SARS-CoV-2 One-Step RT-qPCR Kit II, RdRp and N genes, IVD

Viral RNA dependent RNA polymerase (RdRp) and Nucleocapsid phosphoprotein (N) genes

REF MD04871, 96 reactions
MD04872, 4 x 96 reactions

For professional* in vitro *diagnostic use only

CE IVD

EN

Instructions for Use

MD0487_IM_en

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NZYtech genes & enzymes
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Contents

1. Introduction

Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2), previously named 2019-nCoV, is the causative agent of 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 Kit II, RdRp and N genes, 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. 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 Kit II, RdRp and N genes, IVD, provides the complete set of reagents and probes to qualitatively detect the SARS-CoV-2 genome, through common real-time PCR platforms (see required instrument specifications in **Section 6**). The virus RNA dependent RNA polymerase (RdRp) and the Nucleocapsid phosphoprotein (N) genes have previously been identified as highly specific markers for SARS-CoV-2. This NZYtech kit targets specific regions in the RdRp and N genes of SARS-CoV-2 genome 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 November 2021, 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 external controls (positive low titer control provided with the kit and negative control), as described below. The positive control consists of nucleic acid fragments containing the three target sequences detected by the kit (SARS-CoV-2 RdRp and N genes, and human RNase P (RP)). 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 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 two highly specific primers/probe sets exploiting the so-called TaqMan® principle. During this process, the probes specifically anneal to two regions of the SARS-CoV-2 genome, namely RdRp (within the Orf1ab polyprotein gene) and N genes. An additional primers/probe set acts as an endogenous internal control to detect nucleic acids of the human RP gene, assessing sample quality. To allow identifying amplification of the three specific targets in a single reaction, SARS-CoV-2 RdRp and N genes and human RP specific probes are differently labelled, with HEX™, FAM™ and Cy5™ reporter dyes, respectively. Note that this panel contains a duplex assay in two distinct optical channels, HEX (alternatively VIC or JOE) and FAM, to report the performance of the two SARS-CoV-2 amplifications. 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 II, RdRp and N genes, 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	
			MD04871	MD04872
SARS-CoV-2 MMix II, ROX (RdRp & N)*	NZYSupreme One-step RT-qPCR Master Mix, ROX*	1050 µL	1	4
SARS-CoV-2 PPMix II (RdRp & N)	SARS-CoV-2/RP primers/probe Mix II	205 µL	1	4
SARS-CoV-2 POS (RdRp & N)	SARS-CoV-2(RdRp & N genes)/RP Positive Control (1 x 10^4 copies/µL)	105 µL	1	4
NTC	No-Template Control	105 µL	1	4

**Despite most real-time PCR instruments reading ROX dye allow users to run experiments and analyse data without ROX, the inclusion of this internal passive reference dye prevents data misinterpretation and allows to detect and diagnosis errors.*

5. Storage, Stability and Handling Conditions

SARS-CoV-2 One-Step RT-qPCR Kit II, RdRp and N genes, 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/RP primers/probe Mix II should be stored protected from light. Particularly, do not expose the SARS-CoV-2 MMix II ROX (RdRp & N) to direct sunlight after combining with primers/probe mix.
- 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™/JOE™/VIC™ and Cy5™ fluorescent dyes (at emission wavelengths of 520, 556/555/554 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 Kit II, RdRp and N genes, 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 control contains a high copy number of templates; it 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 the SARS-CoV-2 POS (RdRp & N) last to avoid cross contaminations.**

9.1 Reaction set-up

1. Prepare a RT-qPCR mix enough for the number of SARS-CoV-2/RP 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 II, ROX (RdRp & N)(**)	10	*n* x 10.5
SARS-CoV-2 PPMix II (RdRp & N)	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 two more for the No-template and Positive controls, respectively.

