RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT

Qualitative detection of SARS-CoV-2 RNA and mutations K417N, L452R and E484K by multiplex fluorescent RT-qPCR

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CE IVD

INTENDED USE: *IN VITRO* DIAGNOSTICS

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I. Product name

RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69). Qualitative detection of SARS-CoV-2 RNA and K417N, L452R and E484K mutations by multiplex fluorescent RT-qPCR.

II. Intended use of the product

RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69) is optimised to perform as both a 1st and/or 2nd intention diagnosis of SARS-CoV-2, while allowing for the suspect determination of variants (Beta, Delta/Kappa and Omicron). This is achieved through simultaneous detection of highly conserved sequences of the **ORF1ab** and **N** genes of SARS-CoV-2 as well as S gene mutations **K417N**, **L452R** and **E484K**, by multiplex RT-qPCR, on patient nasopharyngeal and salivary samples.
RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69) contains :

- An enzymatic solution allowing, in 1 hour and 30 minutes, to carry out the reverse transcription (RT) reaction and real-time PCR amplification.
- A positive control consisting of inactivated viruses (SARS-CoV-2 variant Delta and Beta) and human cell line RNA. It is used to validate the extraction, RT and PCR steps.
- A negative control (qualified human cell line RNA without the presence of SARS-CoV-2 or other pathogens). It is used to validate the extraction, RT and PCR steps.

RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69) is optimised and validated for the diagnosis of SARS-CoV-2 in 1st and/or in 2nd intention. It allows the detection of the ORF1ab/N genes of the initial strain (Wuhan-Hu-1/2019) and the alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), Kappa (B.1.617.1) and Omicron (BA.1, BA.2) **(Table 1)**, on nasopharyngeal and salivary swabs from patients with signs and symptoms suggestive of SARS-CoV-2 infection (e.g. fever and/or symptoms of acute respiratory illness), from patients who have had contact with an individual who has contracted the virus, patients likely to be infected as a result of their presence in a gathering or cluster, or who are to be screened by the health authorities, and patients who are about to travel, or have travelled, outside of France or abroad.

Table 1 Variants detected by the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* (Appolon Bioteck, KSCV96AB69).

Gene: mutation	Alpha B.1.1.7	Beta B.1.351	Gamma P.1	Delta B.1.617.2	Kappa B.1.617.1	Omicron BA.1, BA.2
ORF1ab/N	+	+	+	+	+	+
S: K417N		+				+
S: L452R				+	+	
S: E484K		+	+			

The medical management of the patient should be combined with the clinical findings and biological data, the patient's history and epidemiological information otherwise available. Positive results cannot exclude bacterial infection or co-infection with viruses other than SARS-CoV-2. Negative results do not exclude infection with SARS-CoV-2. They should not be used as the sole basis for patient management decisions.

RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69) is intended for use by medical laboratories duly trained and authorised in good laboratory diagnostic practice procedures. It is exclusively reserved for medical biology laboratories with a structure that complies with the recommendations of the advising health authority, and is recommended to be used by qualified staff trained in molecular biology techniques, in particular real-time PCR. Laboratories are required to report all results to the relevant health authorities.

RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69) is CE-IVD marked according to European Directive 98/79/EC and complies with the requirements of ISO 13485.

III. Product description

The *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING* KIT (Appolon Bioteck, KSCV96AB69) allows the simultaneous detection of ORF1ab/N genes and S gene mutations (K417N, L452R and E484K) of SARS-CoV-2 allowing the screening of different variants including Beta (B.1.351), Delta (B.1.617.2) / Kappa (B.1.617.1) and Omicron (BA.1, BA.2). An independent specific signal, derived from the amplification of a human control mRNA (RNase P), demonstrates the correct execution of the different technical steps from sample processing to result reporting (RNA extraction, reverse transcription and PCR).

The *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* (Appolon Bioteck, KSCV96AB69) allows 96 tests to be performed. It consists of tubes with different cap colours. Included in the box (**Table 2**):

- RT-qPCR mix: 1 tube with translucent cap.
- Positive control: 1 red cap tube.
- Negative control: 1 blue-capped tube.
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Table 2 Composition of the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING* KIT *(Appolon Bioteck, KSCV96AB69)*.

Name of the component	Composition	Quantity	Volume per tube	Targets (Fluorescence)	Storage	Conservation
RT-qPCR mix	RT-qPCR reaction mixture : • Primers and probes specific to the N gene, ORF1ab of SARS-COV-2 and the K417N, L452R and E484K mutations • Specific primers and probe for the human RNase P gene mRNA • Tris-HCl, KCl, $(NH_4)_2SO_4$, $MgCl_2$, dNTPs, UDG... • Enzymes (reverse transcriptases and heat-resistant DNA polymerase).	1 tube (96 tests) Translucent cap	1500 µL	ORF1ab/N (Cy5.5) K417N (HEX) L452R (FAM) E484K (ROX) RNase P (Cy5)	**-25** °C / -15 °C	12 months
Positive control	3 inactivated and quantified SARS-CoV-2 variants (Beta, Delta and Omicron) and cells from a qualified human line without the presence of SARS-CoV-2 or other pathogens.	1 tube Red cap	150 µL			
Negative control	Cells of a qualified human line without the presence of SARS-CoV-2 or other pathogens, in a virus inactivation and preservation solution.	1 tube Blue Cap	150 µL			

IV. Required equipment not included in the kit

IV.1. Viral collection and transport material

- Collection material for nasopharyngeal swabs :
  - Sterile dry swabs.
  - Viral transport and inactivation medium (Appolon Bioteck, MTL02-AB69).

- Saliva collection equipment :
    - Saliva collection kits with sterile collection tube and funnel.
    - Sampling pipette and tube containing ready-to-use inactivator buffer.

RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69) is recommended for use with **MTL Viral Transport Medium** (Appolon Bioteck, MTL02-AB69). For other transport media, refer to the supplier's recommendations.

IV.2. Laboratory equipment and reagents

All standard molecular biology laboratory equipment, including but not limited to:

- Freezer -20°C.
- Fridge +4°C.
- Powder-free disposable gloves.
- Laboratory coat.
- Benchtop centrifuge with plate and/or for 1.5 mL and 2.0 mL microtubes.
- Benchtop vortex.
- Adjustable micropipettes P10, P20, P200, P1000.
- Sterile filter tips for 10 µL, 20 µL, 200 µL, 1000 µL.
- Eppendorf microtubes 1.5 mL or equivalent.
- 96 position 0.2 mL real-time PCR strips or plates.
- Specific optical films for PCR (or optical plugs if strips are used).
- Microbiological Safety Station (MSS) for handling Class I or II pathogens.
- Organic hood.
- Cold rack or crushed ice.
- Molecular biology grade water (no nucleic acid activity).
- If using the Genolution NX-48S magnetic bead RNA extraction system: Genolution extraction kit, VN143.
- DNA-RNA decontamination products.

IV.3. RNA extraction systems

RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69) can be used from samples (nasopharyngeal or salivary) collected and transported in MTL viral transport medium (Appolon Bioteck, MTL02-AB69), allowing the extraction of viral RNA. Alternatively, a nucleic acid extraction system can be used prior to testing: we recommend the use of a magnetic bead extraction (Genolution, NX 48S).

The user should ensure that the nucleic acid extraction system used is compatible with real-time RT-qPCR technology by referring to the recommendations for use from the selected supplier.

IV.4. Real-time PCR systems

RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69) is validated for use with the following real-time PCR systems:

- Line-Gene 9600 Plus Real-time PCR Detection System.
- CFX96 Touch™ real time PCR.

For all other systems, please follow the usage recommendations of the selected supplier and carry out a prior technical validation.

V. Principle of the test

RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69) is based on the amplification of specific regions of the SARS-CoV-2 genome, corresponding to the ORF1ab,

N and S genes (Beta (B.1.351), Delta (B.1.617.2) / Kappa (B.1.617.1) and Omicron (BA.1, BA.2)). The reaction is based on the 5' exonuclease property of *Taq* polymerase and its ability to degrade the hydrolysis probes included in the reaction mixture. This results in the release of a specific fluorescence that can be read by optical sensors in the thermal cycler, allowing real-time monitoring of the amplification of the target(s).

