Veterinary SARS-CoV-2 One-Step RT-qPCR Kit, RUO

Catalogue number: MD04841, 96 reactions

Application

NZYTech's Veterinary SARS-CoV-2 One-Step RT-qPCR Kit is designed for the *in vitro* qualitative detection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) genome in biological samples collected from wild and domestic animals. Today, SARS-CoV-2, the cause of COVID-19, is a major pandemic that threatens millions of human lives. It is now well established that SARS-CoV-2 likely had ancestors that originated from bats, followed by transmission to an intermediate host that infects humans. Furthermore, there is a strong evidence that SARS-CoV-2 infects multiple animal species, which increases the possibility of further zoonotic transfers of highly infectious virus variants to humans. Thus, it is now clear that these scattered incidences need to be checked to avoid future transmissions from animals. This kit provides a complete set of reagents and probes for the highly specific detection of SARS-CoV-2 genome in animal samples, including an effective internal control to confirm the efficiency of sample RNA extraction and the absence of PCR inhibitors, among others. Nucleocapsid phosphoprotein (N) gene has been identified as an highly specific marker for SARS-CoV-2 virus and NZYTech's kit targets an highly conserved region of SARS-CoV-2 N gene, through an highly optimized primers/probe set. The primers and probe have 100% homology with >95% of the >10,000 genome sequences available on the GISAID database as of Octobre 2020. In addition, primers and probe targeting SARS-CoV-2 genome display no significant homology with unrelated genomes rendering this kit highly specific as there is no cross reactivity with any other Coronavirus sequenced thus far. 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 revisites SARS-CoV-2 genomic targets and, if required, will release new versions of this kit.

General description

One-step real-time RT-PCR is the fastest and most reliable method to perform an accurate detection of SARS-CoV-2 RNA. NZYTech's Veterinary SARS-CoV-2 One-Step RT-qPCR Kit (RUO) includes all reagents required to detect the presence of SARS-CoV-2 RNA in a variety of animal samples. Extracted and purified RNA from different biological material is reverse transcribed 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, one probe specifically anneals to a region of SARS-CoV-2 N gene, when the sample was extracted from an infected animal, and an additional primers/probe set acts as an internal control to detect β-actin (ACTB gene) nucleic acids. The internal control detection validate the efficacy of the extraction process and the absence of PCR inhibitors potencially present in the animals biological samples. This internal control detection was validated for a variety of animal species (see Table 1). The two probes are differently fluorescence labelled, with FAM and HEX reporter dyes, respectively, to allow the identification of the two target genes in a single reaction. In addition, they are provided in optimized concentrations to make sure amplification of animal mRNA, even when present at very high concentrations, does not limit the efficiency of the SARS-CoV-2 primers/probe set.

Table 1. List of **most common** animal species detected by this kit internal control*.

Common name	Scientific name
Dog	*Canis lupus familiaris*
Cat	*Felis catus*
Tiger	*Panthera tigris*
Cheetah	*Acinonyx jubatus*
Leopard	*Panthera pardus*
Red Fox	*Vulpes vulpes*
Giant Panda	*Ailuropoda melanoleuca*
Ferret	*Mustela putorius furo*
Ermine/Stoat	*Mustela erminea*
Otter	*Lutra lutra*
Sea Otter	*Enhydra lutris*
Golden Hamster	*Mesocricetus auratus*
Horse	*Equus caballus*
River Otter	*Lontra canadensis*
Harbor Seal	*Phoca vitulina*
Tupaia	*Tupaia belangeri*
Meerkat	*Suricata suricatta*
Gorilla	*Gorilla gorilla gorilla*
Tatu	*Dasypus novemcinctus*
Grey mouse Lemur	*Microcebus murinus*
Eurasian red Squirrel	*Sciurus vulgaris*
European Squirrel	*Spermophilus citellus*
Alpine Marmot	*Marmota marmota marmota*
Domestic Guinea Pig	*Cavia porcellus*
Long-tailed Chinchilla	*Chinchilla lanigera*

* A complete list of animal species that might be tested using this kit is availabe at NZYTech website *(www.nzytech.com).*

Kit composition

The kit provides a comprehensive set of reagents sufficient to perform 96 one-step RT-qPCR reactions.