(**) Please notice that a precipitate in the bottom of the master mix tube may be observed. 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-PCR 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 SARS-CoV-2/RNase P wells, according to your experimental plate set-up. The final volume in each well should be 20 µL.
5. For the positive control, add 8 µL of SARS-CoV-2 POS (RdRp & N) instead of RNA template into the positive control well. The final volume should be 20 µL.
6. Cover and seal the plate with an appropriate optical adhesive film before proceeding with the RT-PCR and detection steps.
7. Place the reaction plate in the real-time PCR instrument and run the RT-PCR protocol according to the section below.

9.2 Programming the real-time PCR instrument

The tables below display two optimized thermal protocols (standard and fast) to perform SARS-CoV-2/RP tests using SARS-CoV-2 One-Step RT-qPCR Kit II, RdRp and N genes, IVD, on the platforms referred below.

- **Standard Protocol**

CYCLES	TEMPERATURE	TIME	STEP
1	50 °C	20 min	Reverse Transcription
1	95 °C	2 min	Polymerase activation
40	95 °C	5 s	Denaturation
	60 °C	30 s	Annealing/Extension*

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

Standard protocol for NZYtech SARS-CoV-2 One-Step RT-qPCR Kit II, RdRp and N genes, IVD, was validated for the following Real-Time PCR Systems: Applied Biosystems® 7500 FAST, Applied Biosystems® QuantStudio 5, Applied Biosystems® QuantStudio 6 Pro and 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).

- **Fast Protocol**

CYCLES	TEMPERATURE	TIME	STEP
1	50 °C	10 min	Reverse Transcription
1	95 °C	1 min	Polymerase activation
40	95 °C	5 s	Denaturation
	60 °C	20 s	Annealing/Extension*

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

Fast protocol for NZYtech SARS-CoV-2 One-Step RT-qPCR Kit II, RdRp and N genes, IVD, was validated for the following Real-Time PCR Systems: Applied Biosystems® QuantStudio 5 and 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).

Fluorescent dyes used in this kit and respective detection channels are:

TARGETS	FLUORESCENT DYE**	DETECTION CHANNELS
SARS-CoV-2, RdRp gene	HEX™	JOE, VIC or HEX
SARS-CoV-2, N gene	FAM™	FAM
RNase P	Cy5™	Cy5

***Opening the ROX channel will allow using this internal passive reference dye to prevent data misinterpretation and subsequent errors.*

10. Data Analysis

10.1 Run Validation Criteria

The detection of SARS-CoV-2 RNA is performed by detecting two viral genome regions, which are detected in different fluorescence channels (FAM and HEX), and the human RP control in a third channel (Cy5). 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 (FAM™, HEX™ 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 control: the amplification curves of JOE (for SARS-CoV-2 RdRp gene), FAM (for SARS-CoV-2 N gene) and Cy5 (for RP gene) curves are positive. Positive control is expected to amplify at a Ct<30, both in the FAM, JOE and Cy5 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 one, two or three amplification curves (JOE, FAM and/or Cy5) with a sigmoidal shape, sample contamination may have occurred. Repeat the test following good RT-PCR 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

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

SARS-CoV-2 is not detected if both JOE and FAM amplification curves are not positive (Ct>36) while the RP (Cy5) displays a positive sigmoidal curve (Ct<40).

The test is inconclusive for SARS-CoV-2 if either the JOE or FAM amplification curves do not display a sigmoidal shape with a Ct≤36 while the other SARS-CoV-2 target is positive, 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 RESULT SARS-CoV-2 RDRP GENE, CT (JOE)	**SARS-CoV-2 RESULT** SARS-CoV-2 N GENE, CT (FAM)	**RP RESULT** RP GENE, CT (CY5)	**RESULTS INTERPRETATION**
+ (Ct≤36)	+ (Ct≤36)	+/-	SARS-CoV-2 detected → POSITIVE
+ (Ct≤36)	- (Ct>36)	+/-	SARS-CoV-2 detected only for one target → INCONCLUSIVE
- (Ct>36)	+ (Ct≤36)	+/-	SARS-CoV-2 detected only for one target → INCONCLUSIVE
- (Ct>36)	- (Ct>36)	+ (Ct<40)	SARS-CoV-2 not detected → NEGATIVE
- (Ct>36)	- (Ct>36)	- (Ct>40)	Invalid test, repeat extraction and retest

