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

REF MD04851, 96 reactions

For professional* in vitro *diagnostic use only

CE IVD

EN

Instructions for Use

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NZYtech genes & enzymes
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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 virus types A and B are the dominant types of circulating influenza virus and the most influenza epidemics are related to type A. Both coronaviruses and influenza viruses are highly contagious respiratory diseases transmitted via contact, respiratory droplets (coughing and sneezing) and contaminated surfaces. COVID-19 might be clinically confused with pneumonia caused by Influenza viruses and co-infection carries a poor prognosis. The overlap of the flu season with the pandemic of COVID-19 complicates the clinical management of patients with respiratory symptoms. Early detection of SARS-CoV-2 and Influenza viruses A/B 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 two infections in patients presenting similar symptoms.

2. Intended Use

NZYtech's COVID-19 & Flu A/B Multiplex One-Step RT-qPCR Kit, IVD is a molecular test intended for the rapid qualitative detection of the causative agents of COVID-19 and Influenza (Flu A and Flu B) in human biological samples. However, this kit does not make distinction between Influenza A and B types as they are both detected in the same fluorescence channel for FAM. In addition, other beta coronaviruses or influenza viruses, *e.g.,* Influenza C, are not detected with this kit. This test is intended for use as an aid in the diagnosis of SARS-CoV-2 and Flu in combination with clinical and epidemiological risk factors. A positive result indicates the presence of SARS-CoV-2 and/or Influenza 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 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 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. 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 Multiplex One-Step RT-qPCR Kit, IVD is built to have the broadest detection profile possible whilst remaining specific to the SARS-CoV-2 and Influenza types A and B genomes. It provides the complete set of reagents and probes to detect the three 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 (including complete identity to the Delta (B.1.617.2) and Omicron (B.1.1.529) variants) and Influenza A and B genomes, through a highly optimized primers/probes set. The primers and probes have 100% homology with >95% of the >10000 genome sequences available on the GISAID database, as of November 2022. In addition, primers and probes display no significant homology with unrelated genomes rendering this test highly specific. 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 and Influenza A and B viral RNAs. NZYtech's COVID-19 & Flu A/B Multiplex One-Step RT-qPCR Kit, IVD is a multiplex assay detecting SARS-CoV-2, Influenza A, Influenza B 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 five 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 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 five specific targets in a single reaction, SARS-CoV-2, Influenza A/B and human RNase P specific probes are differently labelled, with Texas Red®, FAM™ and JOE™ 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. In addition, the five 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 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		NUMBER OF VIALS	VOLUME (PER VIAL)
COVID-19/Flu MMix	NZYMultiplex One-step RT-qPCR Probe Master Mix (2x)	1	1050 µL
COVID-19/Flu Primer Mix	COVID-19 & Flu A/B Primer Mix (20x)	1	103 µL
COVID-19/Flu Probe Mix	COVID-19 & Flu A/B Probe Mix (20x)	1	103 µL
COVID-19/Flu Pos 1	COVID-19 & Flu A/B Positive Control 1 (SARS-CoV-2 ORF1ab, Influenza B NS2 and human RP genes)	1	105 µL
COVID-19/Flu Pos 2	COVID-19 & Flu A/B Positive Control 2 (SARS-CoV-2 N, Influenza A M1 and human RP genes)	1	105 µL
NTC	No-template Control	1	105 µL

5. Storage, Stability and Handling Conditions

The COVID-19 & Flu A/B 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 Probe Mix (20x) should be stored protected from light. Particularly, do not expose the NZYMultiplex 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 JOE™ fluorescence dyes (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 Multiplex One-Step RT-qPCR Kit, IVD. Please ensure RNA samples are suitable in terms of purity, concentration and nucleic acid integrity. A A260/280 ratio of ~2 is generally accepted for pure RNA. Since ethanol is a strong PCR inhibitor, it is necessary to completely 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).
- 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 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 MMix (2x)(**)	10	*n* x 10.5
COVID-19/Flu Primer Mix (20x)	1	*n* x 1.05
COVID-19/Flu Probe Mix (20x)	1	*n* x 1.05
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 No-template control (NTC) instead of RNA template into the negative well. The final volume should be 20 µL.
4. For the biological samples, add 8 µL of each RNA sample into the COVID-19/Flu 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 Pos 1 (detects SARS-CoV-2 ORF1ab, Influenza B NS2 and human RP genes) and 8 µL of COVID-19/Flu Pos 2 (SARS-CoV-2 N, Influenza A M1 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 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 number of platforms. However, these conditions may be adapted and validated to suit different machine-specific protocols.