Component	Tubes	Volume
NZYSupreme One-step RT-qPCR Master Mix (2x)	1	1000 µL
SARS-CoV-2/ACTB Primer/Probe Mix (FAM & HEX labelled, respectively)	1	220 µL
SARS-CoV-2/ACTB Positive Control (1 x 10^4 copies/µL)	1	120 µL
RNase/DNase Free Water	1	1000 µL

Storage conditions and Kit stability

This One-Step real-time RT-qPCR Kit is shipped refrigerated. All components should immediately be stored at -85°C to -15°C upon arrival. Minimize the number of freeze-thaw cycles by storing in working aliquots. The SARS-CoV-2/ACTB Primer/Probe Mix should be stored protected from light. Particularly, do not expose NZYSupreme One-step RT-qPCR Master Mix to direct sun light after combining with Primer/Probe Mix. NZYTech does not recommend using the kit after the expiry date.

Required Reagents and Equipment

- Real-time PCR Instrument that detects the emitted fluorescence of FAM and HEX/VIC fluorescent dyes (at emission wavelenghts of 520 and 554-6 nm, respectively).
- RNA extraction kit: we recommend to use NZYTech's NZY Viral RNA Isolation kit (Cat. No. MB40701) or NZY Total RNA Isolation kit (Cat. No. MB13402).
- RNase/DNase free qPCR plasticware: PCR tubes, strips, caps, 96-well plates, adhesive films
- Pipettors and filter tips (RNase/DNase free)
- Vortex and centrifuge

Sample Material

All RNA samples suitable for RT-qPCR amplification may be used with this kit. However, different factors such as sample collection, transport, storage and processing time are critical to achieve optimal results. Please ensure RNA samples are suitable in terms of purity, concentration and nucleic acid integrity. NZYTech provides an internal extraction control that targets animal RNA, which is co-purified with viral RNA, and is amplified with the ACTB primers/probe set. This is useful to assess the efficiency of RNA isolation and/or the presence of inhibitors during sample processing.

Dynamic range of test

Under optimal PCR conditions, NZYTech's Veterinary SARS-CoV-2 One-Step real-time RT-PCR Kit display very high priming efficiencies, of >95%, and can detect less than 50 copies of target template from different samples.

Rational for the test

NZYSupreme One-step RT-qPCR master mix

One-step RT-qPCR combines reverse transcription and PCR amplification in a single reaction tube. This saves significant bench time but also reduces errors. The sensitivity of a one-step RT-qPCR protocol is also greater than a two-step workflow as the entire biological sample is available to the PCR without dilution. NZYSupreme One-step RT-qPCR Master Mix is a highly efficient and robust reaction mix that was optimized for the efficient amplification of different RNA targets in multiplexing experiments.

SARS-CoV-2/ACTB Positive Control (1 x 10^4 copies/µL)

The kit includes a Positive control template that allows controlling the performance of RT-PCR reactions. This control is a synthetic nucleic acid molecule carrying sequences that are homologous to SARS-CoV-2 and β-actin targeted sequences included in the detection assay. The SARS-CoV-2/ACTB Positive Control should be used directly with the SARS-CoV-2/ACTB Primer/Probe Mix each time an array of samples is tested. A positive result indicates that the primers and probe sets for detecting the target SARS-CoV-2 and β-actin genes worked properly in the included master mix in that particular experimental scenario. If a negative result is obtained, the test results are invalid and must be repeated. Care should be taken to ensure that the SARS-CoV-2/ACTB Positive Control does not contaminate any other kit component which would lead to false-positive results. This can be achieved by handling this component in a post-PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the Positive Control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the Positive Control into the well. Each µL of the SARS-CoV-2/ACTB Positive Control contains 1 x 10^4 copies of the SARS-CoV-2 and ACTB genes (thus, each reaction containing 8 µL of Positive Control should measure the presence of 8 x 10^4 copies of the target genes).