For the performance of the test, all necessary components are included in the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* (Appolon Bioteck, KSCV96AB69):

- **RT-qPCR mix**

The reaction solution consists of 6 primer pairs and 6 TaqMan™ probes. Five are specific to the genomic regions of SARS-CoV-2: ORF1ab/N genes and S gene (K417N, L452R and E484K mutations). The hydrolysis probes (TaqMan™) are labelled at their 5' ends with the fluorochromes Cy5.5 (ORF1ab and N genes), HEX (K417N), FAM (L452R) and ROX (E484K). A sixth primer pair and a hydrolysis probe (TaqMan™), labelled with the Cy5 fluorochrome at its 5' end are used in the kit as an internal RT-qPCR control allowing the detection of human RNase P gene mRNA **(Figure 1)**. This internal control is amplified in each sample including the positive and negative controls (provided in the kit) to validate the nucleic acid extraction or lysis process, to assess the inhibition status of the sample and to validate the reverse transcription/PCR (RT-PCR) steps.

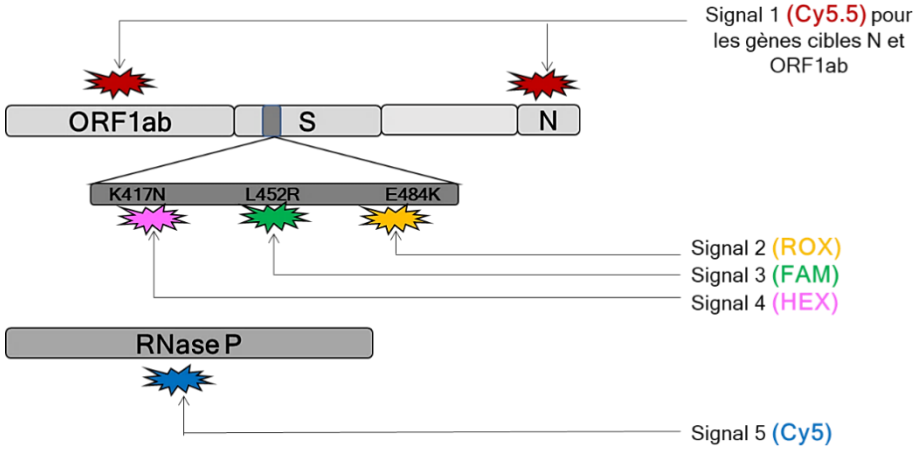

Figure 1 General principle of operation of the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING* KIT *(Appolon BIOTECK: KSCV96AB69).*

- **Positive control**

The positive control allows the validation of the RT-qPCR by the joint detection of the SARS-CoV-2 ORF1ab and N genes, of each of the mutations tested (K417N, L452R, E484K) as well as of the RNase P gene mRNA (endogenous RT-PCR control). It consists of 2 inactivated variants of the SARS-Cov-2 virus, and human lineage cells:

- Variant Beta (B.1.351), with ORF1ab, N and S genes of SARS-CoV-2 and the K417N and E484K mutations of the S gene encoding the Spike protein.
- Delta variant (B.1.617.2), with ORF1ab, N and S genes of SARS-CoV-2 and the L452R mutation of the S gene encoding the Spike protein.
- A qualified human cell line free of SARS-CoV-2 or other pathogens in a viral inactivation and preservation solution comprising RNase P housekeeping gene mRNA.

- **Negative control**

The negative control consists of a human cell line, containing the human RNase P gene, qualified without the presence of SARS-CoV-2 or other pathogens. This control is placed in a virus inactivation and nucleic acid preservation solution.

NB: The positive and negative controls allow the evaluation of the efficiency of the sample preparation, the correctness of the technical process and the absence of inhibitors in the RT-qPCR reaction.

V.1. Types of samples

The types of samples that can be analysed are :

- Nasopharyngeal swabs.
- Saliva samples.

Samples can be taken from patients, for example:

- With signs or symptoms suggestive of SARS-CoV-2 infection (fever and/or symptoms of acute respiratory illness),
- Being a contact case of an individual who has contracted the virus,
- From known SARS-CoV-2 clusters,
- Subject to screening campaigns implemented by the health authorities,
- About to make, or having made, a trip abroad or outside mainland France.

V.2. Collection methods

Reference should be made to the recommendations of the relevant health authorities as well as those of the supplier of the chosen sampling device.

- **Nasopharyngeal swab**
  1- Label the patient's identity on the tube (barcode...).
  2- Immerse the swab in MTL Viral Transport Medium (Appolon Bioteck, MTL02-AB69) immediately after taking the nasopharyngeal swab.
  3- Twist the swab to release the biological material into the MTL collection tube.
  4- Remove the swab from the tube.
  5- Dispose of the swab in a special bin for biohazardous materials.
  6- Close the tube carefully with the dedicated cap.
- **Saliva sampling**
  1- Label the patient's identity on the tube (barcode...).
  2- Collect salivary sputum using the selected saliva collection kit.
  3- Transfer 100 µL of saliva sample to MTL viral transport medium (Appolon Bioteck, MTL02-AB69).
  4- Close the tube carefully with the dedicated cap.

VI. Precautions for use

VI.1. General precautions

- Avoid contact between the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* (Appolon Bioteck, KSCV96AB69) reagents and the skin. In case of skin contact, wash immediately with plenty of water.
- Prepare samples under a microbiological safety cabinet (MSC).
- Unused reagents from the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* (Appolon Bioteck, KSCV96AB69) can be considered non-hazardous and disposed of according to standard laboratory procedures.
- Any contaminated product or infectious sample must be disposed of according to the regulations in force (procedures for the disposal of DASRI).
- Do not use the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* reagents (Appolon Bioteck, KSCV96AB69) after the stated expiry date.

VI.2. Special precautions for molecular biology

Amplification procedures require special facilities and trained personnel to avoid cross-contamination and the spread of amplified genetic material.
The different stages of the procedure should be carried out in separate rooms to avoid any risk of contamination. The transition from one area to another should be unidirectional from the collection area to the reagent preparation area and then to the PCR amplification area.

⚠ **Important:**

- Gowns, pipettes and other small laboratory equipment should be dedicated to each work area.
- Never exchange equipment from one area to another as this may contaminate the laboratory.
- Never introduce amplified material into the reagent or sample preparation rooms.
- Do not substitute reagents between different batches.
- Do not substitute reagents with those of other manufacturers.
- Do not use the reagents if they are received thawed or in a damaged package.
- Avoid repeated freezing/thawing of reagents.
- Reagents must be completely thawed using a cold block (+4°C) before use.
- Thawing is complete within 15 minutes. Keep the reagents on the cold block during the whole handling.
- Wear disposable gloves.

VI.3. Preparation, transport and storage of samples

⚠ **Important:**

- Inappropriate sampling, sample processing, transport and storage conditions may lead to erroneous results.
- Samples must be taken according to the laboratory's instructions.
- The transport of samples must be carried out in accordance with the regulations in force.
- If samples are transported in a transport medium other than MTL (Appolon Bioteck MTL02-AB69), we recommend that you follow the supplier's recommendations for molecular biology applications.
- The performance of the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* (Appolon Bioteck, KSCV96AB69) was demonstrated on nasopharyngeal swabs (nasopharyngeal swab) and salivary swabs (salivary sputum or pipetted specimen) collected on MTL transport medium (Appolon Bioteck, MTL02-AB69) and followed or not by magnetic bead extraction (Genolution, NX-48S)

MTL medium allows collection, inactivation, viral membrane lysis and preservation of viral nucleic acids during transport at +4°C.

The recommended storage conditions for samples after collection are as follows:

- 7 days at +4°C
- 6 months at -20°C (with a maximum of 3 freeze/thaw cycles).

VII. Protocol

VII.1. SARS-CoV-2 RNA extraction protocol

VII.1.1. Chemical lysis extraction in MTL transport medium (Appolon Bioteck, MTL02-AB69)

Prior to collection, record the patient's identifiers on the MTL transport tube (Appolon Bioteck, MTL02-AB69) and follow the instructions below:

Nasopharyngeal swabs collected on swabs:

1. Place the swab in a tube of MTL transport medium (Appolon Bioteck, MTL02-AB69) without touching the edges.
2. Return the collected biological material to the collection tube.
3. Remove the swab from the tube, without touching the sides of the tube.
4. Dispose of the swab in a special bin for biohazardous materials.
5. Close the tube carefully with the dedicated cap.
6. Vortex the sample in its MTL transport medium for 1 minute.
7. Allow the tube to stand, without shaking, for 5 minutes and repeat this process twice.

