SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, IVD

REF MD04911, 96 reactions
MD04912, 4 x 96 reactions

For professional* in vitro *diagnostic use only

CE IVD

EN

Instructions for Use (IFU)

IM-007en

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1. Introduction

Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2), previously named 2019-nCoV, is the causative agent of the ongoing Coronavirus Disease 2019 (COVID-19) and like the closely related SARS coronavirus belongs to the genus *Betacoronavirus* within the family of coronaviruses. Coronaviruses are enveloped, positive, single-stranded large RNA viruses that infect humans, but also a wide range of animals. SARS-CoV-2, thought to be of zoonotic origin, is highly contagious and is primarily transmitted via respiratory droplets (coughing and sneezing). Early detection of SARS-CoV-2 is vital in providing rapid treatment to infected patients and, thus, to reduce the spread of infections. The most common clinical manifestations of COVID-19 include fatigue, fever and lower respiratory symptoms, such as dry cough and dyspnea. Loss of smell and taste can also occur. In the most critical situations, the infection progresses to severe pneumonia with life-threatening complications such as acute respiratory disease syndrome, organ dysfunction and death. Based on current knowledge, a significant proportion of infections are mild or asymptomatic. A percentage of the population is more vulnerable to the severe form of disease, including older adults (60 years and older), smokers and people with chronic diseases such as heart or lung disease, cancer, diabetes and patients with a weakened immune system. Although currently, vaccines are already available to control COVID-19 they do not prevent infection in vaccinated populations.

2. Intended Use

NZYTech SARS-CoV-2 One-Step RT-qPCR III, 5 Targets, IVD, is a molecular real-time reverse transcription polymerase chain reaction (RT-qPCR) test intended for the rapid qualitative detection of SARS-CoV-2 nucleic acids in nasopharyngeal or oropharyngeal swabs samples collected from individuals suspected of COVID-19. This kit was designed to allow robust and efficient detection of the SARS-CoV-2 virus compensating the appearance of variants with significant genetic differences relatively from those currently in circulation. NZYTech SARS-CoV-2 One-Step RT-qPCR III, 5 Targets, IVD, offers the ability to detect 5 viral targets at the same time, allowing that, even in the case of one or more targets becoming unviable due to genetic alterations accumulated in virus, the diagnosis remains effective. A positive result indicates the presence of SARS-CoV-2 RNA but clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Negative results do not preclude SARS-CoV-2 infection and 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 SARS-CoV-2 One-Step RT-qPCR III, 5 Targets, IVD, provides the complete set of reagents and probes to qualitatively detect five regions of SARS-CoV-2 genome, through common real-time PCR platforms (see required instrument specifications in **Section 6**). This NZYTech kit targets specific regions of SARS-CoV-2 genome, particullary, RNA dependent RNA polymerase (RdRp) gene, Nucleocapsid phosphoprotein (N) gene and a specific region in structural envelope protein (E) gene, which is present in all *Sarbecovirus* genomes (including SARS-CoV-2 virus). All target sequences were selected to provide the highest sensitivity of detection. SARS-CoV-2 kit primers and probes have 100% homology with >95% of the >5M genome sequences available on the GISAID database, as of May 2022, including complete identity to the Delta (B.1.617.2) and

Omicron (B.1.1.529) variants. In addition, primers and probes targeting SARS-CoV-2 display no significant homology with unrelated genomes rendering this test highly specific as there is no cross-reactivity with nucleic acids from other respiratory viral and bacterial organisms. An internal control is included to confirm efficient RNA extraction from human biological samples, as well as absence of PCR inhibitors, among others. In addition, the test uses three external controls (two positive low titer controls provided with the kit and negative control), as described below. The SARS-CoV-2 (RdRp, N & E)/RP Positive Control 1 consists of nucleic acid fragments containing three target sequences of SARS-CoV-2 genome located in specific regions of RdRp, N and E genes, and a target sequence located in human RNase P (RP) gene. The SARS-CoV-2 (RdRp, N & E)/RP Positive Control 2 consists of nucleic acid fragments located in the same genes indicated for the SARS-CoV-2 (RdRp, N & E)/RP Positive Control 1, but containing distint target sequences within those genes, and a target sequence located in human RNase P (RP) gene. The natural evolution of SARS-CoV-2 implies that new sequence information will become available after the initial design of this kit, which reflects SARS-CoV-2 adaptation strategies. Thus, NZYTech periodically revisits the five SARS-CoV-2 genomic targets and, if required, will release new versions of this kit.