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 SARS-CoV-2 viral RNA.
- Unsuitable handling of the positive control SARS-CoV-2 POS (RdRp & N).
- 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 II, RdRp and N genes, IVD, performance was carried out on the Applied Biosystems® 7500 FAST, Applied Biosystems® QuantStudio 5, Applied Biosystems® QuantStudio 6 Pro and Bio-Rad® CFX96™ Real-Time PCR Systems using the standard protocol described above. 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 HEX™ channel, corresponding to the human RNase P gene, may be absent or display an atypical form (Figure 1A).

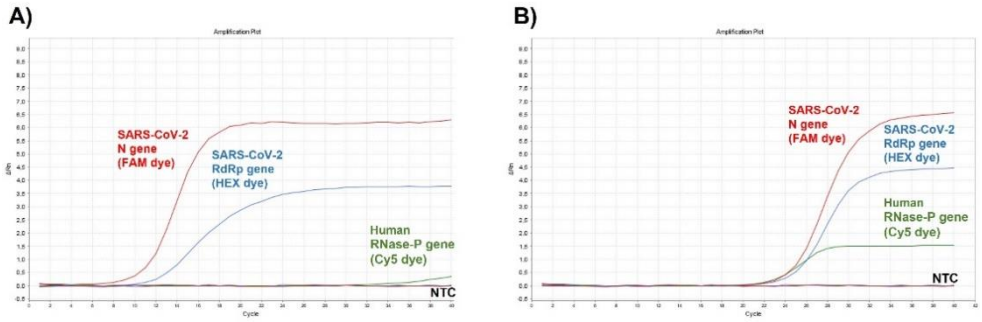

Figure 1. Simultaneous detection of SARS-CoV-2 (RdRp and N genes) and Human RNase-P targets from positive clinical samples with a high (A) and medium (B) SARS-CoV-2 loads. Red curve: detection of the SARS-CoV-2 vRNA target (N gene) through the FAM channel; **Blue curve**: detection of the SARS-CoV-2 vRNA target (RdRp gene) through the HEX channel (*VIC/JOE alternatives*); **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 II, RdRp and N genes, IVD detects 0.25 copies/µL or 250 copies/mL of SARS-CoV-2 viral RNA with a confidence ≥95%, when the standard protocol was used. 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 same LoD was confirmed for the fast protocol.

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 vitro analysis for Cross-Reactivity (Exclusivity) was performed to confirm that NZYtech SARS-CoV-2 One-Step RT-qPCR Kit II, RdRp and N genes, IVD, does not react with other human colonizing microbes and pathogens commonly encountered in clinical specimens. This study was performed by using three commercial respiratory pathogen panels sourced from ZeptoMetrix, notably RP Multimarker Controls (#MDZ001), NATtrol™ Respiratory Pathogen Panel-1 (#NATRPP-1) and NATtrol™ RP Controls, (#NATRPC-NNS). These panels comprise sample polls representative of true clinical human specimens, including Influenza A H3N2 (Brisbane/10/07), Influenza A H1N1 (NY02/2009), Rhinovirus Type 1A, Adenovirus Type 3; Parainfluenza Type 1, Parainfluenza Type 2, Parainfluenza Type 3, Parainfluenza Type 4, Metapneumovirus (Peru 6-2003), *Chlamydophila pneumoniae* (CWL-029), *Mycoplasma pneumoniae* (M-129), Coxsackievirus (Type A1), Influenza A H1N1 (A/New Cal/20/99), Influenza A H1N1 (A/Singapore/63/04), Influenza B (B/Florida/02/06), Respiratory Syncytial Virus A, Respiratory Syncytial Virus B (CH93 (18)-18), Coronavirus (HKU-1 recombinant), Coronavirus (OC43), Coronavirus (NL63), Coronavirus (229E), *Bordetella. pertussis* (A639), *Bordetella pertussis* (A747), *Bordetella holmesii* (F061), *Legionella pneumophila* (Philadelphia) and Human Bocavirus. 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 II, RdRp and N genes, 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 7500 FAST Real-time PCR Instrument.