Saliva samples :

1. Dispense 100 µL of saliva into a tube of MTL transport medium (Appolon Bioteck, MTL02-AB69) without touching the rim.
2. Close the tube carefully with the dedicated cap.
3. Vortex the sample in its MTL transport medium for 1 minute.
4. Allow the tube to stand, without shaking, for 5 minutes and repeat this process twice.

VII.1.2. Nucleic acid extraction with magnetic beads

⚠ RNA extraction from nasopharyngeal and salivary samples can be performed by an automated magnetic bead system or equivalent. This kit has been validated for use with the NX-48S (Genolution) magnetic bead extraction system. For all other machines, it is recommended to follow the recommendations mentioned in the instructions for use of the selected extraction devices and systems.

VII.2. Real-time amplification protocol

⚠ **Important:**

- To avoid contamination, prepare reagents in a dedicated RT-PCR workstation.
- Do not use the same pipette for controls and extracted biological/RNA samples.
- Always use sterile anti-aerosol filter pipette tips.
- Maintain an RNase-free environment.
- Protect the tests from light.
- Once the PCR plate is sealed with an adhesive film, place it immediately into the real-time PCR system.

For each RT-qPCR plate, include the following controls in the test series:

- The positive control (red cap) included in the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING* KIT (Appolon Bioteck, KSCV96AB69), 5 µL per reaction.
- The negative control (blue cap) included in the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING* KIT (Appolon Bioteck, KSCV96AB69), 5 µL per reaction.

- No Template Control (NTC): molecular biology grade water free of DNA, RNA, DNases and RNases.

1. Establish a sample plate plan specifying the identity of patients.
2. Thaw the reagents and for each RT-qPCR run, plan the number of reactions as follows:
    - 1 reaction for each sample (patient) to be tested.
    - 1 reaction for the positive control.
    - 1 reaction for the negative control.
    - 1 reaction for the negative PCR amplification control (NTC, consisting of qualified molecular biology grade water free of DNA, RNA, DNases and RNases).
3. Make the following reaction mixtures, also shown in **Table 3**:
    a. Dispense 15 µL of the amplification mix into each PCR well according to the number of patients and controls.
    b. Add 5 µL of each extracted RNA, following the plate layout.
    c. Add 5 µL of Positive Control (Red cap) to the Positive Control well.
    d. Add 5 µL of the negative control (Blue cap) to the negative control well.
    e. Apply 5 µL of qualified molecular biology grade water free of DNA, RNA, DNases and RNases (negative PCR control: NTC).
    f. Seal the plate with optical adhesive film.
    g. Lightly centrifuge the plate.
    h. Run the thermal amplification profile (**Table 4**) with readings of the Cy5, Cy5.5, FAM, HEX and ROX fluorescence channels (**Table 5**).

Table 3 Volumes to be dispensed per RT-qPCR well.

Product	**Sample volume/well (patient)**	**Volume /wells Negative control**	**Volume /wells Positive control**	**Volume /wells Negative PCR control (NTC)**
RT-qPCR mix - *translucent cap*	15 µL	15 µL	15 µL	15 µL
RNA extracted from the test sample	5 µL	-	-	-
Positive control *red cap*	-	-	5 µL	-
Negative control *blue cap*	-	5µL	-	-
Molecular biology quality water	-	-	-	5 µL
Total volume of reaction	**20 µL**	**20 µL**	**20 µL**	**20 µL**

Table 4 RT-qPCR programme.

Steps	Time	Temperature	Cycles	Fluorescence acquisition
Reverse transcription (RT)	**10 minutes**	**50°C**	**1**	Not applicable
Activation of *Taq* polymerase	**2 minutes**	**95°C**	**1**	Not applicable
Amplification	**15 seconds**	**95°C**	**40**	Cy5 Cy5.5 FAM HEX ROX
	45 seconds	**57°C**		

Table 5 Fluorescence reading channels and associated targets.

Playback channel	Associated target
Cy5	Human RNase P
Cy5.5	ORF1ab / N (SARS-CoV-2)
HEX	K417N (S gene, SARS-CoV-2)
FAM	L452R (S gene, SARS-CoV-2)
ROX	E484K (S gene, SARS-CoV-2)

VIII. Analysis of the results

For any test analysed by the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING* KIT (Appolon Bioteck, KSCV96AB69), the amplification curves should be examined carefully. If the automatic detection threshold appears to be incorrectly set (e.g. too low), it may be necessary to apply a manual detection threshold (e.g. raise the threshold).

A signal is considered positive if it has a Ct ≤ 35 and a sigmoidal amplification pattern: this is indicated by "+" in the following tables.

Alternatively, a signal is considered negative (indicated as "-" in the following tables) if these two conditions are not met.

VIII.1. Fluorescence reading channels

The probes used in the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* (Appolon Bioteck, KSCV96AB69) allow :

- Detection of the SARS-CoV-2 ORF1ab and N genes are acquired by the Cy5.5 reading channel,
- Detection of K417N, L452R and E484K mutations are acquired by HEX, FAM and ROX channels, respectively,
- Detection of human RNase P gene mRNA (endogenous control) through the Cy5 channel.

Figure 2 shows an example of the amplification profile of each of the target genes tested.

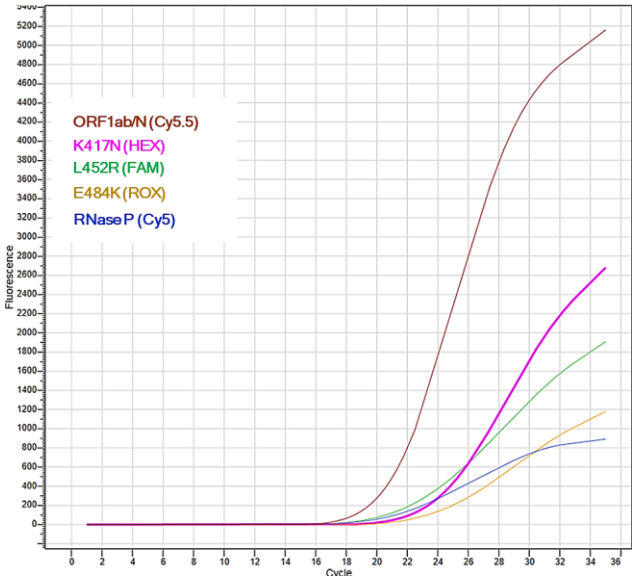

Figure 2 Simultaneous detection of SARS-CoV-2 targets (ORF1ab and N), mutations (K417N, L452R and E484K) and human RNase P mRNA as obtained from the positive control provided.

VIII.2. Validation of controls

For all patients tested with the *SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* (Appolon Bioteck, KSCV96AB69), the amplification curves of the positive and negative controls included in the kit must be integrated into the test flow and carefully reviewed before patient results are reported. A signal is considered positive if it first involves a sigmoidal pattern and a Ct ≤35. Thus, the PCR reaction is considered validated when the criteria listed in **Table 6** are met .

Table 6 Validation criteria for an RT-qPCR series.

	Cy5.5 (ORF1ab/N)	HEX (K417N)	FAM (L452R)	ROX (E484K)	Cy5 (RNase P)	Status of the analysis
Positive control	+	+	+	+	+	Extraction and Validated RT-qPCR
Negative control	-	-	-	-	+	
Negative PCR control (NTC)	-	-	-	-	-	No contamination detected

VIII.3. Interpretation of results

Each sample must be analysed individually. The absence of inhibition for each sample is verified by the presence of an amplification curve in the Cy5 channel corresponding to the endogenous control (RNase P gene). The typical result is summarised in **Figure 3.**

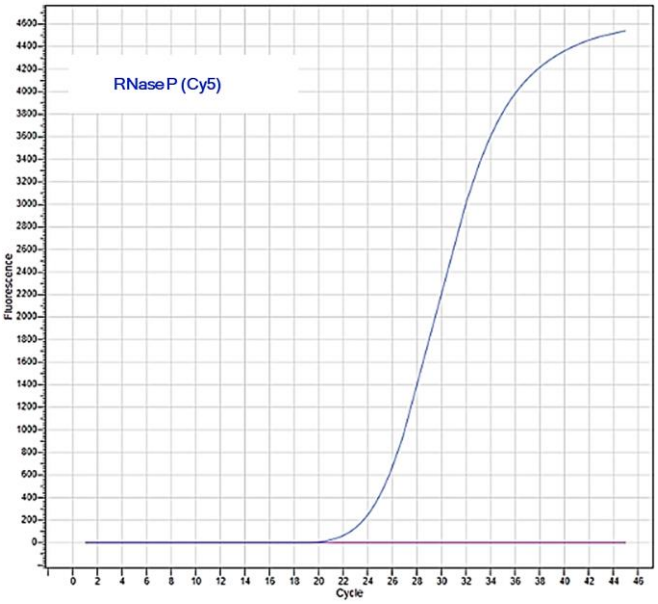

Figure 3 Amplification profile in the 650 nm channel (Cy5) corresponding to the endogenous control (RNase P gene).