One-step RT-qPCR remains the most reliable and sensitive method to perform an accurate detection of SARS-CoV-2 RNA, which is indicative of an human infection. Viral RNA isolated and purified from infected samples is retrotranscribed to cDNA and subsequently amplified in a single reaction using five highly specific primers/probe sets exploiting the so-called TaqMan® principle. To allow identifying amplification of the six specific targets in a single reaction, SARS-CoV-2 RdRp, N and E genes and human RP specific probes are differently labelled, with HEX™, FAM™, Texas Red™ and Cy5™ reporter dyes, respectively. This kit consists in a hexaplex assay in four distinct optical channels: two targets located in RdRp gene are detected in HEX (alternatively VIC or JOE), two targets located in N gene are detected in FAM, one target located in E gene (present in all *Sarbecovirus*) is detect in Texas Red (alternatively JUN), and the human endogenous target is detected in Cy5. Note that this panel includes four SARS-CoV-2 target sequences that emits fluorescence detected in two channels allowing to increase the robustness and sensitivity of SARS-CoV-2 detection. In addition, primers and probes are provided in optimized concentrations to make sure amplification of human mRNA, even when present at very high concentrations, does not limit the efficiency of SARS-CoV-2 primers/probe sets.

4. Kit Composition

NZYTech SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, IVD, provides a comprehensive set of reagents and controls for the qualitative detection of SARS-CoV-2 in a single step.

Kit Component		Volume (per vial)	Number of vials	
			MD04911	MD04912
SARS-CoV-2 MMix III (RdRp, N & E)	NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix (2x)	1050 µL	1	4
SARS-CoV-2 PPMix III (RdRp, N & E)	SARS-CoV-2 (RdRp, N & E)/RP PPMix III (10x)	205 µL	1	4
SARS-CoV-2 POS 1 (RdRp, N & E)	SARS-CoV-2 (RdRp, N & E)/RP Positive Control 1	105 µL	1	4
SARS-CoV-2 POS 2 (RdRp, N & E)	SARS-CoV-2 (RdRp, N & E)/RP Positive Control 2	105 µL	1	4
NTC	No-Template Control	105 µL	1	4

5. Storage, Stability and Handling Conditions

SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, 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. Also proceed with the following indications:

- 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 SARS-CoV-2 PPMix III (RdRp, N & E) should be stored protected from light. Particularly, do not expose the SARS-CoV-2 MMix III (RdRp, N & E) to direct sunlight after combining with SARS-CoV-2 PPMix III (RdRp, N & E).
- If the package that protects the kit arrived damaged, please contact NZYTech.
- Beware of 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 FAM™, HEX™/VIC™/JOE™, Texas Red®/JUN™ and Cy5™ fluorescent dyes (at emission wavelengths of 520, 556, 603 and 670 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), 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 IVD protocol are the starting material for NZYTech SARS-CoV-2 One-Step RT-qPCR III, 5 Targets, IVD. Please ensure RNA samples are suitable in terms of purity, concentration, and nucleic acid integrity. An $A_{260/280}$ ratio of ~2 is generally accepted for pure RNA. Since ethanol is a strong Real-Time 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 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). Detection of SARS-CoV-2 virus 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 the 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 a high copy number of templates; they should be opened and processed away from test samples and kit components to avoid cross-contamination.
- Always use tube NTC to prepare the no template control reaction.
- 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 healthcare professional in the context of the patient medical history and clinical symptoms.
- This test cannot rule out diseases caused by other pathogens.
- 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 ice. After the plate is poured start immediately with 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 SARS-CoV-2 POS 1 (RdRp, N & E) and SARS-CoV-2 POS 2 (RdRp, N & E) 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)**
SARS-CoV-2 MMix III (RdRp, N & E) (**)	10	*n* x 10.5
SARS-CoV-2 PPMix III (RdRp, N & E)	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 No-template and Positive controls (2), respectively.
(**) Please notice that a precipitate in the bottom of the master mix tube may be observed, in particular after multiple freeze/thaw cycles. After master mix is thawed 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 no-template control, add 8 µL of NTC instead of RNA template into the no-template 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 SARS-CoV-2 POS 1 (RdRp, N & E) and 8 µL de SARS-CoV-2 POS 2 (RdRp, N & E) 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 to perform SARS-CoV-2/RP tests using SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, IVD, on the platforms referred below.

