# Bovine coronavirus RT-qPCR Kit

**Catalogue number**
MD05991

**Presentation**
100 reactions

## Description

Bovine coronavirus (BCoV) is a positive single-stranded RNA virus that belongs to the genus *Betacoronavirus* within the *Coronaviridae* family. This virus causes respiratory and enteric infections in cattle and wild ruminants, infecting the upper and lower respiratory tract and intestine. Infection in calves leads to calf enteritis and contributes to the enzootic pneumonia complex. It can also cause winter dysentery in adult cattle. This virus has been found in cattle worldwide. Bovine coronavirus RT-qPCR Kit is designed for the *in vitro* detection of Bovine coronavirus genomes. The kit is built to have the broadest possible detection profile whilst remaining specific to Bovine coronavirus. Thus, this kit has been designed for the specific (inclusivity) and exclusive (exclusivity) *in vitro* detection of this species. The primers and probe sequences have very high (>95%) homology with a broad range of Bovine coronavirus genomes based on a comprehensive bioinformatic analysis with all reference data within the NCBI database at the time of design. Due to the inherent instability of RNA viral genomes, it is not possible to guarantee the detection of all clinical isolates. This kit was meticulously designed and validated to meet the rigorous criteria of a quantitative assay. However, it is important to note that the provided Positive Control is not intended for quantification purposes. We recommend checking NZYtech website for the availability of a suitable Quantitative Standard for an accurate quantification. In alternative, commercially genomic RNA standards can also be used. If required, a complementary kit for the detection of an endogenous gene of the species from which samples are being extracted is available at NZYtech (see https://nzytech.com/en/molecular-diagnostics/). The complementary usage of an Endogenous Detection reaction provides a solid confirmation that nucleic acids were properly extracted from the selected biological matrix. If you require further information or have a specific question about the detection profile of this kit, please send an e-mail to info@nzytech.com and our scientific team will answer your question. This kit is designed to be used by trained users in a suitable molecular biology laboratory environment.

## Shipping & Storage Conditions

This product can be shipped at a range of temperatures from dry ice to room temperature (RT). Although kit components are stable at room temperature, they should be immediately stored at -85°C to -15°C upon arrival. Also proceed with the following recommendations:

- Once the lyophilized components have been resuspended, they should not be exposed to temperatures above -15°C for longer than 30 minutes at a time.
- Minimise the number of freeze-thaw cycles by storing kit components in working aliquots. The kit is stable for six months from the date of resuspension.
- The PPMix must be stored protected from light. Particularly, do not expose the Lyo NZYSupreme One-step RT-qPCR Master Mix (2x) to direct sunlight after combining it with the PPMix.
- 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.

## Components

The kit provides a comprehensive set of reagents sufficient to perform 100 *in vitro* Real-time PCR reactions.

| COMPONENT | # TUBES | VOLUME | CAP COLOR |
|---|---|---|---|
| Lyo NZYSupreme One-step RT-qPCR Master Mix (2x) | 2 | - | Yellow |
| RT-qPCR master mix reconstitution buffer $^\Delta$ | 1 | 1100 µL | Yellow |
| Lyo Bovine coronavirus/IEC PPMix (10x) * | 1 | - | Brown |
| NTC $^\dagger$ | 1 | 760 µL | Neutral |
| Bovine coronavirus Positive Control | 1 | 100 µL | Red |
| Internal Extraction Control (IEC) RNA | 1 | 525 µL | Neutral |

$^\Delta$ for resuspension of Lyo NZYSupreme One-step RT-qPCR Master Mix (2x).

\* Lyo Bovine coronavirus and Internal Extraction Control (IEC) Primers and Probes Mix (PPMix), FAM™ and HEX™/JOE™/VIC™ labelled, respectively.

$^\dagger$ for usage as No Template Control (NTC) and for Primers and Probe Mix (PPMix) resuspension.