The biologist should analyse the amplification curves for each SARS-CoV-2 target tested before reporting the results. **Table 7** summarises the scenarios for the kit results.

Table 7 Interpretation of RT-qPCR results for the sample analysed with *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69).*

ORF1ab/N Cy5.5	K417N HEX	L452R FAM	E484K ROX	RNaseP Cy5	Result	Conclusion
+	-	+	-	+	Presence of the L452R mutation	Presence of the variant Delta or Kappa
+	+	-	-	+	Presence of the K417N mutation	Presence of the variant Omicron
+	+	-	+	+	Presence of K417N and E484K mutations	Presence of the variant Beta
+	-	-	-	+	Presence of ORF1ab and N	POSITIVE SARS-Cov-2
-	-	-	-	+	No SARS-CoV-2 gene is detected	NEGATIVE SARS-CoV-2

VIII.3.1. Positive result

A patient is considered positive when one of the following RT-qPCR patterns is observed (**Figure 4**):

1. SARS-CoV-2 patient (Wuhan-Hu-1/2019): presence of Cy5.5 (ORF1ab/N) and Cy5 (RNase P) amplification curves with Ct≤35: **SARS-CoV-2 patient POSITIVE**
2. **POSITIVE Omicron variant patient**: presence of Cy5.5 (ORF1ab / N), HEX **(K417N**) and Cy5 (RNase P) amplification curves with Ct≤35 (**Figure 4A**).

3. **POSITIVE Beta variant patient**: presence of Cy5.5 (ORF1ab/N), HEX **(K417N**), ROX (**E484K**) and Cy5 (RNase P) amplification curves with Ct≤35 (**Figure 4B**).
4. **POSITIVE Delta / Kappa variant patient**: presence of Cy5.5 (ORF1ab / N), FAM **(L452R**) and Cy5 (RNase P) amplification curves with Ct≤35 (**Figure 4C / 4D**).

NB: In some cases the fluorescence in the Cy5 channel may be absent while the amplification curves in one of the other channels are present, the result must be made positive.

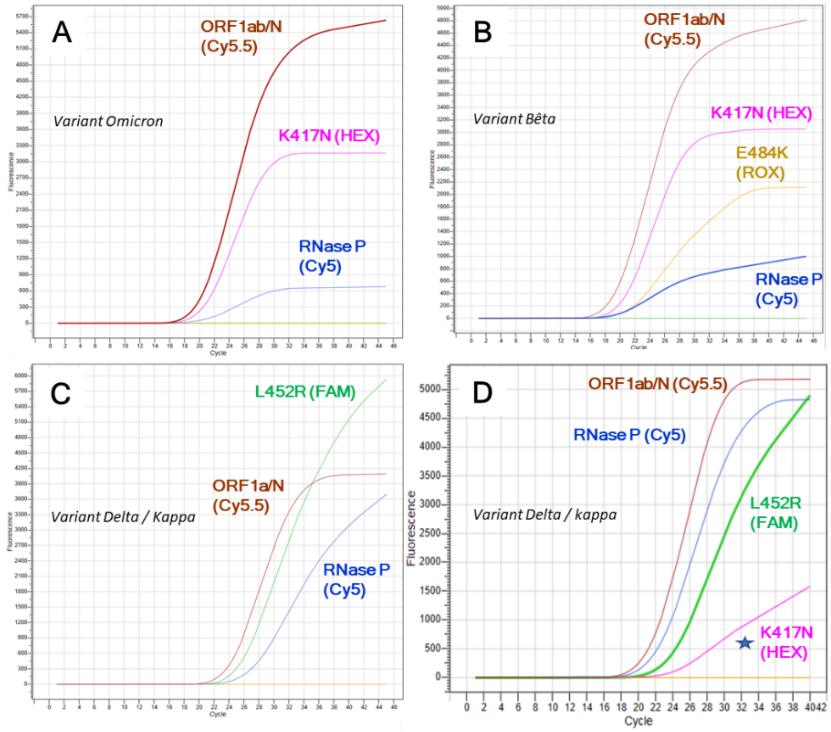

Figure 4 Examples of amplification profiles obtained with positive samples presenting the mutations (A: Omicron, B: Beta, C and D: Delta / Kappa). In some cases, in the presence of the Delta / Kappa variant (L452R mutation) detected in very high intensity, a non-specific signal may appear in HEX (D): This signal should not be taken into account in the result.

VIII.3.2. Negative result

A patient is considered negative when only the amplification curve in the Cy5 channel (RNase P) is detected with a Ct value≤40.

VIII.3.3. Questionable outcome

In the absence of an amplification pattern in the Cy5.5 channel (ORF1ab and N), a result is questionable if an amplification pattern is detected for the other targets tested. In this case, it is recommended that the test be repeated. If the results remain the same, the patient should be considered positive for SARS-CoV-2 for the relevant variant.

VIII.3.4. Invalid result

No amplification curve in all channels is detected.

IX. Limits

- The use of the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING* KIT (Appolon Bioteck, KSCV96AB69) is restricted to personnel properly trained in molecular biology procedures and techniques.
- Failure to follow the instructions in this user manual may lead to incorrect results.

- The performance of the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* (Appolon Bioteck, KSCV96AB69) was established on salivary and nasopharyngeal samples in MTL transport and lysis medium (Appolon Bioteck, MTL02-AB69). Samples should be collected, transported and stored according to appropriate procedures and conditions. Failure to do so may affect the ability of the kit to detect the target sequences of interest.
- Nucleic acid extraction and amplification should be performed using the equipment and methods listed in this user manual. For any other equipment not listed in this manual, we recommend that its effectiveness be assessed by an internal laboratory validation procedure.

False negative results can arise from :

- Non-compliance with specimen collection procedures,
- Non-compliance with the conditions of transport or storage of samples,
- Presence of PCR inhibitors,
- Use of unauthorised or outdated reagents,
- Use of reagents not stored under the conditions specified in the user manual,
- Presence of new mutations in the SARS-CoV-2 genome that have not been identified to date,
- Failure to follow the instructions for using the kit.

False positive results can be caused by :

- Cross-contamination between samples during sample handling,
- Labelling errors,
- Contamination with the positive control during handling.

A negative test result does not prevent subsequent infection with SARS-CoV-2 and should not be the sole basis for the decision to manage the patient. A negative result should be combined with clinical findings, patient history, and epidemiological background.

X. Analytical performance

X.1. Analytical specificity

X.1.1. Inclusiveness

In order to verify the specificity of the primers and probes, an *in silico* sequence homology analysis (Blast N) was performed by comparing the primers and probes to the original genomes deposited in the GISAID database. The results are shown in **Table 8** and show 100% sequence homology for all SARS-CoV-2 strains for at least one of the two conserved markers (Orf1ab and N genes). Any homology less than 100% concerns only one of the two conserved markers whose mismatch(s) do not impact the detection of a SARS-CoV-2 positive patient.

