# NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix (2x)

**Catalogue number:**

MB44201, 2 mL (200 x 20 µL)
MB44202, 5 mL (500 x 20 µL)
MB44203, 20 mL (2000 x 20 µL)

## Description

NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix is an optimized and highly efficient reaction mixture developed for first-strand cDNA synthesis and subsequent real-time PCR in a single tube. This offers great convenience and minimizes the risk of errors and contaminations. The master mix, formulated as a 2× reaction mixture, contains all components necessary for both cDNA synthesis and multiplex qPCR amplification (including enzymes, dNTPs, stabilizers and enhancers). Depending on the real-time thermal cycler used, up to 5-6 targets can be quantified simultaneously in the same well or tube. This master mix was engineered with a dual hot-start enzyme control mechanism to provide the highest detection sensitivity. In addition, the latest developments in PCR enhancers have been incorporated in the NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix, including buffer chemistry and incorporation of highly robust engineered enzymes mixed in an optimized proportion specifically designed for the highest-performance. This master mix does not contain ROX and it was specifically developed for probe-detection technology. For qPCR instruments that require this reference dye, we indicate a concentration range for your assay according to the table shown in the section "ROX reference dye" (ROX not provided).

## Shipping Conditions

The product can be shipped in a range of temperatures from dry ice to blue ice.

## Storage Conditions

This master mix should be stored at -85°C to -15°C in a freezer without defrost cycles in order to guarantee maximal shelf life. Minimize the number of freeze-thaw cycles by storing in working aliquots. The product will remain stable till the expiry date if stored as specified.

## Compatible real-time PCR instruments

The master mix is compatible with real-time PCR instruments that do not require a passive reference signal for data normalization. It has been optimized to be compatible with the following real-time PCR instruments:

Bio-Rad®:
CFX96™; CFX384™; iCycler®; iQ™5; Opticon™; Opticon™ 2

Qiagen (Corbett):
Rotor-Gene™ 3000; Rotor-Gene™ 6000 & Rotor-Gene™ Q

Roche:
Lightcycler® 96; Lightcycler® 480 & Lightcycler® Nano

Applied Biosystems (with optional ROX addition):
7000; 7300; 7700; 7900; 7900HT; 7900HT FAST; StepOne™ & StepOne™plus; 7500; 7500 FAST; QuantStudio™ 6, 7, 12k Flex & ViiA7™

## Protocol

The following protocol serves as a general guideline and a starting point for any One-step RT-qPCR procedure. Optimal reaction conditions (e.g., incubation times, temperatures, and primer/ template concentration) may vary and, in particular conditions, may require further optimization.

**RT-qPCR reaction set-up**: the given volumes are based on a standard 20 µL final reaction mix and can be scale adjusted.

| | | |
|---|---|---|
| NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix (2x) (*1) (*2) | 10 µL | 1× |
| Each forward primer (at 10 µM) | 0.8 µL | 400 nM(¥) |
| Each reverse primer (at 10 µM) | 0.8 µL | 400 nM(¥) |
| Each probe (at 10 µM) | 0.4 µL | 200 nM(¥) |
| RNA Template | up to 8 µL | - |
| Nuclease-free water | up to 20 µL | - |

***(Alternative set-up)*** For easy handling of multiple primers used in multiplex assays, we recommend preparing primer/probe mixes for each target by combining the different sets of primers and probes at required concentrations. For example, for a double gene targeting multiplex qPCR, we recommend preparing two 10x concentrated mixes of primers and probes for both targets and proceed as following *(note: if it is more convenient, alternatively prepare 20x concentrated mixes, and then use 1 µL in the reaction mix)*:

| | | |
|---|---|---|
| NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix (2x) (*1) (*2) | 10 µL | 1× |
| 10x Primer + probe mix - **target 1** *(see Table 1)* | 2 µL | primers: 400 nM(¥); probe: 200 nM(¥) |
| 10x Primer + probe mix - **target 2** *(see Table 1)* | 2 µL | primers: 400 nM(¥); probe: 200 nM(¥) |
| RNA Template | up to 6 µL | - |
| Nuclease-free water | up to 20 µL | - |

