨ **The experimental approach indicates that the 95% $LD_{RT\text{-}PCR}$ for *E gene* target (last dilution giving at least 23 positive results / 24) is 20 copies / RT-PCR.**

Data obtained for ORF1ab of SARS-CoV-2:

Copies number/RT-PCR	Number of detected replicates			Total number of detected replicates	Frequency of detection
	Series 1	Series 2	Series 3		
40	8/8	8/8	8/8	24/24	100 %
20	8/8	8/8	8/8	24/24	100 %
10	8/8	8/8	8/8	24/24	100 %
5	**8/8**	**8/8**	**8/8**	**24/24**	**100 %**
2.5	6/8	7/8	7/8	14/24	83 %
1.25	4/8	3/8	3/8	10/24	63 %

The statistical value is less than 5 copies by RT-PCR (last dilution with at least 23 replicates detected out of 24). The transcribed RNA of *gene E* is also present in equimolar ratio (e.g. 10 copies of gene E + 10 copies of ORF1ab).

⇨ **The experimental approach indicates that the 95% $LD_{RT\text{-}PCR}$ for *ORF1ab* target (last dilution giving at least 23 positive results / 24) is 5 copies / RT-PCR.**

3) Repetability, Intermediate Fidelity and Robustness of RT-PCR

Repeatability

The repeatability of qRT-PCR was determined from dilutions of an RNA transcript from *E gene* and ORFR1ab from SARS-CoV-2 to obtain three levels of positivity. One independent series of qRT-PCR were performed with analysis of 3 dilutions in duplicate in each series, without varying either the thermal cycler or the manipulator. The protocol used is detail at page 10. The coefficient of variation (CV) of repeatability of Ct values was then determined. The coefficient of variation (CV) of repeatability of 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 is the repeatability deviations, and M is the overall mean of the values in the series.
As before, repeatability was evaluated when each target was present with an equimolar ratio (1 copy of *gene E* + 1 copy of ORF1ab for example).

Data obtained (Ct values) for *E gene* target of SARS-CoV2 :

	Repetability (Ct)			Repetability CV %
Level 1	22.84	22.81	22.82	0.07
Level 2	29.25	29.52	29.45	0.48
Level 3	33.26	33.15	33.06	0.30

⇨ **The coefficient of variation of repeatability for *E gene* target per level varies from 0.07 to 0.48%.**

Data obtained (Ct values) for *ORF1ab* target of SARS-CoV2 :

	Repetability (Ct)			Repetability CV %
Level 1	21.10	21.23	21.31	0.50
Level 2	27.83	27.88	28.02	0.35
Level 3	31.23	31.75	31.57	0.84

⇨ **The coefficient of variation of repeatability for *ORF1ab* target per level varies from 0.35 to 0.84%.**

Intermediate Fidelity

The intermediate fidelity of qRT-PCR was determined from dilutions of an RNA transcript from *E gene* and ORFR1ab from SARS-CoV-2 to obtain three levels of positivity.

Three independent series were carried out on two thermal cyclers, by two manipulators, by analysing for each series the 3 dilutions in triplicate. The protocol used is detail at page 10.

At the end of these three series, the coefficient of variation of intermediate fidelity of the values of Ct can be determined, subject to positioning the threshold line according to common criteria. The calculation formula used is: by dividing the standard deviations by the mean according to the formula :

$$CV_{\text{Intermediate Fidelity}} = \frac{Sr}{M} * 100$$

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

As previously, intermediate precision was evaluated when each target is present with an equimolar ratio (1 copy of *gene E* + 1 copy of ORF1ab for example).

Data obtained (Ct values) for *E gene* target of SARS-CoV2 :

	Intermediate Fidelity (Ct)			Intermediate Fidelity CV %
Level 1	22.84	22.76	23.67	2.18
Level 2	26.12	26.20	27.19	2.25
Level 3	29.52	29.33	30.26	1.65

⇨ **The coefficient of variation of intermediate fidelity for *E gene* target per level varies from 1.65 to 2.25%.**

Data obtained (Ct values) for *ORF1ab* target of SARS-CoV2 :

	Intermediate Fidelity (Ct)			Intermediate Fidelity CV %
Level 1	21.31	21.34	22.65	3.52
Level 2	28.02	27.95	29.06	2.19
Level 3	31.75	31.68	32.55	1.51

⇨ **The coefficient of variation of intermediate fidelity for *E gene* target per level varies from 1.51 to 3.52%.**

Robustness of RT-PCR

The robustness of qRT-PCR with the TRISTAR program was evaluated by analysing the 3xLD_{RT-PCR} level, on six replicates per series and by varying the critical parameters of qRT-PCR, compared to the reference conditions detailed page 10:

Variation of critical parameters	
Reference Conditions	5 µl of Nucleic acids (NA) extracts 15 µl of Master Mix Annealing-Elongation of primers at 60°C for 45 seconds
for 10 µl of Master Mix, ± 10% of volume of NA	4.5 µl and 5.5 µl
for 10 µl of Master Mix, ± 1°C of temperature of primers annealing and elongation	59 and 61°C
for 10 µl of Master Mix, ± 10% the duration of primer annealing and elongation step	40 and 50 seconds

