MB442_IFU_EN_V2601

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

Catalogue number	Presentation
MB44201	2 x 1 mL (200 rxns of 20 µL)
MB44202	5 x 1 mL (500 rxns of 20 µL)
MB44203	20 x 1 mL (2000 rxns of 20 µL)
MB44205	1 x 50 mL (5000 rxns of 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 also supports various types of assays, from duplex to hexaplex detection, providing experiment flexibility. Formulated as a 2× reaction mixture, it 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 multiple 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 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 of "Technical Notes" (ROX not provided).

Shipping & Storage Conditions

The product can be shipped in a range of temperatures from dry ice to blue ice. Upon arrival, all components should be stored at -85 °C to -15 °C in a constant temperature freezer to guarantee maximal shelf life. Minimize the number of freeze-thaw cycles by storing it in working aliquots. Do not expose the master mix to direct sunlight. The product will remain stable till the expiry date if stored as specified.

Components

COMPONENT	SKU	TUBES/BOTTLES	VOLUME
NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix	**MB44201**	1	1 mL
	MB44202	5	1 mL
	MB44203	20	1 mL
	MB44205	1	50 mL

Note: Consider preparing multiple aliquots of the master mix to reduce freeze/thaw cycles and minimize the risk of contamination.

Specifications

Compatibility with real-time PCR instruments

NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix is compatible with instruments that do not require a passive reference signal for data normalization. It was optimized to be compatible with the following real-time PCR instruments:

Bio-Rad®: CFX96™; CFX OPUS, 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™

Sensitivity

NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix (2x), ensures high sensitivity in nucleic acid detection in a Hexaplex assay designed to detect SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) and the Nucleocapsid phosphoprotein (N) genes, Influenza A and B Matrix (M1) and Nonstructural 2 (NS2) specific genes, the RSV (subtypes A and B) L gene and the human ribonuclease P (RNase P, RP) gene.

Standard Protocol

Recommendations before starting

- Nucleic acid manipulation: 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 working in an RNase-free area (Nucleases & Nucleic Acid Cleaner, Cat. No. MB48301, or DNA & RNA Cleaner, Cat. No. MB46201, can help remove RNases from surfaces and materials).
- Handling instructions: 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. Any tubes containing amplified PCR product mustn't be opened in the PCR set-up area. Use sterile filtered tips. Minimize exposure by keeping reaction and components capped whenever possible.
- Controls: For verification of the absence of contamination, prepare a mixture sample without a DNA template (negative control). Additionally, include a positive control to serve as a reference for ensuring the correct functioning of the qPCR reaction and detection system. The positive control should exhibit the expected amplification and/or fluorescence signal, confirming the assay's ability to accurately detect the target sequence. To avoid incorrect results caused by unwanted fluorescent substances, consider adding a No-Amplification Control tube. This tube should have the sample but not the enzyme master mix. Elevated fluorescence in the No-Amplification Control compared to the No-Template Control suggests potential fluorescent contaminants in either the sample or the thermal cycler's heat block.
- Validation of each primer/probe pair: Before any multiplex assay, single qPCR reactions might be performed to test the functionality and specificity of each primer/probe pair.

Procedure

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 template concentration) may vary and, in particular conditions, may require further optimization.

Two approaches can be used to set up the experiment: 1) adding the primers and probes individually, or 2) adding primers and probes as pre-prepared mixture solutions. Please select the protocol that is more convenient for your experiments:

Protocol 1 – Set up adding primers and probes individually

1. Thaw the master mix at room temperature or on ice. Mix the master mix thoroughly by flicking the tube and inverting it.
   Note: *A precipitate may form at the bottom of the master mix tube. For optimal performance, the solution should be fully thawed and gently mixed before use. In this case, do not centrifuge the master mix before pipetting. Please note that although mixing homogenizes the mixture, it will not reduce turbidity or dissolve the precipitate. Master mix activity is not affected by its apparent turbidity.*
2. In a clean reaction setup area, prepare the RT-qPCR reaction mixture (without template) according to the table below (please notice that the given volumes are based on a standard 20 µL final reaction mix and can be scale adjusted):
   Note 1: *If setting up more than one reaction, prepare a reaction mixture volume 10% greater than the total required for the number of reactions to be performed. Include sufficient reactions for the No-Template and positive controls.*
   Note 2: *If necessary, prepare a No-Amplification Control by adding nuclease-free water instead of the RT-qPCR master mix.*
   Note 3: *We recommend performing replicates of all reactions.*

