COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD

REF MD04901, 96 reactions
MD04902, 4 x 96 reactions

For professional* in vitro *diagnostic use only

CE IVD

EN

Instructions for Use (IFU)

IM-006en

VERSION 03/2022, October 2022

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1. Introduction

The ongoing Coronavirus Disease 2019 (COVID-19) pandemic, first identified in China and rapidly disseminating in most countries, has caused morbidity and mortality at an unprecedent scale globally, which has resulted in global healthcare crises and strained health resources. The causative agent, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), is a novel *Betacoronavirus* with phylogenetic similarity to SARS-CoV. Seasonal influenza, also named seasonal flu, is a contagious viral infection of the respiratory tract with a major global cause of morbidity, mortality and burden on health-care services. However, its fatality rate is much lower than that of COVID-19. The Influenza (Flu) types A and B are the dominant types of circulating influenza viruses, and the most influenza epidemics are related to type A. Zoonotic transmission of avian or swine influenza directly to humans as well as transmission of reassortant virus has caused notable human intermittently pandemics over the last decades. Infections with influenza B (Flu B) virus are generally restricted to humans and less frequently cause epidemic. Human respiratory syncytial virus (RSV) is the most important pediatric viral respiratory pathogen worldwide. RSV is a member of the pneumovirus genus of the paramyxovirus family. This ubiquitous highly infectious agent emerges each year in seasonal epidemics and nearly everyone is infected at least once with in the first 2 years of life. RSV disease is responsible for considerable morbidity and mortality and lacks an approved vaccine or highly effective antiviral therapy. Rapid diagnosis and isolation are needed to avoid nosocomial transmission and to initiate appropriate treatment.

Respiratory infections can “act synergistically,” meaning that potential interactions between SARS-CoV-2 and other respiratory viruses could increase disease severity. All these viruses are highly contagious and transmitted via contact, respiratory droplets (coughing and sneezing) and contaminated surfaces. COVID-19 might be clinically confused with pneumonia caused by RSV or Influenza viruses and co-infection carries a poor prognosis. Further, the wider circulation of other respiratory viruses will apply selection pressures on SARS-CoV-2 and could lead to the emergence of new variants of concern. So, a crucial action is needed to stop a “lethal triple mix” of COVID-19, Flu, and RSV. COVID-19, Flu, and RSV infections are often difficult to differentiate between based on symptoms alone and each of the viruses are highly contagious. As we enter the flu season will be welcome a to test for common respiratory illnesses alongside COVID-19.

Early detection of SARS-CoV-2, RSV and Influenza types A and B viruses is vital in providing rapid treatment to infected patients with respiratory diseases and, thus, to reduce the spread of infections. Combination testing for both COVID-19 and influenza will be beneficial, as a single sample could be used to distinguish the three infections in patients presenting similar symptoms providing the solution for management of influenza-like illness patients.

2. Intended Use

NZYTech´s COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD is a molecular test intended for the rapid qualitative detection of the causative agents of COVID-19, RSV (subtypes A and B) and Influenza (Flu A and Flu B) in human biological samples. However, this kit does not make distinction between Influenza type A and type B as they are both detected in the same fluorescence channel for FAM. In addition, other betacoronaviruses or Influenza viruses, *e.g.,* Influenza C, are not detected with this kit. This test is intended for use as a primary screening in the diagnosis of SARS-CoV-2, Flu A/B and RSV in combination with clinical and epidemiological risk factors. A positive result indicates the presence of SARS-CoV-2 and/or Influenza A/B and /or RSV viral RNA although clinical correlation with patient history and other diagnostic information

is necessary to determine patient infection status. Negative results do not preclude infection and thus the outcome of the test should not be used as the sole basis for patient management decisions. This kit is intended for use by laboratory trained personnel, specifically instructed in real-time PCR techniques and *in vitro* diagnostics.

