MB337_IFU_EN_V2601

Multiplex PCR NZYProof 2× Green Master Mix

Catalogue number	Presentation
MB33701	2 x 1.25 mL (100 rxns of 50 µL)
MB33702	4 x 1.25 mL (200 rxns of 50 µL)

Description

Multiplex PCR NZYProof 2× Green Master Mix is a premixed ready-to-use solution designed for the simultaneous amplification of multiple DNA fragments (up to 15 targets) in a single tube with high accuracy. The Master Mix contains an engineered proofreading DNA polymerase displaying a hot-start-like PCR capacity and high processivity (fast polymerisation), while reducing errors due to its 3'→5' exonuclease activity. The enzyme is inactive at room temperature, avoiding extension of non-specifically annealed primers or primer-dimers, and thus ensuring highly specific and sensitive multiplex PCR amplification. In addition, the master mix contains dNTPs and reaction buffer at optimal concentrations for efficient multiplexing of targets ranging in size from 70 bp to 2.5 kb over a broad range of primer and template concentrations. $MgCl_2$ final concentration is 1.5 mM, allowing the implementation of a comprehensive variety of PCR protocols. Reactions assembled with Multiplex PCR NZYProof 2× Green Master Mix may be directly loaded onto agarose gels, which simplifies the workflow. There are two dyes (blue and yellow) in the mix that allow monitoring the progress of electrophoresis. Multiplex PCR NZYProof 2× Green Master Mix is not suitable when direct fluorescent or absorbance readings are required without prior purification of the amplified DNA from PCR. We recommend using the master mix version without dyes – Multiplex PCR NZYProof 2× Colourless Master Mix (Cat. No. MB338) – or purifying the PCR product using NZYGelpure (NZYtech, Cat. No. MB011) before performing any other protocol.

Resulting PCR products have blunt-ends and are suitable for cloning with NZYtech´s NZY-blunt PCR cloning kit (NZYtech, Cat. No. MB121).

Shipping & Storage Conditions

The product can be shipped 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 the master mix in working aliquots. The Mix may be stored at 4 °C for up to 7 days. The product will remain stable till the expiry date if stored as specified.

Components

COMPONENT	SKU	TUBES/BOTTLES	VOLUME
Multiplex PCR NZYProof 2× Green Master Mix	**MB33701**	2	1.25 mL
	MB33702	4	1.25 mL

Standard Protocol

Recommendations before starting

- Product usage and reaction requisites:
  - Ensure that the DNA fragments you want to simultaneously amplify differ in size sufficiently enough to be distinguished through electrophoresis.
  - Primers producing a single product of correct size are pivotal for successful multiplex assays. Thus, special care must be taken when designing multiplex PCR primers. See below general recommendations for primer design.
  - Prior to any multiplex assay, we recommend performing single PCR reactions to test the functionality and specificity of each primer pair.
  - For easy handling of multiple primers used in multiplex assays, we recommend preparing a primer mix by combining all primers at equimolar concentrations.
  - The enzyme provided in the Multiplex PCR NZYTaq 2× Green Master Mix is inactive at room temperature, but its functional activity is restored during the 5-min incubation step at 95 °C.
- Nucleic acid manipulation: Stringent precautionary measures must be imposed to mitigate the risk of carry-over contamination of DNA. We recommend using DNase-free plasticware/reagents and working in a DNase-free area (Nucleases & Nucleic Acid Cleaner, Cat. No. MB48301, or DNA & RNA Cleaner, Cat. No. MB46201, can help remove DNases 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.
- Preventing Contamination: Implement stringent laboratory practices to avoid false positives caused by contaminants. Use sterile equipment, clean workstations regularly, and monitor assay integrity by including No-Template Controls (NTC) in each PCR run.

FOR REFERENCE USE ONLY

Protocol

The following protocol serves as a general guideline and a starting point for standard multiplex PCR amplifications. Depending on the complexity of the PCR reaction (such as multiplex reactions with more than 10 targets, amplification of fragments longer than 2 kb or when using very low amounts of starting template), reaction conditions (e.g., incubation times and temperatures, concentration of primers and/or template DNA) vary and may need to be optimised.