Suggested RT-PCR Run Settings

CYCLES	**TEMPERATURE**	**TIME**	**STEP**
1	50 °C	20 min	Reverse Transcription
1	95 °C	3 min	Polymerase activation
40	95 °C	5 s	Denaturation
	60 °C	1 min	Annealing/Extension*

* *Depending on the equipment used select the proper detection channel.*

Fluorescent Dyes & Detection Channels

TARGETS	FLUORESCENT DYE	DETECTION CHANNELS
RdRp & N targets (SARS-CoV-2)	Texas Red®	Texas Red
Flu A M1 target (Influenza A)	FAM™	FAM
Flu B NS2 target (Influenza B)	FAM™	FAM
RNase P	JOE™	Joe or HEX/VIC
COVID-19/Flu Pos 1	Texas Red® & FAM™ & JOE™	Texas Red & FAM & JOE/HEX/VIC
COVID-19/Flu Pos 2	Texas Red® & FAM™ & JOE™	Texas Red & FAM & JOE/HEX/VIC

NZYtech's COVID-19 & Flu A/B Multiplex One-Step RT-qPCR Kit, IVD was validated for the following Real Time PCR Systems: Applied Biosystems® 7500 FAST, Applied Biosystems® QuantStudio 5, and Bio-Rad® CFX96™. 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 three fluorescence signals (Texas Red, FAM and JOE) 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) and JOE (RNase P) curves are positive. Positive controls are expected to amplify at Ct<32, in the three channels. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

Negative control (No template control): no amplification is detected. If the negative control has amplification curves (Texas Red, FAM and JOE) 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

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 (JOE) 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 (JOE) assay.

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

The **test is invalid** if the SARS-CoV-2, Influenza A/B, 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 RESULT (TEXAS RED)	**INFLUENZA A/B RESULT** (FAM)	**RNASE P RESULT** (JOE)	**RESULTS INTERPRETATION**
+ (Ct≤35)	- (Ct>35)	+ (Ct≤40)	SARS-CoV-2 detected → POSITIVE
+ (Ct≤35)	- (Ct>35)	- (Ct>40)	SARS-CoV-2 detected → POSITIVE
- (Ct>35)	+ (Ct≤35)	+ (Ct≤40)	Influenza A/B detected → POSITIVE
- (Ct>35)	+ (Ct≤35)	- (Ct>40)	Influenza A/B detected → POSITIVE
+ (Ct≤35)	+ (Ct≤35)	+ (Ct≤40)	SARS-CoV-2 and Influenza A/B detected → POSITIVE
+ (Ct≤35)	+ (Ct≤35)	- (Ct>40)	SARS-CoV-2 and Influenza A/B detected → POSITIVE
- (Ct>35)	- (Ct>35)	+ (Ct≤40)	SARS-CoV-2 and Influenza A/B not detected → NEGATIVE
- (Ct>35)	- (Ct>35)	- (Ct>40)	Invalid test, repeat extraction and test

Note 1: *NZYtech recommends repeating the analysis for all samples showing an ambiguous or atypical curve that does not allow a clear interpretation.*

Note 2: *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 outside of viraemic phase.
  - Failure to follow procedures in this handbook.
  - Use of unauthorised extraction kit or real-time PCR platform.

- False positive results may be caused by:
  - Unsuitable handling of samples containing high concentration of viral RNA.
  - 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), Influenza A and/or Influenza B (Figure 1C), or both (Figure 1D), 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, Influenza A and Influenza B nucleic acids at different copy numbers, individually or 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 Multiplex One-Step RT-qPCR Kit, IVD detects 0.25 copies/µL of SARS-CoV-2, 0.75 copies/µL of Influenza A and 1.25 copies/µL of Influenza B RNA 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, 0.75 copies/µL or 750 copies/mL for Influenza A and 1.25 copies/µL or 1250 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 negative oropharyngeal swab matrix spiked independently. The capacity of NZYtech's COVID-19 & Flu A/B Multiplex One-Step RT-qPCR Kit, IVD to detect the virus at different loads is presented in Figure 2.