Cycles	Temperature	Time	Step
1	50 °C	10 min	Reverse Transcription
1	95 °C	2 min	Polymerase activation
40	95 °C	5 s	Denaturation
	60 °C	60 s	Annealing/Extension*

**Depending on the equipment used select the proper detection channel. Collect signals through FAM, HEX/JOE/VIC, Texas Red/JUN and Cy5 channels.*

Fluorescent Dyes & Detection Channels

Targets	Fluorescent dye	Detection Channels
SARS-CoV-2, RdRp gene	HEX™	HEX/VIC/JOE
SARS-CoV-2, N gene	FAM™	FAM
SARS-CoV-2, E gene	Texas Red®	Texas Red/JUN
RNase P gene	Cy5™	Cy5

NZYTech's SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, IVD, was validated for the following Real-Time PCR Systems: Applied Biosystems® 7500 FAST, Applied Biosystem® QuantStudio 5, Roche LightCycler® 480 II e Bio-Rad® CFX96™. If other equipment is used, the kit should be validated by the user by using previously characterised samples (both positive and negative).

10. Data Analysis

10.1 Run Validation Criteria

The detection of SARS-CoV-2 RNA is performed by detecting five viral genome regions, which are detected in three fluorescence channels (FAM, HEX and Texas Red), and the human RP control in a fourth channel (Cy5). 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 (FAM, HEX, Texas Red and Cy5) are determined automatically by the software with manual adjustments in case this is required. Before analysing samples results, we recommend to verify 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 curves of HEX (two targets for SARS-CoV-2 RdRp gene), FAM (two targets for SARS-CoV-2 N gene), Texas Red (one target for SARS-CoV-2 E gene) and Cy5 (for RP gene) are positive. Positive controls are expected to amplify at a Ct<32, in the four channels. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

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

If the controls are according to expected, the test is **valid**. Please proceed with the interpretation of the 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 SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, IVD, uses the following Ct cutoff values for results interpretation:

Ct value	Results interpretation
Ct ≤36, Amplification	**Detected (+) → POSITIVE**
Ct>36, No Amplification	**Not Detected (-) → NEGATIVE**

SARS-CoV-2 is detected if HEX, FAM and Texas Red amplification curves display a sigmoidal shape with a Ct≤36, regardless of the result obtained for the RP (Cy5) assay.

SARS-CoV-2 is detected if HEX and FAM amplification curves display a sigmoidal shape with a Ct≤36, regardless of the result obtained for E (Texas Red) and RP (Cy5) assays.

SARS-CoV-2 is detected if HEX and Texas Red amplification curves display a sigmoidal shape with a Ct≤36, regardless of the result obtained for N (FAM) and RP (Cy5) assays.

SARS-CoV-2 is detected if FAM and Texas Red amplification curves display a sigmoidal shape with a Ct≤36, regardless of the result obtained for RdRp (HEX) and RP (Cy5) assays.

SARS-CoV-2 is not detected if FAM, HEX and Texas Red curves are not positive (Ct>36), while the RNase P (Cy5) assay displays a positive sigmoidal curve (Ct≤40).

The test is inconclusive for SARS-CoV-2 if only one amplification curve (FAM, HEX or Texas Red) displays a sigmoidal shape with a Ct≤36, while the others SARS-CoV-2 targets are negative, regardless of the result obtained for the RP (Cy5) assay. The test should be repeated with nucleic acid re-purified from the sample.

The test is invalid if the SARS-CoV-2 and RP assays are all 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 RdRp gene, (HEX)	**SARS-CoV-2** N gene, (FAM)	**SARS-CoV-2** E gene, (Texas Red)	**RP** RP gene, (Cy5)	**Results interpretation**
+	+	+	+ / - *	**SARS-CoV-2 detected → POSITIVE**
+	+	-	+ / - *	**SARS-CoV-2 detected → POSITIVE**
+	-	+	+ / - *	**SARS-CoV-2 detected → POSITIVE**
-	+	+	+ / - *	**SARS-CoV-2 detected → POSITIVE**
+	-	-	+ / - *	**SARS-CoV-2 detected only for one target → INCONCLUSIVE**
-	+	-	+ / - *	**SARS-CoV-2 detected only for one target → INCONCLUSIVE**
-	-	+	+ / - *	**SARS-CoV-2 detected only for one target → INCONCLUSIVE**
-	-	-	+	**SARS-CoV-2 not detected → NEGATIVE**
-	-	-	-	**Invalid test, repeat extraction and retest**

***A high concentration/load of detectable viral RNA in the sample can lead to reduced or absent control (RP, Cy5) signs.*

Note: NZYTech recommends repeating the analysis for all samples showing an ambiguous or atypical curve that does not allow a clear interpretation. 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 SARS-CoV-2 viral RNA.
- Unsuitable handling of the SARS-CoV-2 POS 1 (RdRp, N & E) and SARS-CoV-2 POS 2 (RdRp, N & E).
- Unsuitable handling of amplified product (post-amplification plate).