Negative control

To validate any positive findings, a negative control reaction should be included every time the kit is used. To perform this, RNase/DNase Free Water should be used instead of RNA template. A negative result for all channels (i.e. FAM and HEX/VIC) indicates that the reagents have not become contaminated while setting up the run.

One-step real-time RT-PCR Detection Protocol

Beware that all pipetting steps and experimental plate set-up should be performed on ice. After the plate is prepared proceed 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. Previous to the experiment, start by gently mixing the reaction tubes provided, centrifuge for 5 seconds to collect contents at the bottom of the tube and place tubes on ice. **Pipet Positive Control last to avoid contamination events.**

1. Reaction set-up

1.1. Prepare the reaction mix for SARS-CoV-2/β-actin assays according to the table below that specify the volumes for 1 and *n* reactions (*n*, number of reactions*).

(*) To calculate the total number of reactions needed for each assay, count the number of samples and add two more for the Negative and Positive controls.

Component	1 reaction volume (µL)	*n* reactions volume + 5% (µL)
NZYSupreme One-step RT-qPCR Master Mix (2x)	10	*n* x 10,5
SARS-CoV-2/ACTB Primer/Probe Mix	2	*n* x 2,1
Final Volume	**12**	***n* x 12,6**

1.2. Pipette 12 µL of the RT-PCR mix into individual wells according to your real-time PCR experimental plate set-up.
1.3. For the Negative Control, add 8 µL of RNase/DNase Free Water instead of RNA template into the well. The final volume in each well should be 20 µL.
1.4. For the biological samples, add 8 µL of each RNA sample into the corresponding wells, according to your experimental plate set-up. The final volume in each well should be 20 µL.
1.5. For the Positive Control, add 8 µL of SARS-CoV-2/ACTB Positive Control (8 x 10^4 SARS-CoV-2 & β-actin copies) instead of RNA template into the positive control well. The final volume should be 20 µL.

2. Thermal cycling

2.1. Seal the reaction plate, and briefly spin down before proceed with the RT-PCR and detection steps.
2.2. Place the reaction plate in the real-time PCR instrument and run the RT-PCR protocol according to the section below.

One-step real-time RT-PCR suggested thermal cycling conditions

NZYSupreme One-step RT-qPCR Master Mix (2x) is an optimized and highly efficient reaction mixture developed for RT-PCR. The table below displays a standard protocol optimized on a number of platforms. However, these conditions may be adapted to suit different machine-specific protocols.

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*

*Fluorogenic data should be collected during this step through the FAM and HEX/VIC channels.

Data analysis

Before analysing sample 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 of FAM (SARS-CoV-2) and HEX (ACTB) curves are positive. Positive control is expected to amplify at a Ct<28, both in the FAM and HEX/VIC channels. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

- **No Template Control:** no amplification is detected. If the negative control has one or two amplification curves (FAM and/or HEX) with a sigmoidal shape, sample contamination may have occurred. Repeat the test following good RT-PCR practices.

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

If each one 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.

Interpretation of the results:

The **sample is positive for SARS-CoV-2** if the FAM amplification curve displays a sigmoidal shape with a Ct<35, regardless of what result is obtained for the ACTB (HEX) assay.