1. Gently mix and briefly centrifuge the master mix after thawing.
On ice, in a sterile, nuclease-free microcentrifuge tube, prepare a reaction mixture for the appropriate number of samples to be amplified. A single reaction mixture should combine the following components (for a 50 µL reaction):

	1 REACTION
Multiplex PCR NZYProof 2× Green Master Mix	25 µL
Primer Mix	0.2 µM [1]
DNA Template	5 ng-0.5 µg
Nuclease-free water	up to 50 µL
FINAL VOLUME =	50 µL

[1] Recommended final primer concentration is 0.2 µM. However, the primer concentration may be optimised in a range of 0.1-0.3 µM. If a same primer is used to amplify more than one target, it may be beneficial to use this primer at a 0.4 µM final concentration.

2. Gently mix and centrifuge briefly to spin down the contents.

3. Perform PCR using the following cycling parameters:

CYCLES	**TEMP.**	**TIME**	**STAGE**
1	95 °C	3 min	Initial Denaturation
25-40 †	94 °C	30 sec	Denaturation
	*	30-60 sec	Annealing
	72 °C	30 sec/kb ¥	Extension
1	72 °C	5-10 min	Final Extension

(*) Care must be taken when selecting the annealing temperature because a low annealing temperature may yield nonspecific PCR products while a higher value is likely not to amplify the target. The optimal annealing temperature should be optimised for the primer mixture. We recommend to use 3-5°C below the lowest melting temperature (Tm) of the primer mixture. If necessary, perform a gradient PCR to find the optimal annealing temperature.

† 30 cycles is optimal for most protocols. A larger number of cycles may be required to increase PCR sensitivity when using low levels of template DNA. Smaller number of cycles may be used when amplifying microsatellites or short amplicons.

¥ Calculate extension time based on the size of the largest fragment you want to amplify.

4. Analyse the PCR products by agarose gel electrophoresis and visualise with GreenSafe Premium (NZYtech, Cat. No. MB132) or any other mean. When separating samples through agarose gel electrophoresis, please consider that both concentration and grade of agarose are important when working with closely sized fragments. NZYtech offers a vast portfolio of agaroses with different specifications to cover a wide range of needs. Please visit www.nzytech.com to identify the agarose that most suits your experiment

Technical Notes

Primers: Special care must be taken when designing multiplex PCR primers. In general, primers should range in length from 20–34 bases and should contain 40–60% of GC residues. Minimize the number of consecutive G's in the primers. Preferably, G and C residues should be distributed uniformly along the primer. Avoid sequences that might produce internal secondary structures. Also, avoid significant homology between primers. The 3´-ends of the primers should not be complementary to avoid the production of primer-dimers. Avoid three G or C nucleotides in a row near the 3´-end of the primer, as this may result in non-specific primer annealing, and increased synthesis of undesirable reaction products. All primers should have nearly identical melting temperatures (Tm). The recommended Tm of all primers is between 55-60 °C and should not differ by more than 5-6 °C. To design primers for multiplex PCR, it is important to adjust the amplification conditions for each primer pair so that they produce a single PCR product of correct size. Another critical factor for successful multiplexing is the quality of primers. Primers should be of high-quality, purchased desalted or purified. We recommend dissolving primers in TE buffer and to prepare small-volume aliquots of a primer mix containing all primer pairs at equimolar concentrations. Store at -20 °C and avoid repeated freezing and thawing.

Template: The optimal amount of starting material may vary depending on the template DNA quality and complexity, as well on the range of targets size. In general, we recommend using 500-20 ng of genomic DNA template. Lower amounts of DNA template (typically 20-1 ng) can be used for amplification of lambda or plasmid DNA or even 50-10 ng for amplification of multicopy chromosomal genes.

PCR controls: The reliability of multiplex PCR results can be significantly affected by contaminating DNA or unintended amplification events. It is therefore strongly recommended to include a non-template control (NTC) in every experiment, in which the template DNA is replaced with nuclease-free, PCR-grade water. This control helps detect reagent contamination, primer-dimer formation, or cross-contamination introduced during reaction setup.

FOR REFERENCE USE ONLY

In addition to the NTC, a critical step in multiplex assay development is to validate each primer pair in singleplex reactions before combining them. Testing primers individually confirms their specificity, efficiency, and expected amplicon size under the chosen cycling conditions. Only after each target performs reliably on its own should the primers be combined in a multiplex format. This stepwise validation helps distinguish true multiplex-related issues (e.g., primer competition or cross-interactions) from problems inherent to individual primer sets.