	1 REACTION VOLUME	**FINAL CONCENTRATION**
NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix (2x) (1)(2)	10 µL	1×
10 µM forward primer	0.8 µL	400 nM (3)
10 µM reverse primer	0.8 µL	400 nM (3)
10 µM probe	0.4 µL	200 nM (3)
FINAL VOLUME =	up to 12 µL(4)	-

(1) If a slight precipitate is observed at the bottom of the master mix tube, please refer to the note above (step 1).
(2) The master mix does not contain ROX, but, if required, the 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 the instructions described in the section "Technical Notes" below.
(3) Refer to the section of "Technical Notes" below for more details about primers and probes final concentrations in the reaction.
(4) If using smaller volumes for the primers & probe, supplement the volume up to 12 µL with Nuclease-free Water.

3. Gently mix and centrifuge briefly to spin down the contents.
4. Pipette 12 µL of the Mix into each well, according to your experimental plate/strip/tube configuration.
5. Pipette template (samples and controls):
   a. Add up to 8 µL of RNA sample/positive control in each respective well. If using less volume than 8 µL, supplement with Nuclease-free Water up to 8 µL. In total, the final volume of the reaction will be 20 µL.
   b. Add up to 8 µL of Nuclease-free Water for the negative control to achieve a final volume of 20 µL.

Note: *To avoid cross-contamination, we strongly recommend pipetting the template at last, preferably in a work separate area.*

6. Cover and seal the plate/strip/tube with appropriate caps or optical adhesive film before proceeding with the real-time PCR detection steps.
7. Centrifuge briefly to spin down the contents and eliminate any air bubbles from the reaction mixtures.
8. Place the reaction plate/strip/tube within the real-time PCR instrument and run the general protocol defined below. These conditions might be adapted to suit your specific needs, within sensible limits.

Protocol 2 – Set up adding primers and probes as pre-prepared mixtures

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 mixtures of primers and probes (or alternatively 20x concentrated mixes) for both targets (Table 1) and proceed as follows:

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. Thaw the master mix at room temperature or on ice. Mix the master mix thoroughly by flicking the tube and inverting it.
   Note: *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 mix components are thawed and resuspended/homogenized before use. In this case, do not spin down the master mix before pipetting.*
2. In a clean reaction setup area, prepare the RT-qPCR reaction mixture according to the table below (please notice that the given volumes are based on a standard 20 µL final reaction mix and can be scale adjusted):
   Note 1: *If setting up more than one reaction, prepare a reaction mixture volume 10% greater than the total required for the number of reactions to be performed. Include sufficient reactions for the No-Template and positive controls.*
   Note 2: *If necessary, prepare a No-Amplification Control by adding nuclease-free water instead of the RT-qPCR master mix.*
   Note 3: *We recommend performing replicates of all reactions.*

	1 REACTION VOLUME	FINAL CONCENTRATION
NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix (2x) (1)(2)	10 µL	1×
10x primer + probe mix – **target 1** (*see Table 1*)(5)	2 µL	Primers: 400 nM (3) Probe: 200 nM (3)
10x primer + probe mix – **target 2** (*see Table 1*)(5)	2 µL	Primers: 400 nM (3) Probe: 200 nM (3)
FINAL VOLUME =	up to 14 µL(4)	-

   (1) If a slight precipitate is observed at the bottom of the master mix tube, please refer to the note above (step 1).
   (2) The master mix does not contain ROX, but, if required, the 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 the instructions described in the section of "Technical Notes" below.
   (3) Refer to the section of "Technical Notes" below for more details about primers and probes final concentrations in the reaction.
   (4) If using smaller volumes for the primers & probe, supplement the volume up to 14 µL with Nuclease-free Water.
   (5) If it is more convenient, alternatively prepare 20x concentrated mixes, and then use 1 µL in the reaction mix.
3. Gently mix and centrifuge briefly to spin down the contents.
4. Pipette 14 µL of the Mix into each well, according to your experimental plate/strip/tube configuration.
5. Pipette template (samples and controls):
   a. Add up to 6 µL of RNA sample/positive control in each respective well. If using less volume than 6 µL, supplement with Nuclease-free Water up to 6 µL. In total, the final volume of the reaction will be 20 µL.
   b. Add up to 6 µL of Nuclease-free Water for the negative control to achieve a final volume of 20 µL.