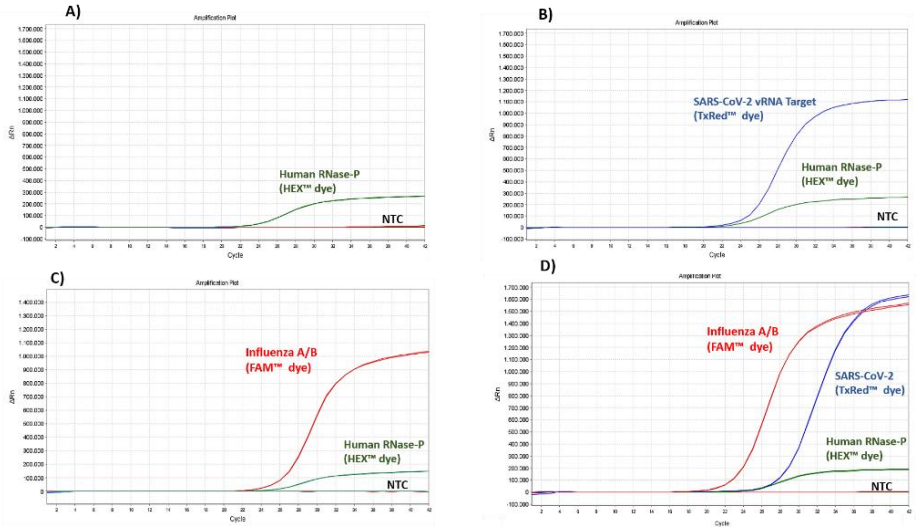

Figure 1. Detection of SARS-CoV-2, Influenza A/B and Human RNase-P targets from negative clinical samples (A) or clinical samples infected with SARS-CoV-2 (B), Influenza A and/or Influenza B (C), or both SARS-CoV-2 and Influenza A/B (D). Blue curve, detection of the SARS-CoV-2 vRNA targets through the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

Analytical sensitivity of the COVID-19 & Flu A/B Multiplex One-Step RT-qPCR Kit, IVD in the context of a co-infection scenario was evaluated by performing a series of serial dilution experiments using mock co-infection specimens for each of the two viral targets. To create the mock co-infection specimens, exactly 10^4 copies of Influenza A and 10^4 copies of Influenza B nucleic acids were added to the SARS-CoV-2 standard curve. In contrast, 10^4 copies of SARS-CoV-2 were added to the Influenza A and Influenza B standard curves. Quadruplicate samples of three kit batches (making a total of 12 replicates per dilution) were tested with the COVID-19 & Flu A/B 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 and Influenza A were unaltered in case of co-infection. However, LoD of Influenza B changed to 1.75 copies/µL or 1750 copies/mL in case of a co-infection.

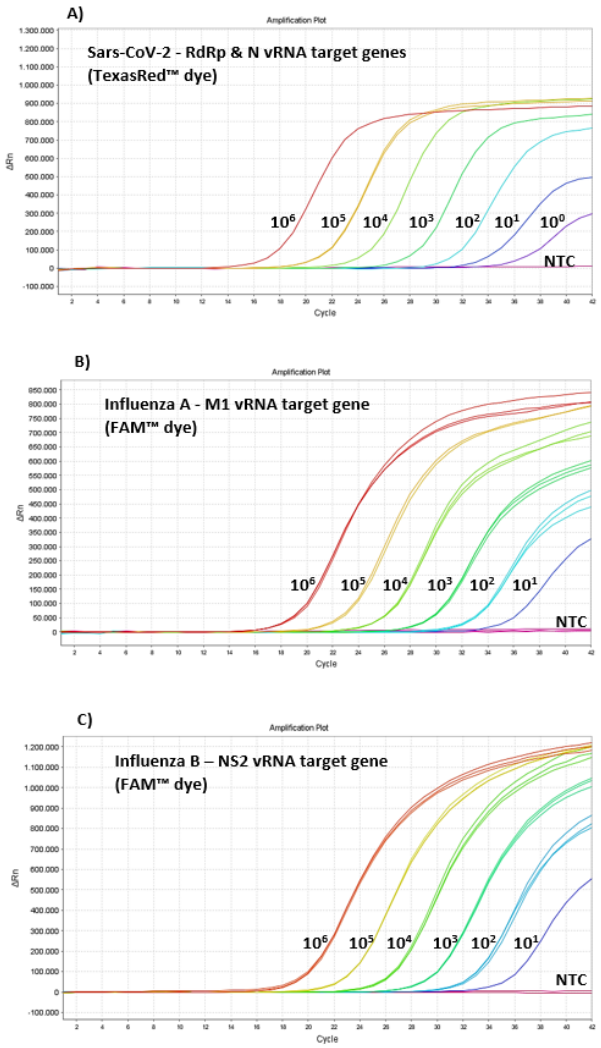

Figure 2. Sensitivity of the COVID-19 & Flu A/B Multiplex One-Step RT-qPCR Kit, IVD. Amplification plot (cycle number *versus* fluorescence - ΔRN) of 1:10 serial dilutions of the SARS-CoV-2 (A), Influenza A (B) and Influenza B (C) vRNA. NTC, No Template Control (negative control).

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 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 virus strains and exhibited no reactivity with non-related species. In addition to *in silico* analysis, COVID-19 & Flu A/B 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.

11.4 Precision

Assay precision for the NZYtech's COVID-19 & Flu A/B Multiplex One-Step RT-qPCR Kit, IVD was determined by the repeated testing of positive samples representing two viral load levels, 200 (low viral load) and 2000 (medium viral load) 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 (200 and 2000 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 (200 and 2000 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 60 replicates of each sample (200 and 2000 copies per reaction) using 3 different kit batches with 20 replicates per batch.