## Reagents, Materials and Equipment Required but Not Provided

- Real-time PCR Instrument that detects FAM™ and HEX™/JOE™/VIC™ fluorescent dyes (emission wavelengths of 520 and 556/555/554 nm, respectively).
- RNA extraction kit: we recommend using NZYtech's RNA extraction kits which are constantly fine-tuned to optimize Molecular Diagnostic applications.
- Quantitative Standard for quantification of *Bovine coronavirus*-containing samples.
- RNase/DNase-free qPCR plasticware: PCR tubes, strips, caps, 96-well plates, and adhesive films (also available at NZYtech).
- Pipettes and filter tips (RNase/DNase-free).
- Disposable gloves.
- Vortex and centrifuge.

## Sample Material

All nucleic acid samples that are suitable for PCR amplification can be used with this kit. However, procedures for the collection of biological sample material, shipping conditions, storage and processing times influence the quality of nucleic acids and may need to be optimized. Please ensure the samples are suitable in terms of purity, concentration, and RNA integrity.

This kit provides a RNA Internal Extraction Control (termed IEC) that can be co-purified and then co-amplified with the target nucleic acid. This is useful for checking the efficiency of RNA extraction and/or the presence of PCR inhibitors contaminating the sample nucleic acids. In addition, we recommend running at least one negative control with the samples (see below). To prepare a negative control, replace the template RNA sample with the No Template Control (NTC).

## The Dynamic Range of the Test

Under optimal PCR conditions this NZYtech Molecular Diagnostic Real-time RT-qPCR Kit displays high priming efficiencies (>95%) and can detect at least 100 copies of the target template per reaction using different sample matrices.

## Rational for the test

### One-step RT-qPCR

One-step RT-qPCR combines the reverse transcription and amplification reactions in a simple closed tube protocol. This saves significant bench time but also reduces errors. NZYtech Bovine coronavirus RT-qPCR Kit includes all reagents to qualitatively identify the presence of *Bovine coronavirus*. The isolated and purified RNA is amplified in a single reaction using a highly Bovine coronavirus-specific primers and probe set, exploiting the so-called TaqMan® principle. During this process, both primers and probe specifically anneal to a selected target region of the *Bovine coronavirus* genome. The fluorogenic probe, which consists of a DNA sequence labelled with a 5'-dye and a 3'-quencher, is degraded during PCR amplification and the reporter dye and quencher are separated, increasing fluorescence. This can be detected on a wide range of real-time PCR platforms.

### Negative Control

To validate any positive findings, a negative control reaction should be included every time the kit is used. To perform this, a reaction should be performed using the No Template Control (NTC) solution provided, instead of the RNA template. A negative result for the two channels (FAM and HEX/JOE/VIC) indicates that the reagents have not become contaminated while setting up the run.

### Positive control (PC)

The kit includes a positive control template (PC) that allows the confirmation of a correct PCR setup. Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that primers and probe used to detect the Bovine coronavirus specific target are working properly. In contrast, if a negative result is obtained, the overall test results are invalid, and the test must be repeated. Care should be taken to ensure that the PC 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. In addition, care should also be taken to avoid cross-contaminating experimental samples when adding the PC to the run. This can be avoided by correctly sealing negative controls and all other samples before pipetting the PC into the positive control well. If such sealing is not possible, we suggest pipetting the PC into a well located the furthest possible from the negative control and biological sample wells. Please note that the PC in the kit is a representative sequence associated with the designed target region and does not contain the organism's entire genome.

### Internal Extraction Control (IEC) RNA

When performing RNA extraction, it is often advantageous to have an exogenous source of RNA template that is spiked into the lysis buffer. This Internal Extraction Control (IEC) RNA is then co-purified with the sample RNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the IEC RNA also indicate that PCR inhibitors are not present at a compromising concentration. The set of primers and probe that detect IEC RNA is supplied in combination with Bovine coronavirus-specific PPMix. Amplification of IEC RNA does not interfere with the detection of the *Bovine coronavirus* target gene even when it is present at low-copy numbers. IEC RNA is detected through the HEX/JOE/VIC channel and should result in a Cq value <28.