Raw data with TRISTAR Amplification program:

		AriaMx™ (Agilent Technologies,)						
		Reference conditions	15 µl MM 4.5 µl NA	15 µl MM 5.5 µl NA	15 µl MM 59°C	15 µl MM 61°C	15 µl MM 40 sec.	15 µl MM 50 sec.
E gene target of SARS-CoV-2	Replicate 1	33.43	33.63	32.66	34.43	34.10	33.87	33.07
	Replicate 2	32.78	33.37	32.90	33.47	33.40	33.95	34.01
	Replicate 3	33.47	32.71	33.14	33.17	33.82	34.26	33.55
	Replicate 4	33.30	32.76	33.13	33.67	32.99	34.36	33.08
	Replicate 5	32.98	33.74	32.27	33.42	34.70	33.48	33.43
	Replicate 6	32.91	33.84	33.65	32.96	33.43	34.57	33.78
ORF1ab target of SARS-CoV-2	Replicate 1	33.34	34.15	32.45	34.79	34.17	33.39	33.39
	Replicate 2	33.43	35.17	32.08	34.16	33.31	34.36	34.36
	Replicate 3	32.16	35.62	32.38	34.60	34.81	34.03	34.03
	Replicate 4	32.39	34.36	33.22	33.13	32.84	33.46	33.46
	Replicate 5	33.00	34.85	32.81	34.40	34.43	34.29	34.29
	Replicate 6	32.22	34.41	32.80	33.59	34.04	33.01	33.01

Variations of ± 10% nucleic acid volume, ± 1°C of primer annealing temperature, and ± 10% of elongation time did not affect the analytical sensitivity of the qRT- PCR with the classical program since the five replicates of the LD_{RT-PCR} level provided a detected signal in 100% of cases.

⇨ **Robustness of Bio-T kit® TriStar Covid-19 with TRISTAR Amplification Program is confirmed for 15 µl of Master-Mix with variations of nucleic acids volume, time or primer annealing and elongation temperature.**

Characterization of the complete method for oral and nasal swab.

1) Repetability and Intermediate Fidelity of the complete method

Repeatability of the method

The repeatability of complete method including sample extraction with BioExtract® Premium Mag and qRT-PCR with Bio-T kit® Tristar Covid-19 was determined using three positives samples coming from patient previously diagnosis with an official method. These patients have been informed that their sample were used for RT-PCR validation and have given their consent. These samples were collected and handled by qualified staff member in accordance with the instructions for use of the sampling device, then stored at ≤ -16°C until their use. Each sample were extracted in one serie of extraction with 3 replicates of extraction by serie. Each obtained extracted nucleic acid were analysed with Bio-T kit® Tristar Covid-19 on AriaMx™ thermal cycler. The protocol is detailed in the extraction manual of the Bio-T kit® TriStar Covid-19 and page 10. The Coefficient of Variation of Repeatability of Ct values was then evaluated. The Coefficient of Variation (CV) of repeatability of 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 is the repeatability deviations, and M is the overall mean of the values in the series.

Experimental design

Number of extraction serie	Number of replicates by extraction series	Number of qRT-PCR by extraction serie
1	3	1

Data obtained (Ct values):

Target	Positivity level	Repetability (Ct)			Repetability	
		Replicate 1	Replicate 2	Replicate 3	Mean (Ct)	CV%
E gene of SARS-CoV-2	Level 1	22.22	22.26	22.28	22.25	0.14
	Level 2	25.52	25.21	25.42	25.38	0.62
	Level 3	31.95	31.73	31.95	31.88	0.40
ORF1ab of SARS-CoV-2	Level 1	22.28	22.22	22.37	22.29	0.34
	Level 2	25.30	25.37	25.50	25.39	0.40
	Level 3	31.96	31.64	31.82	31.81	0.50
Exogenous IPC	Level 1	25.54	24.94	25.15	25.29	1.17

⇨ **For the BioExtract® Premium Mag extraction method, the coefficient of variation for repeatability ranges from 0.14 to 0.62% for the SARS-CoV-2 *E gene,* from 0.34 to 0.50% for the ORF1ab of SARS-CoV-2 and is at 1.17% for the IPC.**

Intermediate Fidelity

The intermediate fidelity of complete method including sample extraction with BioExtract® Premium Mag and qRT-PCR with Bio-T kit® Tristar Covid-19 was determined using the same sample used in the previous paragraph. Each sample were extracted in two series of extraction with 3 replicates of extraction by serie. Each obtained extracted nucleic acid were analysed with Bio-T kit® Tristar Covid-19 on AriaMx™ thermal cycler according to the protocol detailed page 10. At the end of these two series, the coefficient of variation of intermediate fidelity of the values of Ct can be determined, subject to positioning the threshold line according to common criteria. The calculation formula used is: by dividing the standard deviations by the mean according to the formula :

$$CV_{\text{Intermediate Fidelity}} = \frac{Sr}{M} * 100$$

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

Experimental design

Number of extraction serie	Number of replicates by extraction series	Number of qRT-PCR by extraction serie
2	3	1

