

Endogenous Control D Real-time PCR Kit

SKU	Presentation
MD05811	48 reactions

Description

Validation of biological templates subjected to molecular testing, through the analysis of extracted nucleic acids, is highly advantageous when employing extremely sensitive techniques that are prone to contamination, such as a qPCR and RT-qPCR. To achieve this relevant goal, NZYtech developed a range of Real-Time qPCR kits to detect a variety of different species currently subjected to molecular diagnostic testing. NZYtech Endogenous Control D Real-time PCR Kit is designed for the *in vitro* detection of the beta (β)-actin gene (*actB*) of several mammals, in particular ruminants such as cattle and sheep. The most common species detected by this kit are listed below (**Table 1**). The list of detectable species is, however, more extensive and is fully available at nzytech website (www.NZYtech.com). This kit is ideal to validate the usage of proper biological samples through nucleic acid analysis and can be used in combination with other NZYtech Molecular Diagnostic kits (www.nzytech.com).

Table 1. List of **most common** species detected by Endogenous Control D Real-time PCR Kit.

Common name	Scientific name
Cattle	<i>Bos taurus</i>
Buffalo	<i>Bison bison bison</i>
Zebu Cattle	<i>Bos indicus</i>
Wild Yak	<i>Bos mutus</i>
Bactrian Camel	<i>Camelus bactrianus</i>
Arabian Camel	<i>Camelus dromedarius</i>
Red Deer	<i>Cervus elaphus</i>
Hamster	<i>Cricetinae gen. sp.</i>
House Mouse	<i>Mus musculus</i>
Domestic Sheep	<i>Ovis aries</i>
Brown Bear	<i>Ursus arctos</i>

Note: A complete list of species that might be validated using this kit is available at NZYtech website (www.nzytech.com).

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 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 48 *in vitro* Real-time PCR reactions.

COMPONENT	TUBES	VOLUME	CAP COLOR
Lyo NZYSupreme qPCR master mix (2x)	1	-	Yellow
qPCR master mix reconstitution buffer ^Δ	1	1100 μ L	Yellow
Lyo Endogenous control D PPMix (10x) *	1	-	Brown
NTC †	1	760 μ L	Neutral
Endogenous control D DNA	1	110 μ L	Green

^Δ for resuspension of Lyo NZYSupreme qPCR master mix (2x).

* Lyo Endogenous control D Primers and Probes Mix (PPMix), FAM™ labelled.

† 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™ fluorescent dye (emission wavelengths of 520 nm).
- DNA extraction kit: we recommend using NZYtech's DNA extraction kits which are constantly fine-tuned to optimize Molecular Diagnostic applications.
- 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 DNA integrity.

We recommend running at least one negative control with the samples (see below). To prepare a negative control, replace the template DNA sample with the No Template Control (NTC).

The Dynamic Range of the Test

Under optimal PCR conditions this NZYtech Molecular Diagnostic Real-time PCR Kit displays high priming efficiencies (>95%) and can detect at least 10 copies of the target template per reaction using different sample matrices.

Rational for the test

Real-time PCR

NZYtech Endogenous Control D Real-time PCR Kit includes all reagents for the detection of beta (β)-actin gene (actB) from several organisms. The isolated and purified DNA is amplified in a single reaction using a highly 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 beta (β)-actin gene (actB). 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 DNA template. A negative result for FAM channel 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. Endogenous control D DNA should be used directly in the reaction (see below). 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 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.

Standard Protocol

Procedures before starting

To help preventing any carry-over DNA 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 Endogenous control D Positive Control (PC) and the Internal Extraction Control (IEC) DNA, 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-DNA kit components.*

2. In the clean reaction set-up area, reconstitute the **Lyo NZYSupreme qPCR master mix (2x)** with 525 μL of **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 qPCR master mix (2x) (Yellow)	525

3. Reconstitute the **PPMix** with 110 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 Endogenous control D PPMix (10x) (Brown)	110

Procedure

1. qPCR reaction mixture

Prepare the qPCR reaction mixture according to the table below that specifies the volumes for 1 and n reactions (n , number of reactions).

COMPONENT	1 REACTION VOLUME (μL)	n REACTIONS * VOLUME (μL)
Lyo NZYSupreme qPCR master mix (2x) (Yellow) **	10	$n \times 10$
Lyo Endogenous control D PPMix (10x) (Brown)	2	$n \times 2$
NTC (Neutral)	3	$n \times 3$
Final Volume	15	$n \times 15$

* Include sufficient reactions for the negative and positive(s) controls. For negative control use NTC. For positive control use Endogenous control D DNA. 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.

2. Reaction setup

- 2.1. Pipette 15 μL of each 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 DNA 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 DNA 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 positive control reaction (**mandatory**), add 5 μL of Endogenous control D DNA into the corresponding well. The final volume in each well should be 20 μL .

Suggested thermal cycling conditions

Lyo NZYSupreme qPCR master mix (2x) is an optimized and highly efficient reaction mixture developed for real-time PCR. 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	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.

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 across serial dilutions, meeting specified acceptance criteria for assay performance.

Data analysis

Before analysing sample results, we recommend verifying if the real-time PCR test is valid. Thus, for each plate, please confirm if the results for Positive and Negative controls performed as expected, according to the following criteria:

- **Positive control (PC):** the amplification curve of FAM is positive. The positive control is expected to amplify with a Cq 26±5. *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 Cq (FAM)	Negative Control Cq > 40	Positive Control Cq < 32	Result
≤ 38	-	+	POSITIVE result
-	-	+	NEGATIVE result