3. Principles of the Assay

NZYTech´s COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD provides the complete set of reagents and probes to qualitatively detect the SARS-CoV-2 and/or Influenza and/or RSV genomes, through common real-time PCR platforms (see required instrument specifications in **Section 6**). SARS-CoV-2 is identified by detecting RT-qPCR targets located in RdRp and N genes, whereas RSV (subtypes A and B) is identified by detecting targets located in L gene. In contrast, Influenza A and Influenza B viruses are detected through the amplification of targets located in M1 and NS2 genes, respectively. NZYTech´s COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD is built to have the broadest detection profile possible whilst remaining specific to the SARS-CoV-2, RSV, Influenza types A and B genomes. It provides the complete set of reagents and probes to detect the four viral genomes, including an effective internal control to confirm efficient sample RNA extraction and absence of PCR inhibitors, among others. This kit targets highly conserved regions of SARS-CoV-2, RSV (subtypes A and B) and Influenza A and B genomes, through highly optimized primers/probes set. In addition, primers and probes display no significant homology with unrelated genomes rendering this test highly specific, and no cross-reactivity with organisms that can be found in the respiratory tract will be observed. The natural evolution of the viruses detected by this kit implies that new sequence information will become available day by day, which reflects well known viral adaptation strategies. Thus, NZYTech periodically revisits viral genomic targets and, if required, will release new versions of this kit. One-step real-time RT-qPCR is the fastest and most reliable method to perform an accurate detection of SARS-CoV-2, Influenza A and B, and RSV viral RNAs. NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD is a multiplex assay detecting SARS-CoV-2, Influenza A, Influenza B, RSV and Human nucleic acids (acting as an internal positive control). Extracted and purified RNA is transcribed to cDNA and subsequently amplified in a single reaction using six highly specific primers/probe sets, namely those detecting SARS-CoV-2 RNA dependent RNA polymerase (RdRp) and the Nucleocapsid phosphoprotein (N) genes, Influenza A and B Matrix (M1) and Nonstructural 2 (NS2) specific genes, and the RSV L gene and the human ribonuclease P (RNase P, RP) gene. The kit exploits the so-called TaqMan® principle. During this process, probes specifically anneal to their target genes and upon DNA amplification, through two flanking primers, are subjected to degradation leading to the separation of the reporter dye from the quencher thus resulting in an increase in fluorescence. Detection of the internal control (the human RNase P gene) validates the efficacy of the extraction process as well as the absence of PCR inhibitors potentially present in the human biological samples. To allow identifying the amplification of the six specific targets in a single reaction, SARS-CoV-2, Influenza A/B, RSV and human RNase P, specific probes are differently labelled, namely with Texas Red®, FAM™, HEX™ and Cy5 reporter dyes, respectively. Note that this panel contains a duplex assay in the Texas Red® (SARS-CoV-2 RdRp and N specific target genes) and FAM™ (Influenza A and Influenza B specific target genes) channels. This allows reporting an additive performance of assays for SARS-CoV-2 detection but precludes distinguishing between Influenza A/B infections. Also, this test is not intent to differentiate the Influenza A virus subtypes, nor the influenza B virus lineages or the RSV subgroups. If the differentiation of specific Influenza virus strains and RSV subtypes is necessary, additional tests will be required. In addition, the six primers/probe sets are provided

in optimized concentrations guaranteeing that amplification of lower abundant nucleic acids is not compromised when other viral targets are present at higher concentrations.

4. Kit Composition

NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD provides a comprehensive set of reagents sufficient to perform 96 RT-qPCR reactions in a single step.

Kit Component		Volume (per vial)	Number of Tubes	
			MD0490 1	MD0490 2
COVID-19, Flu A/B, RSV MMix	NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix (2x)	1050 µL	1	4
COVID-19, Flu A/B, RSV PPMix	COVID-19, Flu A/B, RSV Primer & Probe Mix (10x)	205 µL	1	4
COVID-19, Flu A/B, RSV POS 1	COVID-19, Flu A/B, RSV Positive Control 1	105 µL	1	4
COVID-19, Flu A/B, RSV POS 2	COVID-19, Flu A/B, RSV Positive Control 2	105 µL	1	4
NTC	No-template Control (RNase/DNase free water)	105 µL	1	4

5. Storage, Stability and Handling Conditions

The NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD is shipped refrigerated. All components should immediately be stored at -85°C to -15°C upon arrival. When in use, the kit components should be returned to the freezer promptly after use to minimise the time at room temperature.

- Minimise the number of freeze-thaw cycles by storing in working aliquots. If appropriate, kit components may be aliquoted into smaller volumes after thawing.
- The COVID-19, Flu A/B, RSV Primer & Probe Mix (10x) should be stored protected from light. Particularly, do not expose the NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix to direct sun light after combining with probe mix.
- If the package that protects the kit arrived damaged, please contact NZYTech.
- Beware to the expiry date indicated on the packaging. NZYTech does not recommend using the kit after the expiry date. On this date, the kit must be discarded following the disposal instructions in **Section 8.2**.

6. Materials and Instrumentation Required but Not Provided

- Real-time PCR Instrument that detects Texas Red, FAM and VIC/HEX fluorescence channels (at emission wavelengths of 615, 520 and 556 nm, respectively). See in **Section 11** the instrument models for which the kit was validated.
- Equipment and consumables for isolating viral RNA from respiratory specimens.
- RNase/DNase free qPCR plasticware: PCR tubes, strips, caps, 96-well plates, adhesive films.
- Pipettors and filter tips (RNase/DNase free).
- Disposable gloves.
- Vortex and centrifuge.