Intercalating Dyes: The master mix contains both a blue and a yellow dye. The blue dye migrates at the same rate as a 3–5 kb DNA fragment in a 1% (w/v) agarose gel. The yellow dye migrates faster than primers (<50 bp) in a 1% (w/v) agarose gel. This mix is not suitable for direct fluorescence or absorbance measurements without prior purification of the amplified DNA from the PCR.

Quality control assays

Genomic DNA contamination

The product must be free of any detectable DNA contamination as evaluated through PCR.

Nuclease assays

0.2-0.3 µg of pNZY28 plasmid DNA are incubated with Multiplex PCR NZYProof 2× Green Master Mix for 14-16 hours at 37 °C. Following incubation, the DNA is visualised on a GreenSafe Premium-stained agarose gel. There must be no visible nicking or cutting of the nucleic acid.

Functional assay

Multiplex PCR NZYProof 2× Green Master Mix is tested for performance in a PCR assay for the simultaneously amplification of 12 fragments (sizes ranging between 200-2500 bp) from human genomic DNA. The resulting PCR products are visualised as single bands in a 2.5% agarose gel (ultrapure grade) stained with GreenSafe Premium.

Troubleshooting

Troubleshooting is often a systematic, meticulous process where varying one parameter at a time and evaluating impacts can unveil the root cause of issues. These adjusted suggestions, incorporating a blend of specificity and exploratory approaches, aim to enhance the clarity and actionability of your troubleshooting guide. Should any other technical or procedural aspects require attention, your feedback and additional information will always be welcomed.

NO PRODUCT AMPLIFICATION OR LOW YIELD
• **Inadequate annealing temperature**
The reaction mix composition may affect the melting properties of primers and DNA. Adjust the annealing temperature to accommodate the primer with the lowest melting temperature (3 ° to 5 °C lower than Tm). Optimize annealing temperature using temperature gradient PCR. Increase primer concentration for lower yield amplicons.
• **Inadequate cycling conditions**
Increase extension time and/or number of cycles.
• **Presence of PCR inhibitors**
Some DNA isolation procedures, particularly genomic DNA isolation, can result in the co-purification of PCR inhibitors. Reduce the volume of template DNA in reaction or dilute template DNA prior to adding to the reaction. Diluting samples even 1:10,000 has been shown to be effective in improving results, depending on initial DNA concentration.
• **Inadequate amount of template DNA**
Titrate template amount. We recommend starting with 20-500 ng of genomic DNA.
• **Template DNA damaged or degraded**
An intact, high-quality template is essential to achieve a reliable amplification, especially from large DNA fragments. Extreme care must be taken in the preparation and handling of DNA. Always use purified high-quality DNA as template.
• **Contamination with DNases**
Ensure that all labware, including pipettes, tubes, and containers, is clean and free from residual DNase contamination. Disinfect laboratory surfaces with the RNase & DNase Cleaner (NZYtech, Cat. No. MB463). Use autoclaved or sterile equipment whenever possible. Use DNase-free water. Change gloves frequently.
• **Concentration of Mg^{2+} is too low**
Mg^{2+} is included in the Master Mix at a final concentration of 2.5 mM, which is sufficient for most targets. For some targets, higher Mg^{2+} concentration may be required. Titrate from 2.5 mM to 4 mM (final concentration) in 0.25 mM increments. (Note: $MgCl_2$ is not provided).
PRESENCE OF NON-SPECIFIC BANDS
• **Non-specific annealing of primers**
Adjust annealing conditions and/or design another pool of primers, avoiding complementary sequences. Ensure that each primer pair will originate a single product without primer-dimers in individual PCR reactions. If necessary, reduce primer concentration.

FOR REFERENCE USE ONLY

• **Primer degradation**
Check the quality and concentration of primer solutions. We recommend preparing small volume working aliquots from the stock solution. Avoid using primers subjected to multiple freezing-thawing cycles.
• **Primer cross-reactivity**
Redesign primers (check for complementarity between primer pairs). Check for 3' complementarity (avoid ≥3 consecutive matching bases), strong overall complementarity between different primer pairs and hairpin structures. Ensure that primers have similar melting temperatures (ideally within ~1-2°C, not differing by more than 5-6 °C).
• **Inadequate cycling conditions**
Reduce the number of cycles.

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

FOR REFERENCE USE ONLY