POTENTIAL INTERFERENT	ACTIVE INGREDIENT	FINAL CONCENTRATION IN SAMPLE	INTERFERENCE YES (Y) NO (N)
Isotonic Sea Water (Rhinomer)	NaCl	15% v/v	N
Throat spray, oral anaesthetic, 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	10 µg/mL	N
Mouth analgeic, anti-inflamatory and antiseptic (Pyralvex)	Rhubard extract, Salicylic acid	5% v/v	N
Antifungal and Antibacterial Oralpharyngeal 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 (Mucosolvan)	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 II, RdRp and N genes, 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 48 replicates of each sample (15 and 150 copies per reaction), for 4 days with 12 replicates of each concentration per day (a total of 120 assays 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 (15 and 150 copies per reaction) using 3 different kit batches with 20 replicates per batch.

Precision of NZYtech SARS-CoV-2 One-Step RT-qPCR Kit II, RdRp and N genes, IVD.

VARIABLE TESTED		RDRP GENE (COPIES/REACTION)		N GENE (COPIES/REACTION)	
		15	150	15	150
REPEATABILITY	n	12	12	12	12
	Mean Cq	31.67	28.46	31.59	28.36
	Coefficient of Variation (%)	1.45	0.66	1.18	0.77
	% Replicate Detection	100	100	100	100
DAILY REPRODUCIBILITY	n	48	48	48	48
	Mean Cq	32.26	28.89	32.30	28.89
	Coefficient of Variation (%)	1.73	1.23	1.73	1.33
	% Replicate Detection	98	100	98	100

LOT-TO-LOT REPRODUCIBILITY	n	60	60	60	60
	Mean Cq	32.14	28.81	32.16	28.78
	Coefficient of Variation (%)	1.83	1.29	1.87	1.44
	% Replicate Detection	98	100	98	100
OPERATOR REPRODUCIBILITY	n	36	36	36	36
	Mean Cq	31.59	28.37	31.71	28.47
	Coefficient of Variation (%)	1.92	0.74	1.82	0.84
	% Replicate Detection	100	100	100	100
INTER-INSTRUMENT REPRODUCIBILITY	n	36	36	36	36
	Mean Cq	31.74	28.53	32.31	29.21
	Coefficient of Variation (%)	1.67	1.22	2.02	2.12
	% Replicate Detection	100	100	100	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 36 replicates of each sample (15 and 150 copies per reaction), in three different qPCR instruments (Applied Biosystems® 7500 FAST, Applied Biosystems® QuantStudio 5 and Bio-Rad® CFX96™ Real-Time PCR Systems), in a total of 72 tests per sample.

11.5 Clinical evaluation

The performance of NZYtech SARS-CoV-2 One-Step RT-qPCR Kit II, RdRp and N genes, IVD, using the standard protocol, with collected nasopharyngeal swab samples, was evaluated by two external laboratories. In total, 240 clinical negative and 130 clinical positive samples have been tested. Data revealed that 100% agreement was achieved for all positive and negative samples tested. The fast protocol was also validated with nasopharyngeal positive and negative swab samples. Data revealed that 100% agreement was achieved for all clinical samples tested.

12. Quality Control

All components of NZYtech SARS-CoV-2 One-Step RT-qPCR Kit II, RdRp and N genes, IVD, are tested following the protocols described above. The triplex real-time PCR system allows the detection of targets described for the identification of SARS-CoV-2 viral RNA (RdRp and N genes) and human mRNA (RNase P gene, RP). Positive amplifications are observed for target genes, positive control and internal controls through FAM, HEX/JOE/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: SARS-CoV-2 One-Step RT-qPCR Kit II, RdRp and N genes, IVD

Catalogue Number: MD04871 and MD04872

Intended use: SARS-CoV-2 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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