Data obtained (Ct values):

Target	Positivity level	Intermediate Fidelity (Ct)			Intermediate Fidelity	
		Replicate 1	Replicate 2	Replicate 3	Mean (Ct)	CV%
E gene of SARS-CoV-2	Level 1	22.22	22.47	22.67	22.45	1.00
	Level 2	25.52	25.42	25.88	25.61	0.94
	Level 3	31.95	33.77	33.90	33.21	3.28
ORF1ab of SARS-CoV-2	Level 1	22.28	22.37	22.68	22.44	0.93
	Level 2	25.50	25.97	26.11	25.86	1.24
	Level 3	31.96	31.64	34.02	32.54	3.97
Exogenous IPC	Level 1	24.94	25.15	25.53	25.20	1.09

⇨ **For the BioExtract® Premium Mag extraction method, the coefficient of variation for Intermediate Fidelity ranges from 0.94 to 3.28% for the SARS-CoV-2 *E gene,* from 0.93 to 3.97% for the ORF1ab of SARS-CoV-2 and is at 1.09% for the IPC.**

2) Diagnostic Specificity and Sensitivity on known status samples

The diagnostic specificity and sensitivity of the complete method combining BioExtract® Premium Mag with Bio-T kit® TriStar-Covid-19 was analyzed using data submitted by a partner laboratory performing CoV-2 SARS detection tests with our method (extraction described in RT-PCR and extraction manual of the Bio-T kit® Tristar Covid-19, and described on page 10).

20 samples from patients previously adjudicated for SARS-CoV-2 with a reference method (confidential internal method using the detection of ORF1ab and the SARS-CoV-2 *N gene*) were thus analyzed by our partner laboratory. From these 20 sample, 13 are expected to be positive and 7 are expected to be negative. The results are analyzed and expressed:

- For diagnostic specificity (Sp): in percentage of negatives found among the expected negatives.
- For diagnostic sensitivity (Se): in percentage of positives found among the expected positives.

Raw data (Ct values)

BioExtract® Premium Mag						
	Reference	Comment	Ct *E gene*	Ct ORF1ab	Ct IPC	Status Bio-T kit® TriStar Covid-19
Sample 1	POSITIVE		21.72	21.07	28.73	POSITIVE
Sample 2	POSITIVE		14.13	13.84	28.02	POSITIVE
Sample 3	POSITIVE		15.53	14.90	27.73	POSITIVE
Sample 4	POSITIVE		29.72	28.48	27.79	POSITIVE
Sample 5	POSITIVE		32.51	32.86	27.86	POSITIVE
Sample 6	POSITIVE		ND	35.40	27.94	POSITIVE
Sample 7	NEGATIVE	Previously positive patient in virology and serology (IgG+ IgM-)	ND	37.94	27.34	POSITIVE At detection limit
Sample 8	NEGATIVE		ND	ND	28.00	NEGATIVE
Sample 9	NEGATIVE		ND	ND	27.91	NEGATIVE
Sample 10	NEGATIVE		ND	ND	28.68	NEGATIVE
Sample 11	NEGATIVE		ND	ND	27.44	NEGATIVE
Sample 12	POSITIVE		29.75	29.29	27.37	POSITIVE
Sample 13	POSITIVE		19.97	19.96	26.80	POSITIVE
Sample 14	POSITIVE		30.23	29.75	27.12	POSITIVE
Sample 15	POSITIVE		33.97	33.77	27.20	POSITIVE
Sample 16	POSITIVE		33.82	34.04	27.48	POSITIVE
Sample 17	POSITIVE		35.54	32.88	28.42	POSITIVE
Sample 18	NEGATIVE		ND	ND	27.39	NEGATIVE
Sample 19	NEGATIVE		ND	37.05	27.67	POSITIVE At detection limit
Sample 20	POSITIVE		19.62	19.40	27.93	POSITIVE

ND : Non detected– no amplification signal

Data obtained

		Known status	
		+	-
Bio-T kit® TriStar Covid-19	+	13	2
	-	0	5
	Total	13	7

⇨ **On the sample panels analyzed, for the BioExtract® Premium Mag method, the diagnostic specificity is Sp = 75%.**

It should be noted that the Sp can reasonably be established at 86% based on the status of sample 7, which was taken from a patient who was previously positive for SARS-CoV-2 with the reference method, then retested by this method a few days later and was identified as negative. It was analyzed positive in ORF1ab on our method but at the limit of detectability. At this level of positivity, it is likely that this result is not repeatable. In addition, it is also possible that there is a slight difference in sensitivity between our method and the reference method, explaining the discordant result. In this context, sample 7 could be considered positive by the reference method.

⇨ **The diagnostic sensitivity is Se = 100%.**

27 chemin des Peupliers

69570 Dardilly, France

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