7. Sample Collection and Preparation

Different factors, such as protocol for sample collection from human respiratory specimen (nasopharyngeal or oropharyngeal swabs, nasopharyngeal wash/aspirates, nasal aspirates, sputa, throat rinsing fluid and BAL), sample transport, storage and processing time, are critical to achieve optimal results. The collected samples should be tested as soon as possible. Samples should be transported and stored at low temperatures in accordance with biosafety regulations. RNA or total nucleic acids extracted following a CE IVD protocol are the starting material for NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD. Please ensure RNA samples are suitable in terms of purity, concentration and nucleic acid integrity. A $A_{260/280}$ ratio of ~2 is generally accepted for pure RNA. Since ethanol is a strong PCR inhibitor, it is necessary to eliminate it prior to the elution of the nucleic acid during extraction. NZYTech kit integrates an internal RNA extraction control reaction that targets human RNA, which is co-purified with viral RNA. Human RNA is amplified with the RNase P (RP) primers/probe set. This is useful for checking the efficiency of RNA isolation and/or the presence of inhibitors during sample processing.

8. Precautions and Warnings

As in any analytical testing procedure, good laboratory practices are essential. Carefully follow the procedures and guidelines provided in this handbook to ensure that the test is performed correctly. Any deviation from them may result in assay failure or cause erroneous results. Due to high sensitivity of the kit, special care must be taken to keep reagents and PCR amplification mixes free from contamination.

8.1 Safety Information

Before using the kit, please consult the Safety Data Sheet (SDS) that is available at NZYTech website (**www.nzytech.com**). This kit detection should be performed only by staff trained in the relevant technical and safety procedures in appropriately equipped laboratories. International and national guidelines on laboratory biosafety should be followed in all circumstances.

8.2 Handling and Procedural Requirements

- Only for professional *in vitro* diagnostic use.
- Do not use this kit after expiration date.
- Do not use the test components, if kit sealing is damaged.
- Do not interchange reagents of different production lots.
- No reagents of other manufacturers should be used along with reagents of this test kit.
- DNase/RNase free disposable plasticware and pipettes should be used in all procedures.
- Use DNase/RNase free filter tips throughout the protocol to prevent aerosol and liquid contamination.
- Sample preparation, reaction set up and amplification should be performed in different working areas.
- Positive controls contain high copy number templates; they should be opened and processed away from test samples and kit components to avoid cross-contamination.
- Always use the water provided in the kit (NTC – No-template Control/DNase free water).
- At the end of each testing, clean work surfaces and equipment with a DNA/RNA remover.

- Handle post-amplification plates with care and dispose them immediately after the end of the testing; plates should always be discarded into a proper biohazard container after use.
- Biological samples must be handled as if they are infectious following proper biosafety precautions.
- Residues of chemicals and preparations are generally considered as hazardous waste. The disposal of this kind of waste is regulated through national and regional laws and regulations.
- All results should be interpreted by a health-care professional in the context of patient medical history and clinical symptoms.
- A negative result for any PCR test does not conclusively rule out the possibility of infection.
- Follow good laboratory practices, wear protective clothing, permanently wear disposable powder-free gloves, use goggles and mask. Do not eat, drink or smoke in the working area.

9. Testing Procedure

Please read the instructions for use carefully before performing the assay. Beware that all pipetting steps and experimental plate set-up should be performed on benchtop coolers or ice. After the plate is poured start immediately to the one-step RT-qPCR protocol. Prolonged incubation of reaction mixes at room temperature can lead to PCR artefacts that reduce the sensitivity of detection. Prior to the experiment, start to gently mix the reaction tubes provided, centrifuge for 5 seconds to collect contents at the bottom of the tube and place tubes on ice. **We strongly recommend pipetting COVID-19, Flu A/B, RSV Positive Controls 1 and 2 last to avoid cross contaminations.**

9.1 Reaction set-up

1. Prepare a RT-qPCR mix enough for the number of tests to be performed with a 5% additional volume for pipetting losses. Proceed according to the table below that specify the volumes for 1 and *n* tests (where *n* corresponds to the total number of reactions):

Component	**1 test volume (µL)**	***n* tests (*) volume + 5% (µL)**
COVID-19, Flu A/B, RSV MMix (2x)(**)	10	*n* x 10.5
COVID-19, Flu A/B, RSV Primer & Probe Mix (10x)	2	*n* x 2.1
Final Volume	**12**	***n* x 12.6**

(*) To calculate the total number of reactions needed for each assay, count the number of samples and add three more for the Negative and the two Positive controls.
(**) Please notice that a precipitate in the bottom of the master mix tube may be observed, in particular after multiple freeze/thaw cycles. To ensure optimal performance, please make sure all components are thawed and resuspended prior to use. In this case do not spin the master mix before pipetting.

2. Pipette 12 µL of the RT-qPCR mix into individual wells according to your real-time PCR experimental plate set-up.
3. For the negative control, add 8 µL of NTC instead of RNA template into the negative control well. The final volume should be 20 µL.
4. For the biological samples, add 8 µL of each RNA sample into the sample wells, according to your experimental plate set-up. The final volume in each well should be 20 µL.

5. For the two positive controls, add 8 µL of COVID-19, Flu A/B, RSV POS 1 (detects SARS-CoV-2 ORF1ab, Influenza B NS2, RSV L and human RP genes) and 8 µL of COVID-19, Flu A/B, RSV POS 2 (SARS-CoV-2 N, Influenza A M1, RSV L and human RP genes) instead of RNA template into the positive control wells. The final volume should be 20 µL.
6. Cover and seal the plate with an appropriate optical adhesive film or caps before proceeding with the RT-qPCR and detection steps.
7. Place the reaction plate in the real-time PCR instrument and run the RT-qPCR protocol according to the section below.