**Table 1:** Concentrated assay mixtures of primers and probes with suggested concentrations for a standard qPCR reaction (¥)

| Mix (final) | Primer Forward | Primer Reverse | Probe |
|---|---|---|---|
| **10x** | 4 µM | 4 µM | 2 µM |
| **20x** | 8 µM | 8 µM | 4 µM |

*(*1) The master mix does not contain ROX, but, if required, addition of this internal passive reference dye can be conducted in a separate step. The final concentration will vary according to the qPCR instrument used. Please follow instructions described in the section "ROX reference dye" below.*

*(*2) Please notice that a precipitate in the bottom of the master mix tube may be observed, in particular after multiple freeze/thaw cycles. To ensure optimal performance, please make sure all components are thawed and resuspended/homogenized prior to use. In this case do not spin down the master mix before pipetting..*

*(¥) See section of "General considerations" below for more details about primers and probes final concentrations in the reaction.*

**Testing and Ct values:** When comparing this RT-qPCR master mix with a mix from another supplier we strongly recommend amplifying from a 10-fold template dilution series. Loss of detection at low template concentration is the only direct measurement of sensitivity. An early Ct value is not an indication of good sensitivity, but rather an indication of speed.

**ROX reference dye:** NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix (2x) is compatible with most thermocyclers available in the market and can include ROX passive reference dye (not provided) to normalize non-PCR-related fluctuations in fluorescence. If ROX addition is required for your qPCR platform, an optimal quantity of this dye should be included in your master mix. The recommended amount of ROX for the most common qPCR instruments is stated in the table below:

| qPCR instrument | Volume of ROX per 1 mL of Master Mix (2x) (*) |
|---|---|
| **Applied Biosystems**: 7000/7300/7700/7900/7900HT/7900HT FAST/ StepOne™/StepOne™plus | 20-29 µL (500-725 nM) |
| **Applied Biosystems**: 7500/7500FAST/QuantStudio™ 6, 7, 12k Flex/ViiA7™ | 2-4 µL (50-100 nM) |
| **Bio-Rad®:** CFX96™/CFX384™/iCycler®/iQ™5/Opticon™/ Opticon™ 2 **Qiagen:** Rotor-Gene™ 3000/6000/Q **Roche:** Lightcycler® 96/480/Nano | Not required |

*(*) For different volumes please scale-up or scale-down the volume of ROX accordingly. Please notice that ROX is not provided by NZYTech.*

## Suggested thermal cycling conditions

NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix was optimized for the amplification of RNA fragments up to 200 bp under different RT-qPCR cycling conditions. The table below displays a standard cycling setup optimized on several platforms. However, these conditions may be adapted to suit different machine-specific protocols.

| Cycles | Temp. | Time | Main reaction |
|---|---|---|---|
| 1 | 50 °C | 10-20 min (*) | Reverse Transcription |
| 1 | 95 °C | 2-5 min | Polymerase activation |
| 40 | 95 °C | 5 sec | Denaturation |
| | 60 °C | 30 sec-50 sec | Annealing/Extension |

*(*) Reverse transcriptase has a high processivity and may take as little as 10 minutes to synthesize cDNA. However, in specific situations increasing reaction time up to 20 minutes may be beneficial.*

## General considerations

The inherent chemical instability of RNA and the ubiquitous presence of RNases require that particular care should be taken while working with RNA. We recommend using RNase-free plasticware/reagents and work in an RNase-free area (RNase Cleaner, Cat. No. MB16001, can help removing RNases from surfaces and materials). In addition, to help prevent any carry-over DNA contamination, you should assign independent areas for reaction set-up, PCR amplification and any post-PCR gel analysis. It is essential that any tubes containing amplified PCR product are not opened in the PCR set-up area.