## Standard Protocol

## Procedures before starting

To help preventing any carry-over RNA contamination, we recommend assigning independent areas for reaction set-up, the addition of samples and PC, PCR amplification and any post-PCR gel analysis. Also keep the kit components containing nucleic acids, specifically the Bovine coronavirus Positive Control (PC) and the Internal Extraction Control (IEC) RNA, separated from the remaining components of the kit and in the positive control setup area. It is essential that any tubes containing amplified PCR product are not opened in the PCR setup area. We also recommend the use of RNase/DNase-free plasticware/reagents, filter tips (preferably of low retention) and a clean area to work. Prepare the kit contents as described below:

1. Pulse-spin all Lyo tube components in a centrifuge before opening. This will ensure that the lyophilized qPCR master mix (2x) and PPMix remain at the base of the tube, avoiding spilling upon opening the tubes.
   **Note**: *never pulse tubes containing PC and IEC in the same centrifuge used for non-RNA kit components.*
2. In the clean reaction set-up area, reconstitute the **Lyo NZYSupreme One-step RT-qPCR Master Mix (2x)** with 525 µL of **RT-qPCR master mix reconstitution buffer**, as stated below. Flick gently until complete resuspension and spin. Do not replace the reconstitution buffer with water or any other buffer. The master mix is then ready to use as a 2x qPCR master mix.

| COMPONENT | VOLUME (µL/per tube) |
|---|---|
| Lyo NZYSupreme One-step RT-qPCR Master Mix (2x) (Yellow) | 525 |

3. Reconstitute the **PPMix** with 210 µL of the supplied **NTC**. To ensure complete resuspension, vortex the tube thoroughly until complete resuspension and spin. Do not replace the NTC as the reconstitution agent with water or any other buffer. The PPMix is then ready to use as 10x qPCR PP mix.

| COMPONENT | VOLUME (µL) |
|---|---|
| Lyo Bovine coronavirus/IEC PPMix (10x) (Brown) | 210 |

## Nucleic Acids Extraction

The **Internal extraction control (IEC) RNA** can be added either to the RNA lysis/extraction buffer or to the biological sample once it has been resuspended in the lysis buffer.

**Note**: *Do not add the Internal Extraction Control RNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.*

1. Add 4 µL of the **IEC RNA** to the RNA lysis/extraction buffer that will be added to each sample **(4 µL of IEC RNA/sample)**.
2. Proceed to RNA extraction according to the manufacturer's protocols.

## Procedure

### 1. RT-qPCR reaction mixture

Prepare the RT-qPCR reaction mixture according to the table below that specifies the volumes for 1 and *n* reactions (*n*, number of reactions). NZYtech highly recommends performing qPCR analyses in duplicates, which increases the probability of detection of the pathogen and facilitates the interpretation of results.

| COMPONENT | 1 REACTION VOLUME (µL) | *n* REACTIONS [*] VOLUME (µL) |
|---|---|---|
| Lyo NZYSupreme One-step RT-qPCR Master Mix (2x) (Yellow) [**] | 10 | *n* x 10 |
| Lyo Bovine coronavirus/IEC PPMix (10x) (Brown) | 2 | *n* x 2 |
| NTC (**Neutral**) [***] | 3 | *n* x 3 |
| **Final Volume** | **15** | *n* x 15 |

[*] Include sufficient reactions for the negative and positive(s) controls. For negative control use NTC. Positive controls include the Bovine coronavirus Positive Control (**mandatory**) and the Internal Extraction Control (IEC) RNA (**optional**: include this positive control in case IEC was added during sample extraction to confirm the correct function of the IEC detection reaction). We strongly recommend performing replicates of all reactions.

[**] Please note that a precipitate in the bottom of the master mix tube may be observed after resuspension, 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.

[***] In case you prefer to use the IEC RNA as an amplification control (instead of adding it during the extraction protocol), add 2 µL of NTC and 1 µL of IEC RNA per reaction and adjust the volumes needed depending on the number of reactions (*n*).