9.2 Programming the real-time PCR instrument

The table below displays a standard protocol optimized on a few platforms. However, these conditions may be adapted and validated to suit different machine-specific protocols.

Suggested RT-qPCR Run Settings

Cycles	Temperature	Time	Step
1	50 °C	10 min	Reverse Transcription
1	95 °C	3 min	Polymerase activation
40	95 °C	5 s	Denaturation
	60 °C	30 s	Annealing/Extension*

*Fluorogenic data should be collected during this step through channels Texas Red, FAM, VIC/HEX and Cy5.

Fluorescent Dyes & Detection Channels

Targets	Fluorescent dye	Detection Channels
COVID-19 (SARS-CoV-2)	Texas Red®	Texas Red/JUN
Flu A/B (Influenza A/Influenza B)	FAM™	FAM
RSV	HEX™	VIC/HEX or JOE
RNase P	Cy5™	Cy5

NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD was validated for the following Real Time PCR Systems: Applied Biosystem® 7500 FAST, Applied Biosystem® QuantStudio 5, Roche Life Science LightCycler® 96 System and Bio-Rad® CFX Opus. If other equipment is used, the kit should be validated by the user by using previous characterised samples (both positive and negative).

10. Data Analysis

10.1 Run Validation Criteria

Data analysis is performed by the software of the instrument. Considering performance differences in different real-time PCR instruments, thresholds for the four fluorescence signals (Texas Red, FAM, VIC/HEX and Cy5) are determined automatically by the software with manual adjustments in case this is required. Before analysing samples results, we recommend verifying if the real-time PCR test is valid. Thus, for each plate, please confirm if the results for Positive and Negative controls performed as expected, according to the following criteria:

Positive controls: the amplification of FAM (Influenza B in Control 1 and Influenza A in Control 2), Texas Red® (SARS-CoV-2 ORF1ab gene in Control 1 and N gene in Control 2), VIC/HEX (RSV L gene) and Cy5 (RP gene) curves are positive. Positive controls are expected to amplify at Cts < 32, in the four channels. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

Negative control (no template reaction): no amplification is detected. If the negative control has amplification curves (Texas Red, FAM; VIC/HEX and Cy5) with a sigmoidal shape, sample contamination may have occurred. Repeat the test following good RT-qPCR practices.

If the controls are according with expected, the test is **valid**. Please proceed with interpretation of results for the tested samples.

If any of the controls do not exhibit the expected performance, the assay was compromised or executed improperly and should be considered **invalid**.
Please, repeat the test
If the problem persists, contact the manufacturer

10.2 Test Results Interpretation

NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD uses the following Ct cutoff values for assay targets for results interpretation:

Ct value	Results interpretation
Amplification Ct ≤35	**Detected (+) → POSITIVE**
***No Amplication* Ct>35**	**Not Detected (-) → NEGATIVE**

SARS-CoV-2 is detected if the Texas Red amplification curve displays a sigmoidal shape with a Ct≤35, regardless of what result is obtained for the RNase P (Cy5) assay.

Influenza A and/or Influenza B is detected if the FAM amplification curve displays a sigmoidal shape with a Ct≤35, regardless of what result is obtained for the RNase P (Cy5) assay.

RSV is detected if the VIC/HEX amplification curve displays a sigmoidal shape with a Ct≤35, regardless of what result is obtained for the RNase P (Cy5) assay.

SARS-CoV-2, Influenza A and/or Influenza B, RSV are not detected if Texas Red, FAM and VIC/HEX curves do not amplify or amplify at Ct>35, while the RNase P (Cy5) assay displays a positive sigmoidal curve (Ct≤40).

The **test is invalid** if the SARS-CoV-2, Influenza A/B, RSV and RNase P assays are negative. The test should be repeated with nucleic acid re-purified from the sample.

The following table summarises the interpretation of principal results (evaluate the overall shape of the amplification curves; **only sigmoidal amplification curves are indicative of true amplification**).

SARS-CoV-2 (Texas Red)	**Influenza A/B** (FAM)	**RSV** (VIC/HEX)	**RP** (Cy5)	**Results interpretation**
+	-	-	+/-*	**SARS-CoV-2 detected → POSITIVE**
-	+	-	+/-*	**Influenza A/B detected → POSITIVE**
-	-	+	+/-*	**RSV → POSITIVE**
+	+	-	+/-*	**SARS-CoV-2 and Influenza A/B deteted → POSITIVE**
+	-	+	+/-*	**SARS-CoV-2 and RSV detected → POSITIVE**
-	+	+	+/-*	**Influenza A/B and RSV detected → POSITIVE**
+	+	+	+/-*	**SARS-CoV-2, Influenza A/B, RSV detected → POSITIVE**
-	-	-	+/-*	**SARS-CoV-2, Influenza A/B and RSV not detected → NEGATIVE**
-	-	-	-	**Invalid test, repeat extraction**

** Internal Control detection on the Cy5 channel is not required for positive results on the Texas Red, FAM, or HEX detection channels. A high concentration/load of detectable viral RNA in the sample can lead to reduced or absent internal control signal.*

Note: Interpretation of results must account for the possibility of false negative and false positive results.