   Note: *To avoid cross-contamination, we strongly recommend pipetting the template at last, preferably in a work separate area.*
6. Proceed according to Protocol 1 (steps 6 to 8).

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 1-step RT-qPCR setup optimized on several platforms. However, these conditions may be adapted to suit different equipment-specific protocols.

CYCLES	TEMP.	TIME	STAGE
1	50 - 55°C	10 - 20 min (*)	Reverse Transcription
1	95 °C	2 - 5 min	Polymerase activation
40	95 °C 60 °C	5 sec 30 - 60 sec (**)	Denaturation 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.*
(**) *Amplicons exceeding 200 base pairs in length can be employed, although optimizing extension times may be necessary. An additional increment in the extension step, up to 60 seconds, may also be required for complex multiplexing assays.*

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.

Technical Notes

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 (Tm) of approximately 58-62 °C. The probe Tm 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 70-200 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 150-600 nM. Forward and reverse primers concentration should be equimolar;
- A final probe concentration of 150-200 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 50-300 nM;
- For multiplex RT-qPCR, high probe concentrations 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: The RNA template must be purified and devoid of contamination by RT-qPCR inhibitors (*e.g.* EDTA). The recommended amount of template is dependent upon the source 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 5 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).

$MgCl_2$: 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.

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: 7300/7700/7900/7900HT/7900HT FAST/ StepOne™plus	500-725 nM (20-29 µL) (**)
Applied Biosystems: 7500/7500FAST/QuantStudio™ 5, 6, 7, 12k Flex/ViiA7™	50-100 nM (2-4 µL) (**)
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.*
(**) *The volume calculated considering a ROX reference dye stock at 25 µM.*

Data

NZYSupreme Multiplex One-step RT-qPCR Probe Master Mix (2x) was tested for the simultaneous detection of five SARS-CoV-2 targets plus one human RNAse P (RP) target from a positive nasopharyngeal sample. The resultant amplification plot is exposed in Figure 1 and serves as an example of a multiplex result.

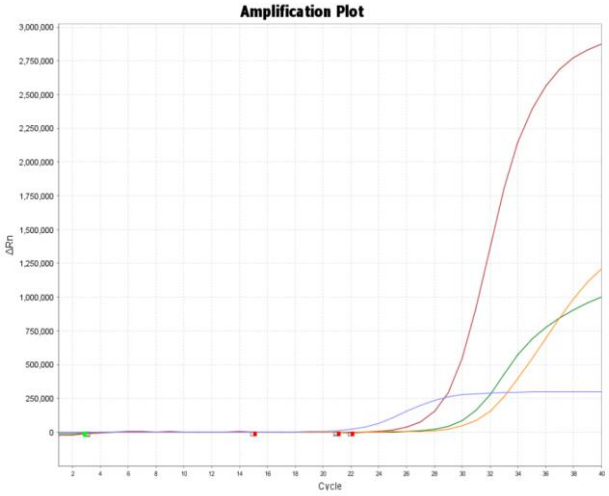

Figure 1. Simultaneous detection of five SARS-CoV-2 targets plus one human RNAseP (RP) target from a positive nasopharyngeal sample. Red curve: detection of the SARS-CoV-2 vRNA target (two targets for N gene) through the FAM channel; Orange curve: detection of the SARS-CoV-2 vRNA target (two targets 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; 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 PCR.

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 activity, processivity, efficiency, sensitivity and heat activation.

This master mix is manufactured under stringent quality standards and complies with ISO 9001 and ISO 13485 certifications for research and diagnostic-grade reagents.