**Primers and probe**: These guidelines refer to the design and set-up of dual labelled probes. Please refer to the relevant literature when using other probe types. The specific amplification, yield and overall efficiency of any real-time RT-PCR can be critically affected by the sequence and concentration of the probes and primers, as well as by the amplicon length. We strongly recommend taking the following points into consideration when designing and running your real-time RT-qPCR experiment:

- Primers should have a melting temperature ($T_m$) of approximately 60 °C. The probe $T_m$ should be approximately 10°C higher than that of the primers;
- ***Use spectrally distinct fluorophores to label each probe***;
- The fragment to amplify should be between 80-200 bp in length and not superior to 300 bp;
- Final primer concentrations of 400 nM are suitable for most probe-based reactions. However, to determine the optimal concentration we recommend titrating in the range 0.2-1 µM. Forward and reverse primers concentration should be equimolar;
- A final probe concentration of 100 nM is suitable for most applications; we recommend that the final probe concentration is at least two-fold lower than the primer concentration; to determine the optimal concentration we recommend titrating in the range 0.1-0.25 µM;
- For multiplex RT-qPCR, probe concentrations higher than 100 nM can result in cross channel fluorescence.
- Prior to any multiplex assay, single qPCR reactions might be performed to test the functionality and specificity of each primer/probe pair.

**Template:** It is important that the RNA template is purified and devoid of contamination by RT-qPCR inhibitors (e.g., EDTA). The recommended amount of template is dependent upon the type of RNA used. Please consider the following points when selecting RNA templates:

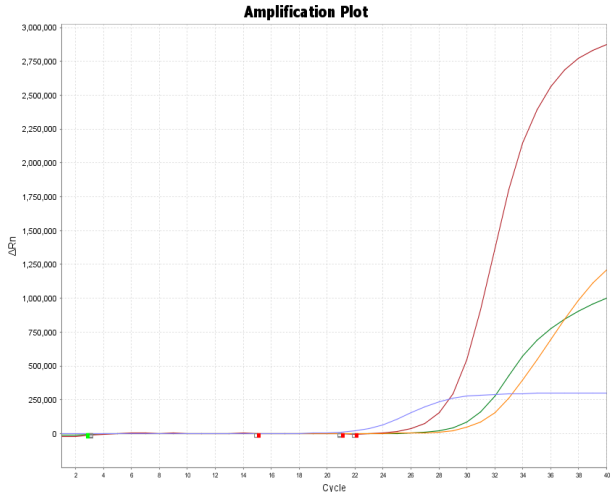
- **Total RNA:** purified total RNA can be used in the range from 1 pg to 1 µg per 20 µL reaction;
- **mRNA:** purified mRNA can be used from 0.01 pg per 20 µL reaction.

To obtain high yield of highly purified RNA we suggest using the NZY Viral RNA Isolation kit (Cat. No. MB407) or NZY Total RNA Isolation Kit (Cat. No. MB134).

**$Mg^{2+}$:** It is not necessary to supplement the reaction mixture with $MgCl_2$ as the NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix already contains an optimized concentration of $MgCl_2$.

**RT-qPCR optimization**: It may be necessary to improve the efficiency of some reactions, such as multiplexing with more than two probes, or if the target amplicon is longer than 200 bp. In these cases, the reverse transcription reaction time can be extended up to 30 minutes; the annealing/extension time can be extended up to 60 seconds.

## Example of a Multiplex Result




**Simultaneous detection of five SARS-CoV-2 targets plus one human RNAseP (RP) target from a positive nasopharyngeal sample.** Red curve: detection of the SARS-CoV-2 vRNA target (two targets for N gene) through the FAM channel; Orange curve: detection of the SARS-CoV-2 vRNA target (two targets for RdRp gene) through the HEX channel; Green curve: detection of the SARS-CoV-2 vRNA target (one target for E gene) through the TxRed channel, and Purple curve: detection of the RP gene through the Cy5 channel.

## Quality control assays

### Genomic DNA contamination

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

### Nuclease assays

To test for DNase contamination, 0.2-0.3 µg of pNZY28 plasmid DNA are incubated with the master mix for 14-16 h at 37 °C. To test for RNase contamination, 1 µg of RNA is incubated with the master mix 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.

### Functional assay

NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix is extensively tested for multiplexing activity, processivity, efficiency, sensitivity and heat activation.

*V2205*

## Certificate of Analysis

| Test | Result |
|---|---|
| Genomic DNA contamination | Pass |
| Nuclease assays | Pass |
| Functional assay | Pass |

Approved by:

Patrícia Ponte
Senior Manager, Quality Systems

*For research use only.*

nzytech
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Estrada do Paço do Lumiar, Campus do Lumiar - Edifício E, R/C, 1649-038 Lisboa, Portugal Tel.:+351.213643514 Fax: +351.217151168
www.nzytech.com