Table 8 *In silico* evaluation of the inclusivity of primer and probe pairs for the SARS-CoV-2 target genes N and ORF1ab, and the target mutations K417N, L452R and E484K. (continued on next page)

Genes	Gene Target N			Target gene ORF1ab		
Primers and probes	Direction	Antisens	Probe	Direction	Antisens	Probe
ID Variant (database GISAID)	Percentage of identity (%)					
hCoV-19/Wuhan/Hu-1/2019|EPI_ISL_402125|WUHAN	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/England/C210000873/2020|EPI_ISL_846588|B1.1.7 ALPHA	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/France/ARA-210015597301/2021|EPI_ISL_1406533|B.1.1.7-ALPHA	22/22 (100%)	21/22 (95%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/South - EPI_ISL_660609 B.1.351 BETA	21/22 (95%)	21/22 (95%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/France/ARA-0001078/2021|EPI_ISL_1190781|B.1.1.351 BETA	21/22 (95%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/Japan/IC-0562/2021|EPI_ISL_792681|Gamma GR/501Y.V3	22/22 (100%)	21/22 (95%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/France/ARA-0011076/2021|EPI_ISL_925031|P1-GAMMA	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/Switzerland/GE-32931080/2021|EPI_ISL_864699|C16 DELTA	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/India/MH-ICMR-MCL_5715_5771/2020|EPI_ISL_3473623|B.1.617.2 DELTA	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/France/ARA-CFD210044293601/2021|EPI_ISL_3432335|B.1.1.617.2 DELTA	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/France/PAC-IHU-29249-Nova1M/2021|EPI_ISL_4393324|A.21 DELTA	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/France/PAC-IHU-10104/2021|EPI_ISL_5032149|A.27 DELTA	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/India/GJ-INSACOG-GBRC2187/2020|EPI_ISL_3717677|B.1.1.617.1 KAPPA	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/France/ARA-HCL021224452901/2021|EPI_ISL_8121710|B.1.1.529 OMICRON	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/England/ALDP-2F86D33/2021|EPI_ISL_8166781|B.1.1.529 OMICRON	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/Germany/SH-ChVir26566_61/2021|EPI_ISL_8152515|B.1.1.529 OMICRON	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/Belgium/CHUNamur13894259/2021|EPI_ISL_8189646|B.1.1.529 OMICRON	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/France/ARA-CFD210070385801/2021|EPI_ISL_7780903|BA.1 OMICRON	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/England/ALDP-2F86D33/2021|EPI_ISL_8166781|B.1.1.529 OMICRON	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/Germany/SH-ChVir26566_61/2021|EPI_ISL_8152515|BA.1 GERMANY	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/USA/OH-CDC-STM-0000013-D09/2021|EPI_ISL_884777|B.1.1	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/England/PHEC-U307UFCD/2021|EPI_ISL_3776866|A.23.1	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/Scotland/GCVR-16FD61/2020|EPI_ISL_632246|A2.3	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/France/ARA-210015587201/2021|EPI_ISL_1406608|Q.7	20/22 (91%)	21/22 (95%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/Belgium/ULG-11353/2021|EPI_ISL_872085|B.1.221 CLADE G	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/Australia/NSW1447/2021|EPI_ISL_812521|B.1.517.1	21/22 (95%)	21/22 (95%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/England/QEUH-1002AD0/2021|EPI_ISL_863493 B.1.177.54	22/22 (100%)	22/22 (100%)	23/24 (96%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/France/IDF_PSL_541/2020|EPI_ISL_1015028|B.1.1.1	22/22 (100%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)
hCoV-19/England/ALDP-1496C6E/2021|EPI_ISL_1486041|B.1.1.318	19/22 (86%)	22/22 (100%)	24/24 (100%)	21/21 (100%)	19/19 (100%)	28/28 (100%)

Table 8 (continued): *In silico* evaluation of the inclusivity of primer and probe pairs for the SARS-CoV-2 target genes N and ORF1ab, and the target mutations K417N, L452R and E484K.

Genes	Mutation K417N			L452R mutation		
Primers and probes	**Direction**	**Antisens**	**Probe**	**Direction**	**Antisens**	**Probe**
ID Variant (database GISAID)	**Percentage of identity (%)**					
hCoV-19/Wuhan/Hu-1/2019|EPI_ISL_402125|WUHAN	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/England/C210000873/2020|EPI_ISL_846588|B1.1.7 ALPHA	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/France/ARA-210015597301/2021|EPI_ISL_1406533|B.1.1.7-ALPHA	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/South - EPI_ISL_660609 B.1.351 BETA	22/22 (100%)	25/25(100%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/France/ARA-0001078/2021|EPI_ISL_1190781|B.1.1.351 BETA	22/22 (100%)	25/25(100%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/Japan/IC-0562/2021|EPI_ISL_792681|Gamma GR/501Y.V3	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/France/ARA-0011076/2021|EPI_ISL_925031|P1-GAMMA	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/Switzerland/GE-32931080/2021|EPI_ISL_864699|C16 DELTA	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/India/MH-ICMR-MCL_5715_5771/2020|EPI_ISL_3473623|B.1.617.2 DELTA	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/France/ARA-CFD210044293601/2021|EPI_ISL_3432335|B.1.1.617.2 DELTA	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/France/PAC-IHU-29249-Nova1M/2021|EPI_ISL_4393324|A.21 DELTA	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/France/PAC-IHU-10104/2021|EPI_ISL_5032149|A.27 DELTA	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/India/GJ-INSACOG-GBRC2187/2020|EPI_ISL_3717677|B.1.1.617.1 KAPPA	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/France/ARA-HCL021224452901/2021|EPI_ISL_8121710|B.1.1.529 OMICRON	No sequence data			No sequence data		
hCoV-19/England/ALDP-2F86D33/2021|EPI_ISL_8166781|B.1.1.529 OMICRON						
hCoV-19/Germany/SH-ChVir26566_61/2021|EPI_ISL_8152515|B.1.1.529 OMICRON	22/22 (100%)	25/25(100%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/Belgium/CHUNamur13894259/2021|EPI_ISL_8189646|B.1.1.529 OMICRON	22/22 (100%)	25/25(100%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/France/ARA-CFD210070385801/2021|EPI_ISL_7780903|BA.1 OMICRON	22/22 (100%)	25/25(100%)	23/23 (100%)	19/19 (100%)	23/23 (100%)	20/21 (95%)
hCoV-19/England/ALDP-2F86D33/2021|EPI_ISL_8166781|B.1.1.529 OMICRON	22/22 (100%)	25/25(100%)	23/23 (100%)	No sequence data		
hCoV-19/Germany/SH-ChVir26566_61/2021|EPI_ISL_8152515|BA.1 GERMANY	22/22 (100%)	25/25(100%)	23/23 (100%)	19/19 (100%)	19/19 (100%)	19/19 (100%)
hCoV-19/USA/OH-CDC-STM-0000013-D09/2021|EPI_ISL_884777|B.1.1	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	19/19 (100%)	19/19 (100%)
hCoV-19/England/PHEC-U307UFCD/2021|EPI_ISL_3776866|A.23.1	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	19/19 (100%)	19/19 (100%)
hCoV-19/Scotland/GCVR-16FD61/2020|EPI_ISL_632246|A2.3	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	19/19 (100%)	19/19 (100%)
hCoV-19/France/ARA-210015587201/2021|EPI_ISL_1406608|Q.7	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	19/19 (100%)	19/19 (100%)
hCoV-19/Belgium/ULG-11353/2021|EPI_ISL_872085|B.1.221 CLADE G	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	19/19 (100%)	19/19 (100%)
hCoV-19/Australia/NSW1447/2021|EPI_ISL_812521|B.1.517.1	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	19/19 (100%)	19/19 (100%)
hCoV-19/England/QEUH-1002AD0/2021|EPI_ISL_863493 B.1.177.54	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	19/19 (100%)	19/19 (100%)
hCoV-19/France/IDF_PSL_541/2020|EPI_ISL_1015028|B.1.1.1	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	19/19 (100%)	19/19 (100%)
hCoV-19/England/ALDP-1496C6E/2021|EPI_ISL_1486041|B.1.1.318	22/22 (100%)	24/25 (96%)	23/23 (100%)	19/19 (100%)	19/19 (100%)	19/19 (100%)

Table 8 (continued): *In silico* evaluation of the inclusivity of primer and probe pairs for the SARS-CoV-2 target genes N and ORF1ab, and the target mutations K417N, L452R and E484K.