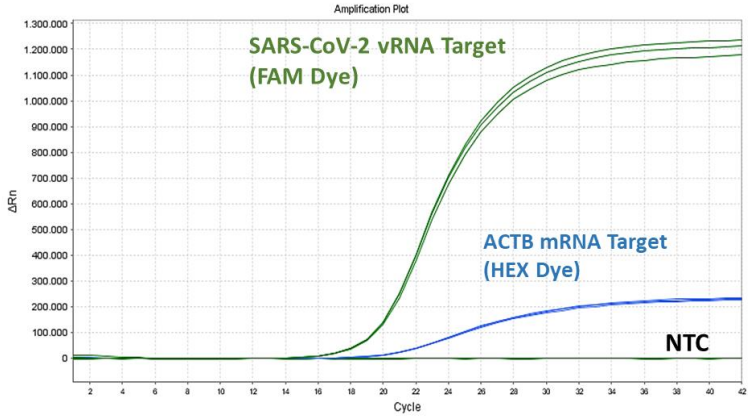
The **sample is negative for SARS-CoV-2** if FAM curve does not amplify or amplify at Ct≥35, while the ACTB displays a positive sigmoidal curve (Ct<40).

The **test is invalid** if the SARS-CoV-2 and ACTB assays are both negatives and should be repeated with nucleic acid re-purified from the sample.

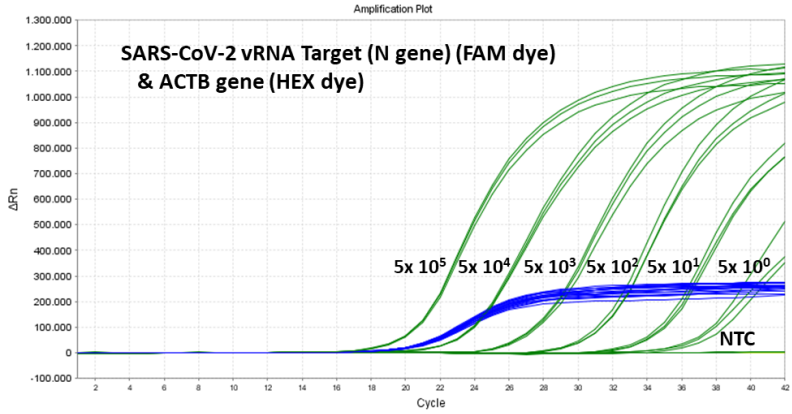
The following table summarizes the interpretation of results (evaluate the overall shape of the amplification curves; only sigmoidal amplification curves are indicative of true amplification).

SARS-CoV-2 result (FAM)	**ACTB result** (HEX)	**Results interpretation**
+ (Ct<35)	+ (Ct<40)	SARS-CoV-2 detected
+ (Ct<35)	- (Ct>40)	SARS-CoV-2 detected
- (Ct≧35)	+ (Ct<40)	SARS-CoV-2 not detected
- (Ct≧35)	- (Ct>40)	Invalid test, repeat extraction

Expected Results



Simultaneous detection of SARS-CoV-2 and animal ACTB targets from a positive animal sample in triplicate. Green curve: detection of the SARS-CoV-2 vRNA target (N gene) through the FAM channel; Blue curve: detection of the ACTB gene through the HEX/VIC channel. Typical No Template Control (NTC) curves are also displayed.



Sensitivity of the Veterinary SARS-CoV-2 One-Step RT-qPCR Kit, RUO. Amplification plot (cycle number versus fluorescence) of 1:10 serial dilutions of the SARS-CoV-2 vRNA Target (N gene) Copy Number Quantification plasmid (from 5x 10^5 copies to 5 copies per reaction) (**curves in green**), in the presence of 5x 10^5 copies of internal control (internal reference) ACTB gene (**curves in blue**). Typical No Template Control (NTC) curves are also displayed. Sample triplicates analysed.

Quality control assay

All components of Veterinary SARS-CoV-2 One-step RT-qPCR Detection Kit (RUO) are tested following the protocols described above. The duplex real-time PCR system allows the detection of targets described for the identification of SARS-CoV-2, precisely the N gene, and the ACTB gene from different species mRNA (see Table 1). Positive amplifications are observed for target genes, positive control and internal controls through FAM and HEX/VIC channels.

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