Negative results do not preclude SARS-CoV-2 infection and 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

Evaluation of the NZYTech SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, IVD, performance was carried out on the Applied Biosystem® 7500 FAST, Applied Biosystem® QuantStudio 5, Roche LightCycler® 480 II e Bio-Rad® CFX96™. If other equipment is used, the kit should be validated by the user by using previously characterised samples (both positive and negative).

11.1 Expected Results

Typical amplification plots observed for clinical samples containing SARS-CoV-2 nucleic acids are presented in Figure 1. The cases represent examples of clinical samples presenting high (Panel A) or medium (Panel B) SARS-CoV-2 loads. Note that in cases of very high SARS-CoV-2 loads the curve of the Cy5 channel, corresponding to the human RNase P gene, may be absent or display an atypical form (Figure 1A).

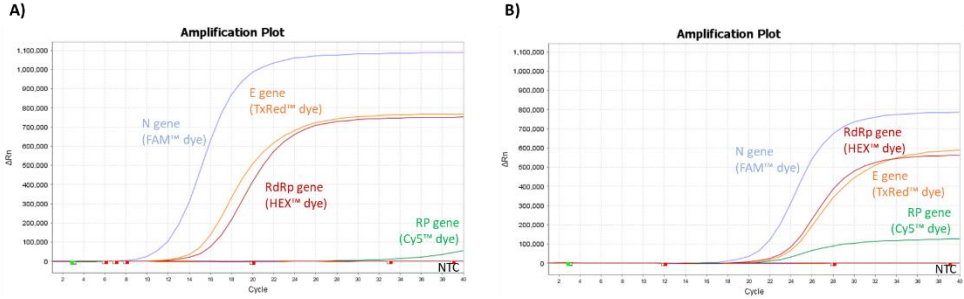

Figure 1. Simultaneous detection of SARS-CoV-2 (RdRp, N and E genes) and Human RNase P targets from positive clinical samples with a high (A) and medium (B) SARS-CoV-2 loads. Red curve: detection of two SARS-CoV-2 vRNA targets (RdRp gene) through the HEX channel; Blue curve: detection of two SARS-CoV-2 vRNA targets (N gene) through the FAM channel; Orange curve: detection of one SARS-CoV-2 vRNA target (E gene) through the Texas Red channel; Green curve: detection of the human RNase P gene through the Cy5 channel.

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 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 3 days, producing 96 replicates for each SARS-CoV-2 concentration tested. Together, the data revealed that NZYTech SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, IVD, detects 0.25 copies/µL or 250 copies/mL of SARS-CoV-2 viral RNA with a confidence ≥95%. 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.

11.3 Inclusivity, Cross-Reactivity and Interfering Substances

Inclusivity and cross-reactivity were evaluated by *in silico* analysis of oligonucleotide probes and primers against pathogens nucleic acids related to SARS-CoV-2 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 virus strains and exhibited no reactivity with non-SARS-CoV-2 species. In addition, *in vitro* analysis for Cross-Reactivity (Exclusivity) was performed to confirm that NZYTech SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, IVD, does not react with other human colonizing microbes and pathogens commonly encountered in clinical specimens. This study was performed by using a commercial respiratory pathogen panel sourced from ZeptoMetrix, notably NATtrol ™ Respiratory Verification Panel (# NATRVP-IDI). This panel comprise sample polls representative of true clinical human specimens, including Influenza A H1N1 (A/New Cal/20/99), Influenza A H3N2 (Brisbane/10/07), Influenza A 2009 H1N1pdm, Influenza B (B/Florida/02/06), Metapneumovirus 8 (Peru 6-2003), Respiratory Syncytial Virus A, Rhinovirus Type 1A, Parainfluenza virus Type 1, Parainfluenza virus Type 2, Parainfluenza virus Type 3, Parainfluenza virus Type 4, Adenovirus Type 3, Coronavirus NL63, Coronavirus 229E, Coronavirus OC43, Coronavirus HKU-1, *M. pneumoniae* M-129, *C. pneumoniae* CWL-029 and *B. pertussis* A639. All tests were run in triplicates.

