NZYSupreme Mutagenesis kit

Catalogue numbers:

MB44701, 10 mutations

MB44702, 10 mutations plus competent cells

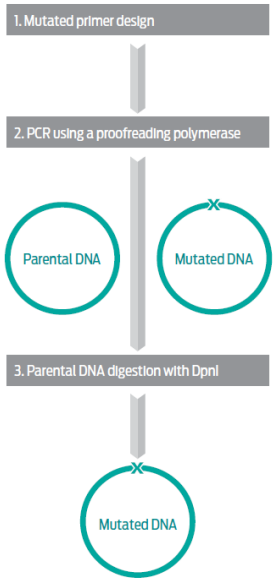

Figure 1. Overview of the NZYSupreme Mutagenesis kit protocol.

Description

NZYSupreme Mutagenesis kit provides a simple and highly efficient method to generate point mutations or to delete/insert single or multiple nucleotides in any type of plasmid DNA using PCR. This mutagenesis kit was recently developed to reduce the working time and to increase the efficiency of DNA editing. The system requires the provision of two synthetic partially complementary oligonucleotide primers containing the desired mutation. Incorporation of these mutated oligonucleotide primers with Supreme NZYProof DNA polymerase, an engineered highly accurate polymerase, generates a mutated plasmid containing staggered nicks, which resists to Dpn I digestion (as the synthetic DNA is not methylated). After Dpn I treatment, the resulting mutated plasmid is recovered through transformation of NZYStar competent cells. For a schematic presentation of the mutagenesis protocol, see Figure 1. DNA isolated from dam$^-$ *Escherichia coli* strains, including JM101 and SCS110, is not a suitable template for the mutagenesis reaction.

Storage temperature

Store competent cells at -80 °C on receipt. Other kit components may be stored at -20 °C. NZYSupreme Mutagenesis kit components are stable for at least six months when stored under the recommended conditions.

Kit components

Component	Quantity
Supreme NZYProof 2x Colourless Master Mix	300 µL
Dpn I (10 U/µL)	500 U
Control plasmid	5 µL
Mutagenesis Control primer mix	10 µL
NZYStar competent cells[a,b]	5×0.20 mL
Competent Cells Control Plasmid (pCCCP) (0.1 ng/µL)[a]	10 µL

[a]only provided in MB44702 kit.

[b]Genotype : *end*A1 *hsd*R17(r_k-, m_k+) *sup*E44 thi -1 *rec*A1 *gyr*A96 *rel*A1 lac[F´ *pro*A$^+$B$^+$ lacI^{q}ZΔM15 :Tn10(Tc^R)].

Guidelines for using NZYSupreme Mutagenesis kit

Primer specifications

Primers should be complementary at the 5'-overhangs and must contain the desired mutation(s) in both sequences. Thus, the two primers should consist of two main parts (Figure 2): (1) a 5'-complementary region of 17 nucleotides (in green), containing the desired point-mutation(s) (indicated as * in Figure 2) in the middle of the sequence; (2) 15-20 non-overlapping nucleotides (in grey) at the 3'-terminus. In case the desired mutation is a codon select the most divergent codon possible.

Primers should have a melting temperature (Tm) of at least 78 °C and should terminate in one or more C or G bases. A minimum GC content of 40% is advisable. Primer purification (FPLC, PAGE or HPLC) is strongly recommended. The mutagenesis protocol uses 125 ng of each oligonucleotide primer.

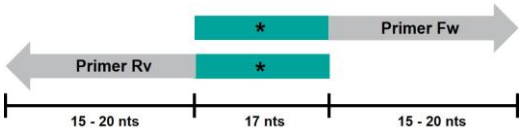

Figure 2. NZYSupreme Mutagenesis kit primer design scheme.

Plasmid specifications

The target plasmid DNA may be isolated from any source and purified using DNA purification kits (as NZYGelpure kit, MB011). No special vectors or restriction sites are required. We recommend starting with 5-25 ng of plasmid template. However, some plasmids may require higher amounts depending on the sequence and quality of the nucleic acid.

Control reaction

Control plasmid and control primer mix are included in the NZYSupreme Mutagenesis kit, allowing blue/white screening of mutagenesis reaction efficiency. The control plasmid, derived from pNZY28 vector (2.88 Kb), contains a premature stop-codon in the gene coding for *lacZα* and thereby forms white colonies on LB-ampicillin agar plates containing X-Gal and IPTG. The control primer mix is designed to revert the premature stop-codon into a functional codon, thus allowing *lacZα* expression. A positive mutagenesis control reaction forms blue colonies on LB-ampicillin agar plates containing X-Gal and IPTG.