Genes	E484K mutation		
Primers and probes	Direction	Antisens	Probe
ID Variant (database GISAID)	Percentage of identity (%)		
hCoV-19/Wuhan/Hu-1/2019|EPI_ISL_402125|WUHAN	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/England/C210000873/2020|EPI_ISL_846588|B1.1.7 ALPHA	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/France/ARA-210015597301/2021|EPI_ISL_1406533|B.1.1.7-ALPHA	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/South - EPI_ISL_660609 B.1.351 BETA	17/17 (100%)	22/22 (100%)	15/15 (100%)
hCoV-19/France/ARA-0001078/2021|EPI_ISL_1190781|B.1.1.351 BETA	17/17 (100%)	22/22 (100%)	15/15 (100%)
hCoV-19/Japan/IC-0562/2021|EPI_ISL_792681|Gamma GR/501Y.V3	17/17 (100%)	22/22 (100%)	15/15 (100%)
hCoV-19/France/ARA-0011076/2021|EPI_ISL_925031|P1-GAMMA	17/17 (100%)	22/22 (100%)	15/15 (100%)
hCoV-19/Switzerland/GE-32931080/2021|EPI_ISL_864699|C16 DELTA	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/India/MH-ICMR-MCL_5715_5771/2020|EPI_ISL_3473623|B.1.617.2 DELTA	15/17 (88%)	22/22 (100%)	14/15 (93%)
hCoV-19/France/ARA-CFD210044293601/2021|EPI_ISL_3432335|B.1.1.617.2 DELTA	15/17 (88%)	22/22 (100%)	14/15 (93%)
hCoV-19/France/PAC-IHU-29249-Nova1M/2021|EPI_ISL_4393324|A.21 DELTA	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/France/PAC-IHU-10104/2021|EPI_ISL_5032149|A.27 DELTA	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/India/GJ-INSACOG-GBRC2187/2020|EPI_ISL_3717677|B.1.1.617.1 KAPPA	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/France/ARA-HCL021224452901/2021|EPI_ISL_8121710|B.1.1.529 OMICRON	15/17 (88%)	20/22 (91%)	13/15 (87%)
hCoV-19/England/ALDP-2F86D33/2021|EPI_ISL_8166781|B.1.1.529 OMICRON	15/17 (88%)	20/22 (91%)	13/15 (87%)
hCoV-19/Germany/SH-ChVir26566_61/2021|EPI_ISL_8152515|B.1.1.529 OMICRON	15/17 (88%)	20/22 (91%)	13/15 (87%)
hCoV-19/Belgium/CHUNamur13894259/2021|EPI_ISL_8189646|B.1.1.529 OMICRON	15/17 (88%)	20/22 (91%)	13/15 (87%)
hCoV-19/France/ARA-CFD210070385801/2021|EPI_ISL_7780903|BA.1 OMICRON	15/17 (88%)	20/22 (91%)	13/15 (87%)
hCoV-19/England/ALDP-2F86D33/2021|EPI_ISL_8166781|B.1.1.529 OMICRON	15/17 (88%)	20/22 (91%)	13/15 (87%)
hCoV-19/Germany/SH-ChVir26566_61/2021|EPI_ISL_8152515|BA.1 GERMANY	15/17 (88%)	20/22 (91%)	13/15 (87%)
hCoV-19/USA/OH-CDC-STM-0000013-D09/2021|EPI_ISL_884777|B.1.1	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/England/PHEC-U307UFCD/2021|EPI_ISL_3776866|A.23.1	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/Scotland/GCVR-16FD61/2020|EPI_ISL_632246|A2.3	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/France/ARA-210015587201/2021|EPI_ISL_1406608|Q.7	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/Belgium/ULG-11353/2021|EPI_ISL_872085|B.1.221 CLADE G	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/Australia/NSW1447/2021|EPI_ISL_812521|B.1.517.1	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/England/QEUH-1002AD0/2021|EPI_ISL_863493 B.1.177.54	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/France/IDF_PSL_541/2020|EPI_ISL_1015028|B.1.1.1	17/17 (100%)	22/22 (100%)	14/15 (93%)
hCoV-19/England/ALDP-1496C6E/2021|EPI_ISL_1486041|B.1.1.318	17/17 (100%)	22/22 (100%)	15/15 (100%)

X.1.2. Exclusive - Cross-reactions *in silico*

The specificity of the *SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT RT-qPCR* primers and probes (Appolon Bioteck, KSCV96AB69) is tested on a panel of genomic sequences from 48 strains (bacterial, viral and fungal). These genomic sequences were obtained from the NCBI Genbank database (https://www.ncbi.nlm.nih.gov/genbank/) and analysed using the *Geneious Prime 2022* software. The results of these analyses are presented in **Table 9** (oligonucleotides for conserved ORF1ab and N genes) and **Table 10** (oligonucleotides for K417N, L452R and E484K mutations) on the following pages. In summary, apart from other coronaviruses, no other organisms studied present a risk of possible *in silico* amplification under the conditions of use of the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* (Appolon Bioteck, KSCV96AB69).

Table 9 *In silico* evaluation of potential cross-reactions of primer and probe pairs for the SARS-CoV-2 target genes ORF1ab and N.

Organism (genome)	Gene Target N			Target gene ORF1ab		
	Direction	Antisens	Probe	Direction	Antisens	Probe
SARS coronavirus BJ01 (AY278488)	19/21 (90%)	14/19 (74%)	27/28 (96%)	20/22 (91%)	20/22 (91%)	18/24 (75%)
SARS coronavirus ZJ01 (AY297028.1)	19/21 (90%)	14/19 (74%)	27/28 (96%)	20/22 (91%)	20/22 (91%)	18/24 (75%)
SARS coronavirus TW1 (AY291451.1)	19/21 (90%)	14/19 (74%)	27/28 (96%)	20/22 (91%)	20/22 (91%)	18/24 (75%)
SARS coronavirus CUHK-L2 (AH013657.2)	19/21 (90%)	14/19 (74%)	27/28 (96%)	20/22 (91%)	20/22 (91%)	18/24 (75%)
SARS coronavirus Urbani (AY278741.1)	19/21 (90%)	14/19 (74%)	27/28 (96%)	20/22 (91%)	20/22 (91%)	18/24 (75%)
SARS coronavirus HKU-39849 (AY278491.2)	19/21 (90%)	14/19 (74%)	27/28 (96%)	20/22 (91%)	20/22 (91%)	18/24 (75%)
SARS coronavirus Tor2 (NC_004718.3)	19/21 (90%)	14/19 (74%)	27/28 (96%)	20/22 (91%)	20/22 (91%)	18/24 (75%)
Human coronavirus OC43 (taxid :31631)	11/21 (43%)	(0%)	(0%)	11/22 (50%)	(0%)	(0%)
Human coronavirus NL63 (taxid :277944)	9/21 (42%)	(0%)	16/28 (57%)	13/22 (59%)	(0%)	(0%)
Human coronavirus 229E (taxid :11137)	12/21 (57%)	(0%)	16/28 (57%)	(0%)	(0%)	(0%)
Human coronavirus HKU1 (taxid :290028)	9/21 (42%)	(0%)	16/28 (57%)	(0%)	(0%)	(0%)
MERS-CoV (taxid :1335626)	13/21 (62%)	10/19 (53%)	16/28 (57%)	11/22 (50%)	(0%)	12/24 (50%)
H1N1 subtype (taxid :114727)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
H3N2 subtype (taxid :119210)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Influenza B virus (taxid :11520)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Influenza C virus (taxid :11552)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
RSV (taxid :12331)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
HBoV4-NI (taxid :1511883)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
HPIV1 (taxid :12730)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
HPIV2 (taxid :1979160)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
HPIV3 (taxid :11216)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
HPIV4 (taxid :1979161)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
HMPV (taxid :162145)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human enterovirus (taxid :1193974)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human adenovirus sp. (taxid :1907210)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human adenovirus 71 (taxid :1643649)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human metapneumovirus (taxid :162145)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human respirovirus 1 (taxid :12730)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human respirovirus 3 (taxid :11216)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human rubulavirus 2 (taxid :1979160)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human rubulavirus 4 (taxid :1979161)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Rhinovirus (taxid :12059)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Chlamydia pneumoniae (taxid:83558)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Haemophilus influenzae (taxid:727)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Streptococcus pneumoniae (taxid:1313)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Streptococcus pyogenes (taxid :1314)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Bordetella pertussis (taxid :520)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Mycoplasma pneumoniae (taxid :2104)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Pneumocystis jirovecii (taxid :42068)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Candida albicans (ASM225980v1)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Pseudomonas aeruginosa (NC_002516)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Staphylococcus epidermidis (AR886665)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Streptococcus salivarius (GQ857551)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)

Table 10 *In silico* evaluation of potential cross-reactions of primer and probe pairs for the target mutations K417N, L452R and E484K.