Additionally, other 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* and *Streptomyces albidoflavus* were also tested. The data, collected using three different kit batches, confirmed that none of the organisms tested interfered with NZYTech SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, IVD, performance by generating false-positive results or an unspecific 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 pentaplicate and results were 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.

Potential Interferent	Active Ingredient	Final concentration in sample	Interference Yes (Y) or No (N)
Isotonic Sea Water (Rhinomer)	NaCl	15% v/v	N
Throat spray, oral anesthetic and analgesic (Strepfen)	Flurbiprofen	5% v/v	N
Nasal wash solution (Allergy spray - Vibrocil)	Fluticasone propionate	5% v/v	N
Nasal Corticosteroids spray (Nasomet)	Mometasone furoate	5% v/v	N
Nasal Corticosteroids spray (Pulmicort)	Budesonide	5% v/v	N
Antimicrobial, systemic (Trobex)	Trobamycin	600 µg/mL	N
Mouth analgeic, anti-inflamatory and antiseptic (Pyralvex)	Rhubard extract, Salicylic acid	5% v/v	N
Antifungal and Antibacterial Oropharyngeal Topic (Daktarin)	Miconazole	5 mg/mL	N
Mouthwash solution antiseptics (Eludril Gé)	Chlorhexidine gluconate, Chlorobutanol hemihydrate	5% v/v	N
Antitussive, Syrup (Codipront)	Codeine, Phenyltoloxamine citrate	5% v/v	N
Whole Blood (human)	-	4% v/v	N
Antiviral drug (Tamiflu)	Oseltamivir	7,5 mg/mL	N
Mucolytic (Mucolsovan)	Ambroxol hydrochloride	5% v/v	N
Nasal drops solution (Nasarox)	Oxymetazoline Chlorhydrate	10 % v/v	N
Antibiotic, nasal ointment (Bactroban)	Mupirocin	5 mg/mL	N
Saliva (human)	-	25% v/v	N
Absolute ethanol	Alcohol	5% v/v	N

11.4 Precision

Assay precision for the NZYTech SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, IVD, was determined by the repeated testing of SARS-CoV-2 nucleic acids representing two viral load levels, 15 (3x LoD) and 150 (30x LoD) copies per reaction (0.75 and 7.50 copies/µL), 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 table displayed below.

11.4.1. Repeatability

Repeatability was assessed by one operator by analysing 12 replicates of each sample (15 and 150 copies per reaction), accounting for a final number of 24 tests performed.

11.4.2. Daily Reproducibility

Daily reproducibility was assessed by one operator by analysing 36 replicates of each sample (15 and 150 copies per reaction), for 3 days with 12 replicates of each concentration per day (a total of 72 assays were performed).

11.4.3. Lot-to-lot Reproducibility

Reproducibility between lots was assessed by one operator through the analysis of 36 replicates of each sample (15 and 150 copies per reaction) using 3 different kit batches with 24 replicates per batch.

Precision of NZYTech SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, IVD

Variable tested		RdRp gene (Copies/Reaction)		N gene (Copies/Reaction)		E gene (Copies/Reaction)	
		15	150	15	150	15	150
Repeatability							
	n	12	12	12	12	12	12
	Mean Cq	32,40	29,07	31,67	28,46	32,89	29,28
	Coefficient of Variation (%)	1,53	1,23	1,94	1,48	2,79	2,24
	% Replicate Detection	100	100	100	100	100	100
Daily Reproducibility	n	36	36	36	36	36	36
	Mean Cq	32,57	29,56	31,87	28,58	31,95	30,24
	Coefficient of Variation (%)	2,31	2,58	2,51	2,76	3,48	3,22
	% Replicate Detection	100	100	100	100	100	100
Lot-to-lot Reproducibility	n	36	36	36	36	36	36
	Mean Cq	32,55	29,39	31,77	28,42	32,13	30,03
	Coefficient of Variation (%)	2,25	2,49	2,68	2,30	3,87	3,24
	% Replicate Detection	100	100	100	100	98	100
Operator Reproducibility	n	36	36	36	36	36	36
	Mean Cq	32,44	29,33	31,88	28,68	32,03	29,66
	Coefficient of Variation (%)	1,56	2,14	1,63	2,24	3,46	3,07
	% Replicate Detection	100	100	100	100	100	100
Inter-instrument Reproducibility	n	48	48	48	48	48	48
	Mean Cq	33,10	29,96	32,08	29,00	32,90	30,55
	Coefficient of Variation (%)	1,98	1,24	2,68	2,83	4,12	3,87
	% Replicate Detection	100	100	100	100	96	100