Mutagenesis protocol

1. Synthesize the two oligonucleotides containing the desired mutation(s) (see Primer specifications section).
2. On ice, in a sterile, nuclease-free microcentrifuge tube, prepare a reaction mixture, combining the following components (for a 50 µL reaction):

Component	Volume
Supreme NZYProof 2x Colourless Master Mix	25 µL
dsDNA template (5-25 ng)*	x µL
Oligonucleotide primer #A (125 ng)*	y µL
Oligonucleotide primer #B (125 ng)*	z µL
Nuclease-free water	up to 50 µL

**Control reaction: To test the efficiency of the system use 1 µL of control plasmid and 2 µL of control primer mix provided.*

3. Gently mix and centrifuge the reactions in a microcentrifuge for 5 seconds. If using a thermal cycler without a heated lid, overlay the reaction mix with 1-2 drops of mineral oil to prevent evaporation during the thermal cycling.
4. Proceed with the amplification following the cycling parameters outlined in Table 1.

Table 1. Cycling parameters for the NZYSupreme Mutagenesis method.

Segment	Cycles	Temperature	Time
1	1	95 ºC	5 min
2	18	95 ºC	30 sec
		64 ºC	30 sec
		68 ºC	30 sec/kb plasmid length
3	1	68 ºC	10 min

Note: *control plasmid is 2.88 kb in size and, therefore, use a 1.5 minutes elongation period for the control reactions.*

5. Place reaction tubes on ice for 2 minutes. Check the efficiency of the amplification by analysing 5 µL of the reaction on a 0.7-1% agarose gel. Proceed with the Dpn I digestion even if a band is not visualized at this stage.
6. Add 5 µL of Dpn I directly into the reaction (below the mineral oil if used). Gently mix, spin down the reaction and incubate at 37 °C for 5-20 minutes to digest the non-mutated template DNA.
7. Transfer 5-10 µL of the Dpn I treated DNA to 100 µL of the ultracompetent cells. NZYStar cells are resistant to tetracycline. If the mutagenized plasmid contains only a *tet*^{R} resistance marker, an alternative tetracycline-sensitive strain of competent cells must be used.
8. To determine the transformation efficiency, add 1 µL (10 ng) competent cells control plasmid DNA to one tube containing 100 µL competent cells. Gently tap tube to mix. Do not mix cells by pipetting.
9. Incubate transformation reaction for 30 min on ice.
10. Heat shock cells at 42 °C for exactly 40 seconds.
11. Place on ice for 2 minutes.
12. Add 900 µL of pre-warmed SOC medium (not provided).
13. Shake the tubes at 200 rpm at 37 °C for 1 hour.
14. Centrifuge at 5000 rpm for 1 min. Remove 900 µL of the supernatant.
15. Re-suspend cells by gently pipetting. Plate 100 µL of cells onto LB agar plates containing the appropriate antibiotic. For control reaction, plate 100 µL of cells onto LB agar plates containing 100 µg/mL ampicillin, 15 µg/mL tetracycline, 100 µg/mL X-gal and 0.5 mM IPTG.
16. For competent cells control plasmid transformation directly plate 100 µL without spinning, onto LB agar plates containing 100 µg/µL ampicillin.
17. Incubate inverted plates overnight at 37 °C.
18. Select 3-5 colonies and analyse by plasmid isolation, PCR, or sequencing.

Additional guidelines

- In case completely overlapping mutated primers are used, the following PCR program should be performed (instead of the one indicated in Table 1):

Table 2. Cycling parameters for the NZYSupreme Mutagenesis method in case of using overlapping nucleotide primers.

Segment	Cycles	Temperature	Time
1	1	95 ºC	2 min
2	18	95 ºC	1 min
		64 ºC	1 min
		68 ºC	1.5 min/kb plasmid length
3	1	68 ºC	15 min

- PCR efficacy may be improved by increasing the amount of template DNA used (to a maximum of 50 ng of plasmid DNA *per* reaction). In this circumstance increase the incubation time with Dpn I to 2 hours.
- The amount of reaction used for transformation may be increased to a maximum of 10 µL to 100 µL of cells.
- False priming and the formation of secondary structures may affect the mutagenesis reaction. Increasing the annealing temperature up to 68 °C may help improving the efficacy of the PCR reaction.

Quality control assays

Functional assay

All components of the NZYSupreme Mutagenesis kit are tested following the mutagenesis protocol described above. The mutagenesis system must generate mutants with an efficiency ≥ 80% of the colonies screened.

V2201

Certificate of Analysis

Test	Result
Functional assay	Pass

Approved by:

Patrícia Ponte
Senior Manager, Quality Systems

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