NZYSupreme Multiplex qPCR Probe Master Mix (2x)

Catalogue number:

MB45201, 2 mL (200 x 20 µL)
MB45202, 5 mL (500 x 20 µL)
MB45203, 20 mL (2000 x 20 µL)

Description

NZYSupreme Multiplex qPCR Probe Master Mix (2x) is an optimized and highly efficient reaction mixture developed for real-time PCR. The master mix, formulated at a 2× concentration, contains all components necessary for multiplex qPCR amplification (including enzyme, dNTPs, stabilizers and enhancers). Depending on the real-time thermal cycler used, assays with up to 5-6 targets can be detected simultaneously in the same well/reaction. This master mix was engineered with an enzyme dual hot-start control mechanism to provide the highest detection sensitivity. In addition, the latest developments in PCR enhancers have been incorporated in the NZYSupreme Multiplex qPCR Probe Master Mix (2x), including buffer chemistry and incorporation of highly robust engineered enzymes mixed in an optimized proportion specifically designed for the highest performance. This master mix was specifically developed for probe-detection technology.

Shipping Conditions

The product can be shipped at 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. The master mix has is compatible with the following real-time PCR instruments:

Bio-Rad®:
CFX96™; CFX384™; CFX Opus; 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™ 5, 6, 7, 12k Flex & ViiA7™

Protocol

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

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

NZYSupreme Multiplex qPCR Probe Master Mix (2x) (*1) (*2)	10 µL	1×
Each forward primer (at 10 µM)	0.8 µL	0.4 µM(¥)
Each reverse primer (at 10 µM)	0.8 µL	0.4 µM(¥)
Each probe (at 10 µM)	0.4 µL	0.2 µM(¥)
Template	up to 8 µL	>1-10 copies
Nuclease-free water	up to 20 µL	-

(Alternative set-up) For easy handling of multiple primers used in multiplex assays, we recommend preparing primers/probes mixes for each target by combining the different sets of primers and probes at required concentrations. For example, for a five-target multiplex qPCR, we recommend preparing one 10× concentrated mix of primers and a 10× concentrated mix of probes contemplating all 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 qPCR Probe Master Mix (2x) (*1) (*2)	10 µL	1×
10x primers mix *(see Table 1)*	2 µL	0.4 µM(¥)
10x probes mix *(see Table 1)*	2 µL	0.2 µM(¥)
Template	up to 6 µL	>1-10 copies
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 qPCR master mix with a mix from another supplier we strongly recommend amplifying 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 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 the fluorescent reporter signal and correct non-PCR-

related fluorescence fluctuations. 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	Final ROX concentration per 1 mL of Master Mix (2x) (*)
Applied Biosystems: 7000/7300/7700/7900/7900HT/7900HT FAST/StepOne™/StepOne™plus	500-725 nM
Applied Biosystems: 7500/7500FAST/QuantStudio™ 5, 6, 7, 12k Flex/ViiA7™	50-100 nM
Bio-Rad®: CFX96™/CFX Opus/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 qPCR Probe Master Mix (2x) was optimized for the amplification of DNA fragments up to 250 bp under different 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	Notes
1	*95 °C	*2-5 min	Polymerase activation
40	95 °C	5 sec	Denaturation
	60 °C	**30 sec – 50 sec	Annealing/Extension

** 2 min for cDNA, up to 3 or 5 min for genomic DNA.*

*** Up to 50s may be necessary for multiplexing with more than two probes.*

General considerations

In order to prevent nucleic acid contamination, we recommend that users assign independent areas for reaction set-up, addition of samples and positive controls, 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 Design: 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 qPCR 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 qPCR experiment:

- Primer pairs should have a compatible melting temperatures (T_m), between 58-60 °C (within 5ºC). 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 80-250 bp in length and ideally at the shorter end of this range. However, with adjusted times it is possible to amplify products up to 300 bp (the size of the amplicon should not exceed 300 bp).
- Final primer concentrations between 0.3-0.4 µM are suitable for most probe-based reactions. However, to determine the optimal concentration we recommend titrating in the range 0.1-0.6 µM. Forward and reverse primers concentration should be equimolar;
- A final probe concentration between 0.15-0.25 µM 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 0.1-0.5 µM;
- Prior to any multiplex assay, single qPCR reactions are imperative to be performed to test the functionality and specificity of each primer/probe pair.

Template: It is important that the nucleic acid template is purified and concentrated according to conventional nucleic acid clean up procedures. In addition, templates must be devoid of any contaminating PCR inhibitors (e.g. EDTA). The recommended amount of template for PCR is dependent upon the type of DNA used. Please consider the following points when using genomic DNA or cDNA templates:

- **Genomic DNA:** use up to 1 µg of genomic DNA in a single PCR. We recommend using NZY Tissue gDNA Isolation kit (Cat. No. MB135) for high yield and purity from both prokaryotic and eukaryotic sources.
- **cDNA:** the optimal amount of cDNA to use in a single PCR depends upon the copy number of the target gene; we suggest using 100 ng cDNA per reaction. However, this amount may be adjusted to a more appropriate concentration. We suggest using NZY First-Strand cDNA Synthesis Kit (Cat. No. MB125) for reverse transcription of purified RNA. To obtain high yield of highly purified RNA we suggest using 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 qPCR Probe Master Mix (2x) already contains an optimized concentration of $MgCl_2$.

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 150 bp. In these cases, the annealing/extension time can be extended up to 60 seconds.

PCR controls: The reliability of the data may be affected by the presence of contaminating DNA. It is strongly recommended to include a no-template control reaction in the qPCR design, replacing template DNA/cDNA with nuclease-free PCR-grade water (DEPC-treated Water, Cat. No. MB43701).

Example of a Multiplex Assay

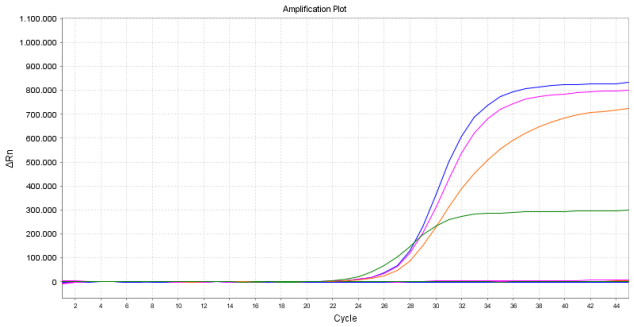

Figure 1: Representative tetraplex fluorescence curves generated by the detection of three Methicillin-resistant *Staphylococcus aureus* (MRSA) targets plus a human endogenous control in a positive clinical sample. Blue curve: detection of *mecA or mecC* targets through the FAM channel; Pink curve: detection of *nuc* target through the TexasRed channel; Orange curve: detection of *SCCmec/orfX junction* target through the VIC/HEX channel, and Green curve: detection of human *RNaseP* target 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 Premium-stained agarose gel. There must be no visible nicking or cutting of the nucleic acids.

Functional assay

NZYSupreme Multiplex qPCR Probe Master Mix (2x) is extensively tested for multiplexing activity, processivity, efficiency, sensitivity and heat activation.

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