Organism (genome)	Mutation K417N			L452R mutation			E484K mutation		
	Direction	Antisens	Probe	Direction	Antisens	Probe	Direction	Antisens	Probe
SARS coronavirus BJ01 (AY278488)	28/30 (93%)	(0%)	28/30 (93%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
SARS coronavirus ZJ01 (AY297028.1)	28/30 (93%)	(0%)	28/30 (93%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
SARS coronavirus TW1 (AY291451.1)	28/30 (93%)	(0%)	28/30 (93%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
SARS coronavirus CUHK-L2 (AH013657.2)	28/30 (93%)	(0%)	28/30 (93%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
SARS coronavirus Urbani (AY278741.1)	28/30 (93%)	(0%)	28/30 (93%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
SARS coronavirus HKU-39849 (AY278491.2)	28/30 (93%)	(0%)	28/30 (93%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
SARS coronavirus Tor2 (NC_004718.3)	28/30 (93%)	(0%)	28/30 (93%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human coronavirus OC43 (taxid :31631)	14/30 (46%)	11/23 (43%)	14/30 (46%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human coronavirus NL63 (taxid :277944)	12/30 (40%)	11/23 (43%)	12/30 (40%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human coronavirus 229E (taxid :11137)	11/30 (36%)	11/23 (43%)	11/30 (36%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human coronavirus HKU1 (taxid :290028)	11/30 (36%)	11/23 (43%)	11/30 (36%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
MERS-CoV (taxid :1335626)	11/30 (36%)	(0%)	11/30 (36%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
H1N1 subtype (taxid :114727)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
H3N2 subtype (taxid :119210)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Influenza B virus (taxid :11520)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Influenza C virus (taxid :11552)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
RSV (taxid :12331)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
HBoV4-NI (taxid :1511883)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
HPIV1 (taxid :12730)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
HPIV2 (taxid :1979160)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
HPIV3 (taxid :11216)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
HPIV4 (taxid :1979161)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
HMPV (taxid :162145)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human enterovirus (taxid :1193974)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human adenovirus sp. (taxid :1907210)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human adenovirus 71 (taxid :1643649)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human metapneumovirus (taxid :162145)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human respirovirus 1 (taxid :12730)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human respirovirus 3 (taxid :11216)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human rubulavirus 2 (taxid :1979160)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Human rubulavirus 4 (taxid :1979161)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Rhinovirus (taxid :12059)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Chlamydia pneumoniae (taxid:83558)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Haemophilus influenzae (taxid:727)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Streptococcus pneumoniae (taxid:1313)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Streptococcus pyogenes (taxid :1314)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Bordetella pertussis (taxid :520)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Mycoplasma pneumoniae (taxid :2104)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Pneumocystis jirovecii (taxid :42068)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Candida albicans (ASM225980v1)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Pseudomonas aeruginosa (NC_002516)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Staphylococcus epidermidis (AR886665)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Streptococcus salivarius (GQ857551)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)

X.1.3. Diagnostic specificity

An exclusivity and cross-reaction analysis of the primers and probes used in the kit was performed by RT-qPCR (Appolon Bioteck, KSCV96AB69) on a panel of viral, bacterial and fungal strains in 3 replicates per panel tested (**Table 11**).

Table 11 RT-qPCR results of exclusivity/cross-reaction tests with primers and probes used (ND: Not Detected).

Organization	Source	Concentration cp/ml	ORF1ab/N gene	Mutation K417N	L452R mutation	E484K mutation
Human coronavirus 229E	Vircell Amplirun total respiratory viral panel (MBTC020)	29000	ND	ND	ND	ND
Adenovirus 4		50000	ND	ND	ND	ND
Influenza A H3N2		29000	ND	ND	ND	ND
Novel Influenza virus A H1N1		41000	ND	ND	ND	ND
Influenza B virus		27000	ND	ND	ND	ND
Parainfluenza 1		50000	ND	ND	ND	ND
Parainfluenza 2		33000	ND	ND	ND	ND
Parainfluenza 3		42000	ND	ND	ND	ND
RSV A		29000	ND	ND	ND	ND
RSV B		38000	ND	ND	ND	ND
Bordetella pertussis	Amplirun total stypical bacterial pneumonia control (MBTC022-R)	37000	ND	ND	ND	ND
Chlamydophila psittaci		20000	ND	ND	ND	ND
Chlamydophila pneumoniae		40000	ND	ND	ND	ND
Coxiella burnetii		35000	ND	ND	ND	ND
Legionella pneumophila		33000	ND	ND	ND	ND
Mycoplasma pneumoniae		35000	ND	ND	ND	ND
Mycobacterium tuberculosis	MBTC013	50000	ND	ND	ND	ND
Streptococcus pneumoniae	DSMZ 20566	$1,6x10^9$	ND	ND	ND	ND
Streptococcus pyogenes	DSMZ 20565	$6,2x10^8$	ND	ND	ND	ND
Candida albicans	DSMZ 3454	Unknown	ND	ND	ND	ND
Pseudomonas aeroginosa	DSMZ 22644	$1,4x10^8$	ND	ND	ND	ND
Staphylococcus epidermidis	DSMZ 20044	$3,9x10^8$	ND	ND	ND	ND

No cross-reactivity with pathogen sequences other than those tested by the RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69).

X.2. Analytical sensitivity

The lowest concentration detected at a 95% confidence interval is considered the limit of detection (LoD) of the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* (Appolon Bioteck, KSCV96AB69). The limit of detection is estimated on 4 replicates (dilutions ranging from 10^5 to 10^2 copies of the different inactivated and quantified SARS-CoV-2 variants, diluted in known Covid-19 negative patient samples). These tests are performed for each of the targets of interest on the following matrices:

- Nasopharyngeal swabs, and
  - Magnetic bead extraction (Genolution, NX-48S Viral NA kit), or
  - RNA extraction by chemical lysis in MTL medium (Appolon Bioteck, MTL02-AB69)
- Saliva samples, and
  - Magnetic bead extraction (Genolution NX-48S Viral NA kit), or
  - RNA extraction by chemical lysis in MTL medium (Appolon Bioteck, MTL02-AB69)

The detection limits according to the nature of the sample and the type of extraction are summarised in **Table 12.**

Table 12: RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69) LoD by sample type and extraction type.

Nature of the sample	Type of extraction	Target	LoD (No. of copies / reaction)
Nasopharyngeal	Nucleic acid extraction with magnetic beads	ORF1ab / N	100
		K417N	100
		L452R	100
		E484K	100
	Extraction by chemical lysis	ORF1ab / N	100
		K417N	100
		L452R	100
		E484K	100
Saliva	Nucleic acid extraction with magnetic beads	ORF1ab / N	100
		K417N	100
		L452R	100
		E484K	100
	Extraction by chemical lysis	ORF1ab / N	100
		K417N	100
		L452R	100
		E484K	100

XI. Clinical performance

The clinical performance of Appolon Bioteck's RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (KSCV96AB69) was established on patient samples characterised by French medical laboratories with a CE-marked SARS-CoV-2 RT-PCR kit other than Appolon Bioteck's.

XI.1. Clinical performance on nasopharyngeal swabs

The clinical evaluation of the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING* KIT (Appolon Bioteck, KSCV96AB69) is performed on nasopharyngeal samples with nucleic acid extraction by magnetic beads (Genolution NX-48SViral NA kit) and chemical lysis (MTL; Appolon Bioteck, MTL02-AB69) of patients tested in two French laboratories.

The study included **117** nasopharyngeal specimens **positive** and **92 negative** for SARS-CoV-2 and its Omicron (K417N) and Delta (L452R) variants with magnetic bead extraction, and **117 positive** and **92 negative** for SARS-CoV-2 and its Omicron (K417N) and Delta (L452R) variants for specimens processed by chemical lysis. The clinical samples obtained are tested to generate positive (PPV) and negative (NPV) predictive values, sensitivity and specificity of the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* (Appolon Bioteck, KSCV96AB69).