• Although the risk of false negative results is mitigated due to the dual target design of the present test, false negative results may be caused by:

- Unsuitable collection, handling and/or storage of samples.
- Sample collected outside viraemic/symptomatic phase.
- Sample degradation.
- Presence of RT-qPCR inhibitors.
- Mutations in the genome of viruses.
- Failure to follow procedures in this handbook.
- Use of unvalidated receiving kits or real-time PCR platforms.

• False positive results may be caused by:

- Unsuitable handling of samples containing high concentration of viral RNA. The high susceptibility of the RT-qPCR method for cross contaminations special care should be taken during RNA isolation
- Unsuitable handling of the positive controls.
- Unsuitable handling of amplified product (post-amplification plate).

Negative results do not preclude infection and the test result should not be used as the sole basis for treatment or other patient management decisions. In addition, this test cannot rule out diseases caused by other bacterial or viral pathogens.

11. Performance Evaluation

This kit performance was validated for the instruments specified in section 9.2 (see above). If other equipment is used, the kit should be validated by the user by using previous characterised samples (both positive and negative).

11.1 Expected Results

Typical amplification plots observed for clinical negative samples (Figure 1A) or samples from patients infected with SARS-CoV-2 (Figure 1B), co-infection with SARS-CoV-2 and Influenza A/B (C) and co-infection with SARS-CoV-2, Influenza A/B, and RSV (D), are presented in Figure 1.

11.2 Limit of Detection (LoD) - Analytical Sensitivity

The analytical sensitivity was defined as the lowest concentration of analyte that could be reliably detected with 95% confidence. This was assessed by testing SARS-CoV-2, RSV, Influenza A and Influenza B nucleic acids at different copy numbers, spiked into RNA extracted from negative oropharyngeal samples, using 3 different kit batches following typical testing reaction conditions. Tests were repeated over 4 days, producing 48 replicates for each concentration tested. Together, the data revealed that NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD detects 0.25 copies/µL of SARS-CoV-2, 0.375 copies/µL of Influenza A, 0.375 copies/µL of Influenza B and 0.375 copies/µL of RSV with a confidence ≥95%.

Thus, the tentative Limit of Detection (LoD) was determined to be 0.25 copies/µL or 250 copies/mL for SARS-CoV-2, 0.375 copies/µL or 750 copies/mL for RSV, 0.375 copies/µL or 375 copies/mL for Influenza A and 0.375 copies/µL or 375 copies/mL for Influenza B. The tentative LoD was confirmed by two different operators using three kit batches in an experiment with a total of 48 replicates of pooled, contrived negative oropharyngeal swabs (OPS) and nasopharyngeal swabs (NPS) specimens were spiked with various concentrations of virus (inactivated) from SARS-CoV-2, Influenza A, Influenza B, RSV A or RSV B. Either NPs or OPS specimens pooled were spiked with various concentrations of virus from the following strains: SARS-CoV-2 (Omicron variant isolate) virus, Influenza A virus: strain A/Michigan/45/2015 (H1N1) pdm09), strain A/Singapore/ INFIMH-16-0019/2016 (H3N2), Influenza B virus strain B/Colorado/06/2017 (Vitoria lineage) and strain B/Phuket/3073/2013 (Yamagata lineage), RSV A and RSV B (strain CH93 (18)-18), at several concentrations and processed through the COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD.