11.4.4 Operator Reproducibility

Operator reproducibility was assessed by testing 24 replicates of each sample (200 and 2000 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.

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

VARIABLE		SARS-CoV-2 (COPIES/REACTION)	
		200	2000
REPEATABILITY	n	12	12
	Mean Cq	28.87	26.35
	Coefficient of Variation (%)	0.95	1.92
	% Replicate Detection	100	100
DAILY REPRODUCIBILITY	n	12	12
	Mean Cq	28.87	26.35
	Coefficient of Variation (%)	0.95	1.92
	% Replicate Detection	100	100
LOT-TO-LOT REPRODUCIBILITY	n	60	60
	Mean Cq	28.86	25.97
	Coefficient of Variation (%)	1.97	2.71
	% Replicate Detection	100	100
OPERATOR REPRODUCIBILITY	n	24	24
	Mean Cq	28.87	25.68
	Coefficient of Variation (%)	2.54	2.20
	% Replicate Detection	100	100
INTER-INSTRUMENT REPRODUCIBILITY	n	24	24
	Mean Cq	29.83	26.89
	Coefficient of Variation (%)	3.29	2.46
	% Replicate Detection	100	100

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

VARIABLE		INFLUENZA A (COPIES/REACTION)	
		200	2000
REPEATABILITY	n	12	12
	Mean Cq	30.47	26.46
	Coefficient of Variation (%)	0.82	0.53
	% Replicate Detection	100	100
DAILY REPRODUCIBILITY	n	48	48
	Mean Cq	30.75	26.51
	Coefficient of Variation (%)	1.39	1.13
	% Replicate Detection	100	100
LOT-TO-LOT REPRODUCIBILITY	n	60	60
	Mean Cq	30.69	26.61
	Coefficient of Variation (%)	1.35	1.13
	% Replicate Detection	100	100
OPERATOR REPRODUCIBILITY	n	24	24
	Mean Cq	30.79	26.61
	Coefficient of Variation (%)	1.55	1.06
	% Replicate Detection	100	100
INTER-INSTRUMENT REPRODUCIBILITY	n	24	24
	Mean Cq	30.00	26.07
	Coefficient of Variation (%)	1.76	1.63
	% Replicate Detection	100	100

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

VARIABLE		INFLUENZA B (COPIES/REACTION)	
		200	2000
REPEATABILITY	n	12	12
	Mean Cq	30.72	26.82
	Coefficient of Variation (%)	0.81	1.08
	% Replicate Detection	100	100

DAILY REPRODUCIBILITY	n	48	48
	Mean Cq	30.78	26.82
	Coefficient of Variation (%)	1.14	3.05
	% Replicate Detection	100	100
LOT-TO-LOT REPRODUCIBILITY	n	60	60
	Mean Cq	30.76	26.82
	Coefficient of Variation (%)	1.08	2.77
	% Replicate Detection	100	100
OPERATOR REPRODUCIBILITY	n	24	24
	Mean Cq	30.87	26.71
	Coefficient of Variation (%)	1.11	0.73
	% Replicate Detection	100	100
INTER-INSTRUMENT REPRODUCIBILITY	n	24	24
	Mean Cq	30.15	26.34
	Coefficient of Variation (%)	2.01	2.04
	% Replicate Detection	100	100

11.4.5 Inter-instrument Reproducibility

Inter-instrument reproducibility was measured by one operator through testing 36 replicates of each sample (200 and 2000 copies per reaction), in two different qPCR instruments (Applied Biosystems® 7500 FAST, Applied Biosystems® QuantStudio 5), in a total of 72 tests per sample.

11.5 Clinical evaluation

The performance of NZYtech's COVID-19 & Flu A/B Multiplex One-Step RT-qPCR Kit, IVD, with collected respiratory clinical samples was evaluated by one external laboratory. In total, 250 clinical samples have been tested, namely 72 positive clinical samples for Influenza A virus (36 for AH1 and 36 for AH3); 58 clinical positive samples for Influenza B virus; 60 positive clinical samples for SARS-CoV-2 e 60 clinical negative. The data revealed an 100% agreement for all positive and negative samples tested.

12. Quality Control

All components of NZYtech's COVID-19 & Flu A/B Multiplex One-Step RT-qPCR Kit, IVD are tested following the protocols described above. The pentaplex 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) as well as human RNase P (RP gene). Positive amplifications are observed for target genes, positive control and internal controls through Texas Red, FAM and HEX/VIC/JOE 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 Multiplex One-Step RT-qPCR Kit, IVD

Catalogue Number: MD04851

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

Classification: Others (not covered by Annex II or not intended to self-testing) according to the EC Directive 98/79/EC

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

17. References

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