The summary of clinical performance results on nasopharyngeal swabs is shown in **Table 13**.

Table 13: Summary of clinical performance results of *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69)* on nasopharyngeal swabs

	Statistics	Value	95% CI
Nucleic acid extraction	Sensitivity n=117	100%	96.90% à 100%
	Specificity n=92	100%	96.07% à 100%
	Positive predictive value (PPV)	**100%**	
	Negative predictive value (NPV)	**100%**	
	Accuracy	100%	98.25% à 100%
Chemical lysis	Sensitivity n=117	100%	96.90% à 100%
	Specificity n=92	100%	96.07% à 100%
	Positive predictive value (PPV)	**100%**	
	Negative predictive value (NPV)	**100%**	
	Accuracy	100%	98.25% à 100%

XI.2. Clinical performance on saliva samples

The clinical evaluation of the *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING* KIT (Appolon Bioteck, KSCV96AB69) is performed on salivary samples with nucleic acid extraction by magnetic beads (Genolution NX-48SViral NA kit) and chemical lysis (MTL; Appolon Bioteck, MTL02-AB69) of patients tested in two French laboratories.

The study included **100** saliva samples **positive** and **91 negative** for SARS-CoV-2 and its Omicron (K417N) and Delta (L452R) variants for saliva samples with magnetic bead extraction, and **100** saliva samples **positive** and **91 negative** for SARS-CoV-2 and its Omicron (K417N) and Delta (L452R) variants for samples treated with chemical lysis.

The clinical samples obtained were tested to generate positive (PPV) and negative (NPV) predictive values, sensitivity and specificity of *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT* (Appolon Bioteck, KSCV96AB69), in comparison with concomitant nasopharyngeal swabs.

The summary of clinical performance results on saliva samples is shown in **Table 14**.

Table 14: Summary of clinical performance results of *RT-qPCR SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT (Appolon Bioteck, KSCV96AB69)* on saliva samples.

	Statistics	Value	95% CI
Nucleic acid extraction	Sensitivity n=100	100%	96.38% à 100%
	Specificity n=91	100%	96.03% à 100%
	Positive predictive value (PPV)	**100%**	
	Negative predictive value (NPV)	**100%**	
	Accuracy	100%	98.09% à 100%
Chemical lysis	Sensitivity n=100	100%	96.38% à 100%
	Specificity n=91	100%	96.03% à 100%
	Positive predictive value (PPV)	**100%**	
	Negative predictive value (NPV)	**100%**	
	Accuracy	100%	98.09% à 100%

XI.3. Clinical performance on sequenced samples

Clinical performance was evaluated on samples with sequenced SARS-CoV-2 genome targets. These samples corresponded solely to nasopharyngeal samples (it was impossible to obtain sequenced salivary samples).

Results are given in **Table 15** for nasopharyngeal samples with nucleic acid extraction by magnetic beads (Genolution NX-48SViral NA kit) and chemical lysis (MTL; Appolon Bioteck, MTL02-AB69) respectively.

Table 15: Results of the *SARS-CoV-2 DETECTION AND VARIANTS TYPING KIT RT-qPCR* clinical study (*Appolon Bioteck, KSCV96AB69)* on sequenced nasopharyngeal swabs, with nucleic acid extraction by magnetic beads and chemical lysis

Sample ID	Reference tested samples	Type of sample (sequencing result)	APPOLON Bioteck result	Reference laboratory result
IC-200	A06003631393	21J Delta	**Positive L452R**	**Concordance**
IC-201	c17gn0050295	21J Delta	**Positive L452R**	**Concordance**
IC-203	f06000031521	21J Delta	**Positive L452R**	**Concordance**
IC-204	M79gn0040172	21J Delta	**Positive L452R**	**Concordance**
IC-205	c17gn0060489	21J Delta	**Positive L452R**	**Concordance**
IC-206	19290050477	21J Delta	**Positive L452R**	**Concordance**
IC-207	f12000034098	21J Delta	**Positive L452R**	**Concordance**
IC-208	c17gn0050127	21J Delta	**Positive L452R**	**Concordance**
IC-210	l52gn0101982	21J Delta	**Positive L452R**	**Concordance**
IC-211	14290031309	21J Delta	**Positive L452R**	**Concordance**
IC-212	A16gn0040044	21K (Omicron)	**Positive K417N**	**Concordance**
IC-213	f10000039722	21K (Omicron)	**Positive K417N**	**Concordance**
IC-214	l58gn0100158	21K (Omicron)	**Positive K417N**	**Concordance**
IC-215	l49gn0101203	21K (Omicron)	**Positive K417N**	**Concordance**
IC-216	l49gn0101250	21K (Omicron)	**Positive K417N**	**Concordance**
IC-217	l52gn0072606	21K (Omicron)	**Positive K417N**	**Concordance**
IC-219	l58gn0100266	21K (Omicron)	**Positive K417N**	**Concordance**
IC-220	l73gn0101397	21L (Omicron)	**Positive K417N**	**Concordance**
IC-222	l72gn0101141	21K (Omicron)	**Positive K417N**	**Concordance**
IC-223	56290100391	21K (Omicron)	**Positive K417N**	**Concordance**

XII. Problem solving

XII.1. Amplification problems

Possible causes	Solutions
Alteration of the amplification mix	• Avoid freeze/thaw cycles. • Ensure that the amplification mix has been refrozen to -20°C immediately after use. • Use a cold block (+4°C) when dispensing the PCR master mix and samples. • RT and Taq polymerase enzymes are sensitive to temperature variations. Do not leave at room temperature. • Check the expiry dates of the batch used.
Non-compliance with sample collection, transport and storage conditions	• Follow the instructions for the preparation, transport and storage of samples. • Check the time between the collection of the sample and its analysis by RT-PCR.
Problem during nucleic acid extraction	• Ensure that samples are homogenised before collection for the extraction step. • Check the protocol and equipment used to extract the samples. • Always carry out preventive maintenance on automatic extractors according to the manufacturer's recommendations.
Reagent or sample distribution error	• Make a plate plan to secure transfers. • Check the calibration of pipettes, which should be subject to regular metrological control. • Ensure that the amplification solution, controls and samples are well homogenised before distribution to the PCR plate wells.
Thermocycler programming error	• Check all the programming parameters of the thermal cycler (channel and detection mode, number of cycles, temperature, time, reaction volume, ...).
Amplification problem	• Check the performance of the Peltier block according to the manufacturer's recommendations. • Perform preventive maintenance on real-time PCR equipment according to the manufacturer's recommendations. • Check that the PCR plate is sealed with the optical film. • Check that the plastic consumable used is the one recommended for the instrument used (half-skirt plate, low profile tubes/plate, ...).
Error in the analysis of the results	• Check the adjustment of the threshold line. • Analyse the amplification curves for each target and for each patient. • Check the fluorescence intensity of the amplification curves.
Misinterpretation of results	• Check that the run validation criteria are met as described in the manual. • Check that the thermal cycler used is compatible and is one of the thermal cyclers validated with the kit. • Compare the results of the extraction control sample with those of the negative extraction control. Dilute the sample if necessary.

XII.2. Contamination problems

Possible causes	Solutions
Contamination during the experiment	• Decontaminate all small laboratory equipment with products suitable for removing nucleic acids. • Change the products used (water, PCR reagent, etc.)
Reagent or sample distribution error	• Make a plate plan to secure transfers. • Check the calibration of pipettes, which should be subject to regular metrological control. • Ensure that the amplification mixes, controls and samples are well homogenised prior to dispatch into the amplification microtubes.
Error in the analysis of the results	• Check the adjustment of the threshold line.

XII.3. Inhibited samples

Possible causes	Solutions
Problem during extraction	• Ensure that samples are well homogenised before collection for nucleic acid extraction. • Control the equipment and solutions used for extraction. • Repeat the extraction. • Always carry out preventive maintenance on automatic extractors according to the manufacturer's recommendations.

XIII. Symbols and logos used

IVD	*In vitro* use only		Do not reuse
	Expiry date		Read the instructions before use
	Danger, refer to attached instructions		Fears moisture
	Do not use if packaging is damaged		Store in a dark place
	Date of manufacture	LOT	Batch number
	Manufacturer	CE	The product complies with European requirements

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