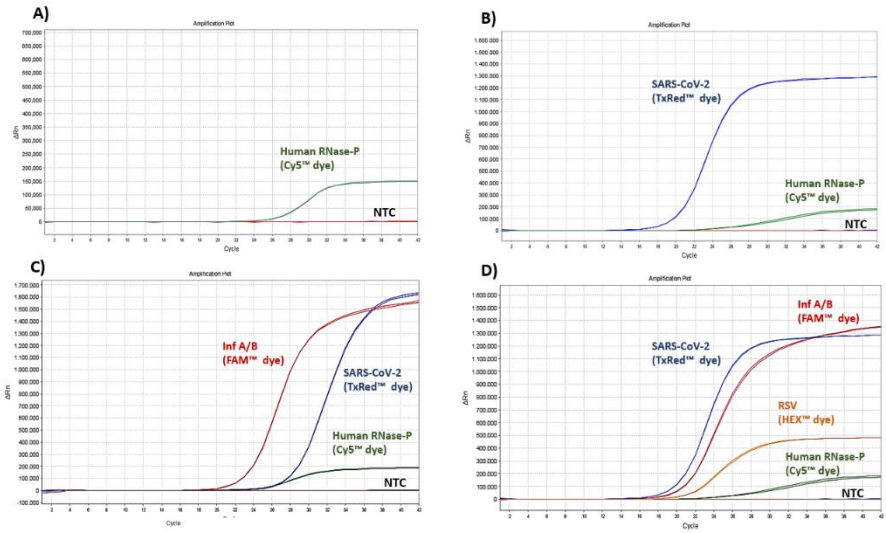

Figure 1. Detection of SARS-CoV-2, Influenza A/B, RSV and Human RNase-P targets from negative clinical samples (A) or clinical samples infected with SARS-CoV-2 (B), co-infection with SARS-CoV-2 and Influenza A/B (C) and co-infection with SARS-CoV-2, Influenza A/B, and RSV (D). Blue curve, detection of the SARS-CoV-2 vRNA targets through the Texas Red/JUN channel. Red curve, detection of Influenza A and/or Influenza B targets through the FAM channel. Orange curve, detection of the RSV L gene through the VIC/HEX/JOE channel. Green curve, detection of the human RNase P gene through the Cy5 channel.

Analytical sensitivity of the NZYTech´s COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD in the context of a co-infection (competitive interference) scenario was evaluated by performing a series of serial dilution experiments using mock co-infection specimens for each one of the viral targets. To create the mock co-infection specimens, exactly 10^3 copies of RSV, 10^3 copies of Influenza A and 10^3 copies of Influenza B nucleic acids were added to the SARS-CoV-2 standard curve. In contrast, 10^3 copies of SARS-CoV-2 and 10^3 copies of RSV and were added to the individually Influenza A and Influenza B standard curves. At last, 10^3 copies of SARS-CoV-2, 10^3 copies of Influenza A and 10^3 copies of Influenza B nucleic acids were added to the RSV standard curve. Quadruplicate samples of three kit batches (making a total of 12 replicates per dilution) were tested with the COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD to determine the sensitivity of the assay when multiple viral targets are present in a sample. The data revealed that LoD of SARS-CoV-2 was unaltered in case of co-infection. However, LoD of RSV changed to 0.75 copies/µL or 750 copies/mL and Influenza B changed to 1.25 copies/µL or 1250 copies/mL in case of a co-infection. For Influenza A the LoD changed to 2.5 copies/µL or 2500 copies/mL in case of a co-infection.

11.3 Analytical Reactivity (Inclusivity) and Analytical Specificity

Inclusivity and cross-reactivity were evaluated by *in silico* analysis of oligonucleotide probes and primers against pathogens related to SARS-CoV-2, Influenza A and Influenza B, RSV and normal pathogens that cause infection with similar symptoms, respectively. Upon *in silico* analysis the assay design was found to detect all SARS-CoV-2, Influenza A and Influenza B, and RSV virus strains and exhibited no reactivity with non-related species. In addition to *in silico* analysis, COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR was performed on nucleic acids of common oral and respiratory tract microbes, including *Bacteroides ovatus*, *Bacteroides thetaiotaomicron*, *Burkholderia vietnamiensis*, *Dickeya dadantii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Mycobacterium intracellulare*, *Mycobacterium mageritense*, *Mycobacterium smegmatis*, *Nocardia nova*, *Pseudomonas mendocina*, *Streptococcus mutans*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptomyces avermitilis*, *Streptomyces albidoflavus*. None of the pathogens tested by the COVID-19 & Flu A/B Multiplex One-Step RT-qPCR Kit (IVD) generated a detectable amplification signal.

The impact of 17 potential interferent substances was assessed in tests consisting of negative nasopharyngeal specimens spiked with SARS-CoV-2 positive specimens at ~3x LoD. Potential interfering substances were added to the contrived samples at concentrations representing the highest levels expected in human respiratory patient samples based on literature data. All tests were performed in triplicate using three kit batches, and results compared to data obtained with a control test that contained no interferents. At the concentrations tested, the results revealed that none of the molecules under test affected the sensitivity of the detection. The table below resumes the data collected under these experiments. All experiments were run on the Applied Biosystems™ 7500 FAST Real-time PCR Instrument (used with 7500 software v2.3).