### 2. Reaction setup
2.1. Pipette 15 µL of each RT-qPCR mix into individual wells according to your real-time PCR experimental plate setup.
2.2. For the negative control reaction (**mandatory**), add 5 µL of NTC instead of the RNA template into the negative control well. The final volume in each well is 20 µL.
   **Note 1:** *Negative controls should be prepared and properly sealed before the addition of the biological samples and positive controls. If this is not possible, avoid pipetting the negative control in adjacent wells to the positive control and biological samples.*
2.3. For the biological sample(s) reaction(s), pipette 5 µL of each extracted RNA sample into the corresponding wells, according to your experimental plate setup. The final volume in each well should be 20 µL.
   **Note 2:** *Seal all biological samples and negative controls before pipetting the PC into the positive control well. If not possible, avoid pipetting the positive and negative controls and the biological samples in adjacent wells.*

**Note 3:** *Up to 8 µl of biological sample can be used. When using an amount other than 5 µl of the sample, the amount of NTC in the reaction mixture must be changed accordingly. Please be aware that a higher volume of sample material may result in partial reaction inhibition.*

2.4. For the <u>Bovine coronavirus positive control</u> reaction (**mandatory**), add 5 µL of Bovine coronavirus Positive Control template into the corresponding well. The final volume in each well should be 20 µL.

2.5. For the <u>IEC positive control</u> reaction (**optional**; just in case IEC RNA was added to the sample during RNA extraction), add 5 µL of Internal Extraction Control (IEC) RNA into the corresponding well. The final volume in each well should be 20 µL.

**Suggested thermal cycling conditions**

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

| CYCLES | TEMPERATURE | TIME | NOTES |
|--------|-------------|------|-------|
| 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 channel. HEX/JOE/VIC channel should be also used in case IEC RNA was added during sample extraction or if it was used as an amplification control.

## Quality Control

**Genomic DNA contamination**

The product must comply with internal standards of DNA contamination as evaluated through real-time qPCR.

**Nucleases assay**

To test for DNase contamination, 0.2-0.3 µg of pNZY28 plasmid DNA are incubated with the kit component in test for 14-16 h at 37 °C. To test for RNase contamination, 1 µg of RNA is incubated with the kit component in test for 1 h at 37 °C. Following incubation, the nucleic acids are visualized on a GreenSafe-stained agarose gel. There must be no visible nicking or cutting of the nucleic acids. To test DNases or RNases contamination of the nucleic acid controls, dilutions of the controls are incubated for 14-16 h at 37 °C and at -20 °C. After incubation, a qPCR/RT-qPCR reaction is performed comparing Ct values of the samples incubated at 37 °C and at -20 °C. There must be a deviation of less than 2 Cts between the two samples.

**Functional assay**

The qPCR/RT-qPCR reactions must ensure the consistent amplification of target DNA/RNA and internal extraction control across serial dilutions, meeting specified acceptance criteria for assay performance.

## Data analysis

Before analysing sample results, we recommend verifying if the real-time RT-qPCR 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:

- **Bovine coronavirus Positive control (PC)**: the amplification curve of FAM (for *Bovine coronavirus* target gene) is positive. The positive control is expected to amplify with a Cq <32. *Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised. Repeat the test.*
- **Positive IEC control (Optional:** in case the IEC RNA was added during sample extraction**)**: the amplification curve of HEX/JOE/VIC, which relates to IEC RNA, is positive. Positive IEC control is expected to amplify with a Cq <28. *Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised. Repeat the test.*
- **Negative control (No Template Control, NTC reaction)**: no amplification is detected. If the negative control has an amplification curve with a sigmoidal shape, sample contamination may have occurred. *Repeat the test following good qPCR practices.*

After verification of the validity of the test, use the following table for the interpretation of principal results (evaluate the overall shape of the amplification curves; only sigmoidal amplification curves are indicative of true amplification).

| Sample Target<br>Cq < 40 (FAM) | Internal Extraction<br>Control<br>Cq < 28 (HEX/JOE/VIC)* | Negative Control<br>Cq > 40 | Positive Control<br>Cq < 32 | Result |
|:---:|:---:|:---:|:---:|:---:|
| + | +/- | - | + | **POSITIVE** result |
| - | + | - | + | **NEGATIVE** result |

*<u>**Internal Extraction Control**</u> (in case IEC RNA was added to the sample during RNA extraction): The Cq value obtained with the IEC in each biological sample will vary significantly depending on the extraction efficiency, the quantity of RNA added to the PCR reaction and the individual machine settings. Cq values < 28 are within the normal range. When amplifying a *Bovine coronavirus* sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.