11.4.4. Operator Reproducibility

Operator reproducibility was assessed by testing 36 replicates of each sample (15 and 150 copies per reaction), by three different operators, with 12 replicates per operator.

11.4.5. Inter-instrument Reproducibility

Inter-instrument reproducibility was measured by one operator by testing 48 replicates of each sample (15 and 150 copies per reaction), in four different qPCR instruments (Applied Biosystems® 7500 FAST, Applied Biosystems® QuantStudio 5, Roche LightCycler® 96 e Bio-Rad® CFX96™), in a total of 96 tests per sample.

11.5 Clinical evaluation

The performance of NZYTech SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, IVD, with collected nasopharyngeal swab samples, was evaluated by one external laboratory. In total, 501 clinical negative and 150 clinical positive samples have been tested. Data revealed that 97.4% of clinical sensitivity and 100% of clinical specificity agreements were achieved for all positive and negative samples tested.

12. Quality Control

All components of NZYTech SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, 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, N and E genes) and human mRNA (RNase P gene, RP). Positive amplifications are observed for target genes, positive controls and internal controls through FAM, HEX, Texas Red 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: SARS-CoV-2 One-Step RT-qPCR Kit III, 5 Targets, IVD

Catalogue Number: MD04911 and MD04912.

Intended use: SARS-CoV-2 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

17. References

Nathan J, Hardenbrook1 and Peijun Zhang (2022). A structural view of the SARS-CoV-2 virus and its assembly. Current Opinion in Virology. 52:123–134.

Swets MC, Russell CD, Harrison EM, Docherty AB, Lone N, Girvan M, Hardwick HE; ISARIC4C Investigators, Visser LG, Openshaw PJM, Groeneveld GH, Semple MG, Baillie JK (2022). SARS-CoV-2 co-infection with influenza viruses, respiratory syncytial virus, or adenoviruses. Lancet 399(10334):1463-1464. doi: 10.1016/S0140-6736(22)00383-X.

Gomez GB, Mahé C, Chaves SS (2021). Uncertain effects of the pandemic on respiratory viruses. Science 372:1043-1044.

Irwin Jungreis, Rachel Sealfon and Manolis Kellis (2021). SARS-CoV-2 gene content and COVID-19 mutation impact by comparing 44 *Sarbecovirus* genomes. Nature Communications. 12:2642.

WHO: Q&A: Influenza and COVID-19 - similarities and differences. 30 September 2021. Available online at https://www.who.int/emergencies/diseases/novel-coronavirus-2019/question-and-answers-hub/q-a-detail/q-a-similarities-and-differences-covid-19-and-influenza.

Gorbalenya, Alexander E.; Baker, Susan C.; Baric, Ralph S.; Groot, Raoul J. de; Drosten, Christian; Gulyaeva, Anastasia A. et al. (2020): Severe acute respiratory syndrome-related coronavirus: The species and its viruses – a statement of the Coronavirus Study Group (14). bioRxiv 2020.02.07.937862; doi: https://doi.org/10.1101/2020.02.07.937862.

Zhou, Peng; Yang, Xing-Lou; Wang, Xian-Guang; Hu, Ben; Zhang, Lei; Zhang, Wei et al. (2020): A pneumonia outbreak associated with a new coronavirus of probable bat origin. In Nature 579 (7798), pp. 270–273. doi: 10.1038/s41586-020-2012-7.

Chhikara, B. S., Rathi, B., Singh, J., Poonam. (2020). Corona virus SARS-CoV-2 disease COVID-19: Infection, prevention and clinical advances of the prospective chemical drug therapeutics. Chem. Biol. 7(1) 63-72.

WHO: Clinical management of severe acute respiratory infection (SARI) when COVID-19 disease is suspected. 13 March 2020. Available online at https://www.who.int/docs/default-source/coronaviruse/clinical-management-of-novel-cov.pdf.

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