Potential Interferent	Active Ingredient	Final concentration in sample	Interference Yes (Y) or No (N)				
			SARS-CoV-2	Flu A	Flu B	RSV A	RSV B
Isotonic Sea Water (Rhinomer)	NaCl	15% v/v	N	N	N	N	N
Throat spray, oral anaesthetic, and analgesic (Strepfen)	Flurbiprofen	5% v/v	N	N	N	N	N
Nasal wash solution (Allergy spray - Vibrocil)	Fluticasone propionate	5% v/v	N	N	N	N	N
Nasal Corticosteroids spray (Nasomet)	Mometasone furoate	5% v/v	N	N	N	N	N
Nasal Corticosteroids spray (Pulmicort)	Budesonide	5% v/v	N	N	N	N	N
Antimicrobial, systemic (Trobex)	Trobamycin	10 µg/mL	N	N	N	N	N
Mouth analgeic, anti-inflamatory and antiseptic (Pyralvex)	Rhubard extract, Salicylic acid	5% v/v	N	N	N	N	N
Antifungal and Antibacterial Oralpharyngeal Topic (Daktarin)	Miconazole	5 mg/mL	N	N	N	N	N
Mouthwash solution antiseptics (Eludril Gé)	Chlorhexidine gluconate, Chlorobutanol hemihydrate	5% v/v	N	N	N	N	N
Antitussive, Syrup (Codipront)	Codeine, Phenyltoloxamine citrate	5% v/v	N	N	N	N	N
Whole Blood (human)	-	4% v/v	N	N	N	N	N
Antiviral drug (Tamiflu)	Oseltamivir	7,5 mg/mL	N	N	N	N	N
Mucolytic (Mucolsovan)	Ambroxol hydrochloride	5% v/v	N	N	N	N	N
Nasal drops solution (Nasarox)	Oxymetazoline Chlorhydrate	10% v/v	N	N	N	N	N
Antibiotic, nasal ointment (Bactroban)	Mupirocin	5 mg/mL	N	N	N	N	N
Saliva (human)	-	25% v/v	N	N	N	N	N
Absolute ethanol	Alcohol	5% v/v	N	N	N	N	N

11.4 Precision

Assay precision for the NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD was determined by the repeated testing of positive samples representing two viral load levels, 3x LoD and 30x LoD copies per reaction, spiked into RNA extracted from negative oropharyngeal samples, using 3 different kit batches, and following typical testing reaction conditions. Precision was evaluated by measuring Cq average, Cq coefficient of variation and % of replicate detection, as described below for each case. The data is resumed in the three tables (one for each target) displayed below.

11.4.1. Repeatability

Repeatability was assessed by one operator by analysing 12 replicates of each sample (3x LoD and 30x LoD copies per reaction), accounting for a final number of 24 tests performed per target.

11.4.2. Daily Reproducibility

Daily reproducibility was assessed by one operator by analysing 48 replicates of each sample (3x LoD and 30x LoD copies per reaction), for 4 days, with 12 replicates of each concentration per day (a total of 96 assays per target were performed).

11.4.3. Lot-to-lot Reproducibility

Reproducibility between lots was assessed by one operator through the analysis of 84 replicates of each sample (3x LoD and 30x LoD copies per reaction) using 3 different kit batches with 28 replicates per batch.

11.4.4. Operator Reproducibility

Operator reproducibility was assessed by testing 24 replicates of each sample (3x LoD and 30x LoD copies per reaction), by four different operators with 6 replicates per operator and per viral load, making a total of 36 replicates per operator, including the 3 kit targets.

11.4.5 Inter-instrument Reproducibility

Inter-instrument reproducibility was measured by one operator through the testing 24 replicates of each sample (3x LoD and 30x LoD copies per reaction), in two different qPCR instruments (Applied Biosystems™ 7500 FAST, Applied Biosystems™ QuantStudio 5), in a total of 48 tests per sample.

Precision of NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD, while detecting SARS-CoV-2 target.

Variable		SARS-CoV-2 (Copies/Reaction)	
		3x LoD	30x LoD
Repeatability	n	12	12
	Mean Cq	33,84	30,75
	Coefficient of Variation (%)	0,61	0,19
	% Replicate Detection	100	100
Daily Reproducibility	n	48	48
	Mean Cq	33,79	30,66
	Coefficient of Variation (%)	2,02	1,62
	% Replicate Detection	100	100
Lot-to-lot Reproducibility	n	84	84
	Mean Cq	33,82	30,59
	Coefficient of Variation (%)	1,96	1,60
	% Replicate Detection	100	100
Operator Reproducibility	n	24	24
	Mean Cq	33,86	30,73
	Coefficient of Variation (%)	1,26	1,13
	% Replicate Detection	100	100
Inter-instrument Reproducibility	n	24	24
	Mean Cq	33,83	30,30
	Coefficient of Variation (%)	2,20	1,49
	% Replicate Detection	100	100

Precision of NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD, while detecting Influenza A target.

Variable		Influenza A (Copies/Reaction)	
		3x LoD	30x LoD
Repeatability	n	12	12
	Mean Cq	34,39	31,26
	Coefficient of Variation (%)	3,31	1,05
	% Replicate Detection	100	100
Daily Reproducibility	n	48	48
	Mean Cq	34,30	30,92
	Coefficient of Variation (%)	2,90	1,52
	% Replicate Detection	100	100
Lot-to-lot Reproducibility	n	84	84
	Mean Cq	34,28	30,76
	Coefficient of Variation (%)	2,55	1,88
	% Replicate Detection	100	100
Operator Reproducibility	n	24	24
	Mean Cq	34,14	30,97
	Coefficient of Variation (%)	1,43	0,57
	% Replicate Detection	100	100
Inter-instrument Reproducibility	n	24	24
	Mean Cq	34,44	30,67
	Coefficient of Variation (%)	3,34	2,02
	% Replicate Detection	100	100

Precision of NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD, while detecting Influenza B target.

Variable		Influenza B (Copies/Reaction)	
		3x LoD	30x LoD
Repeatability	n	12	12
	Mean Cq	33,87	30,63
	Coefficient of Variation (%)	1,85	1,19
	% Replicate Detection	100	100
Daily Reproducibility	n	48	48
	Mean Cq	33,75	30,23
	Coefficient of Variation (%)	2,10	2,56
	% Replicate Detection	100	100
Lot-to-lot Reproducibility	n	84	84
	Mean Cq	33,73	30,23
	Coefficient of Variation (%)	1,99	2,00
	% Replicate Detection	100	100
Operator Reproducibility	n	24	24
	Mean Cq	33,70	30,34
	Coefficient of Variation (%)	1,51	0,65
	% Replicate Detection	100	100
Inter-instrument Reproducibility	n	24	24
	Mean Cq	33,93	30,27
	Coefficient of Variation (%)	2,47	1,29
	% Replicate Detection	100	100

Precision of NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD, while detecting RSV target.

Variable		Influenza B (Copies/Reaction)	
		3x LoD	30x LoD
Repeatability	n	12	12
	Mean Cq	33,66	30,126
	Coefficient of Variation (%)	1,00	0,84
	% Replicate Detection	100	100
Daily Reproducibility	n	48	48
	Mean Cq	33,24	30,05
	Coefficient of Variation (%)	2,55	0,84
	% Replicate Detection	100	100
Lot-to-lot Reproducibility	n	84	84
	Mean Cq	33,50	30,26
	Coefficient of Variation (%)	2,16	1,23
	% Replicate Detection	100	100
Operator Reproducibility	n	24	24
	Mean Cq	33,32	30,18
	Coefficient of Variation (%)	2,85	1,33
	% Replicate Detection	100	100
Inter-instrument Reproducibility	n	24	24
	Mean Cq	33,30	30,20
	Coefficient of Variation (%)	1,19	0,90
	% Replicate Detection	100	100

11.5 Clinical evaluation

The performance of NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD, with collected respiratory clinical samples was evaluated by an independent molecular diagnostic laboratory. In total, 1516 clinical samples have been tested, namely namely 850 negative samples; 293 positive clinical samples for SARS-CoV-2 (from May 2021 to June 2022, this period coverage different waves of SARS-CoV-2); 123 positive clinical samples for Influenza A (41 for AH1, 41 for AH1N1 pdm, 41 for AH3); 82 positive clinical samples for Influenza B (41 for Vitoria lineage and 41 for Yamagata lineage); 168 positive samples for RSV (88 for subtype A and 88 subtype B). The data revealed an 99% agreement for all positive and negative samples tested.

12. Quality Control

All components of NZYTech's COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD are tested following the protocols described above. The hexaplex real-time PCR system allows the detection of targets described for the identification of SARS-CoV-2 viral RNA (RdRp and N genes), Influenza A/B viral RNA (M1 and NS2 genes, respectively), RSV viral RNA (L gene) as well as human RNase P (RP gene). Positive amplifications are observed for target genes, positive control and internal controls through Texas Red, FAM, HEX/VIC and Cy5 channels, according to respective primers/probe set reporter dyes.

13. Technical Support

For Technical support, please contact our dedicated technical support team by Phone: +351 (0) 21 364 35 14 or Email: info@nzytech.com.

14. Trademarks and Disclaimers

All trademarks that appear in this manual are the property of their respective owners.

15. Explanation of Symbols

IVD	*in vitro* diagnostic medical device		Consult instructions for use
REF	Catalogue number		Manufacturer
LOT	Batch code		Use by
	Temperature limitation		Sufficient for
CONTROL +	Positive control		Keep away from the sun light (primer/probe mix)
CONTROL –	Negative control		

16. Conformity Declaration

Product Name: COVID-19, Flu A/B, RSV Multiplex One-Step RT-qPCR Kit, IVD.

Catalogue Number: MD04901 and MD04902.

Intended use: SARS-CoV-2, Influenza A and Influenza B, and RSV qualitative detection.

Manufacturer: NZYTech - Genes & Enzymes,

Estrada do Paço do Lumiar, Campus do Lumiar

Edifício E, R/C,

1649-038, Lisboa

Portugal

We, NZYTech, Lda – Genes & Enzymes, hereby declare that this product, to which this declaration of conformity relates, is in conformity with the following standards and other normative documents ISO 9001:2015 and ISO 13485:2016, following the provisions of the 98/79/EC Directive and of the Regulation (EU) 2017/746 on *in vitro* diagnostic medical devices as transposed into the national laws of the Member States of the European Union.

The product technical file is maintained at NZYTech, Estrada do Paço do Lumiar, Campus do Lumiar - Edifício E, R/C, 1649-038 Lisboa, Portugal.

Joana Brás, PhD

Technical Director

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