MB121_IFU_EN_V2301

NZY-blunt PCR cloning kit

Catalogue number	Presentation
MB12101	24 ligations w/ competent cells
MB12102	24 ligations

Description

NZY-blunt PCR cloning kit was designed to allow the direct cloning of PCR products with blunt ends, which result from PCR amplifications using proofreading DNA polymerases, into a pre-linearized vector. The cloning vector provided by the NZY-blunt PCR cloning kit was prepared by cutting NZYtech´s pNZY28 with EcoRV, followed by dephosphorylation. Multiple restriction sites are introduced within the multiple cloning region of the pNZY28 vector. Vector digestion with EcoRI or BamHI allows the release of the PCR product since the vector cloning region is flanked by recognition sites of both enzymes.

Storage temperature

For MB12101, store competent cells at -85°C to -65°C upon arrival. Other kit components or MB12102 may be stored at -85°C to -15°C. NZY-blunt PCR cloning kit components are stable till the expiry date when stored under the recommended conditions.

System components

MB12101, 24 ligations w/ competent cells:

KIT PART REFERENCE	COMPONENT	CONCENTRATION	AMOUNT
MB12102	Phos Buffer	10×	28 µL
	Phos Enzyme mix	-	28 µL
	Positive control insert	120 ng/µL	8 µL
	Linearized pNZY28 vector	20 ng/µL	28 µL
	T4 DNA Ligase	2 U/µL	28 µL
MB00504	NZYStar Competent Cells [a]	-	12 × 200 µL
	Competent Cells Control Plasmid [b]	0.1 ng/µL	10 µL

[a] ***Genotype of NZYStar competent cells****: endA1 hsdR17(r_k-, m_k+) supE44 thi -1 recA1 gyrA96 relA1 lac[F´ proA$^+$B$^+$ lacI^{q}ZΔM15 :Tn10(Tc^R)]*
[b] ***Antibiotic resistance*** *: ampicillin*

MB12102, 24 ligations:

COMPONENT	CONCENTRATION	AMOUNT
Phos Buffer	10×	28 µL
Phos Enzyme mix	-	28 µL
Positive control insert	120 ng/µL	8 µL
Linearized pNZY28 vector	20 ng/µL	28 µL
T4 DNA Ligase	2 U/µL	28 µL

NZY-blunt cloning protocol

Insert preparation

For optimal cloning efficiencies, gel purification of PCR product using NZYtech´s NZYGelpure kit (MB011) is highly recommended. This kit can also be used for PCR product clean-up which is sufficient in case non-specific amplification or primer-dimer is not apparent.

We recommend using a 1:10 molar ratio of vector:insert, starting with 20 ng of the linearized pNZY28 vector. To calculate the optimal amount of PCR product required, use the following equation:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

Example: If using 20 ng of a vector plasmid with 3 kb, for a 1:10 molar ratio of vector:insert then you will require the following amount of a 500 bp insert:

$$\frac{20 \times 0.5}{3} \times 10 = 33 \text{ ng}$$

Phos reaction

1. On ice, in a sterile, nuclease-free microcentrifuge tube, prepare the following reaction mixture (for a 10 µL reaction):

Phos buffer	1 µL
PCR fragment*	x µL
Phos Enzyme mix	1 µL
Nuclease-free water	up to 10 µL

* ***Control reaction:*** *To test the efficiency of the system use 2 µL of the Positive control insert provided.*

2. Mix the reactions by pipetting and spin to collect contents at the bottom of the tubes.

3. Incubate the reaction at 37 °C for 20 minutes.

4. Heat inactivate the Phos Enzyme mix by incubating at 75 °C for 10 min.

5. Cool the reaction on ice for 2 min.

6. Centrifuge briefly to collect the reaction components.

Ligation reaction

1. On ice, in a sterile, nuclease-free microcentrifuge tube, prepare the following reaction mixture (for a 10 µL reaction):

Product from Phos reaction step	10 µL
Linearized pNZY28 vector (20 ng/µL)	1 µL
T4 DNA Ligase	1 µL

Note: ***It is extremely important not to change the ratio volume of the T4 DNA Ligase/reaction volume to prevent a decrease in the efficiency of the cloning reactions.***

2. Incubate the reactions at room temperature (20-25 °C) for 1 hour to overnight. If a maximum number of transformants is required, incubate the reactions for a long period.

Transformation

1. Thaw the required number of tubes of competent cells on ice. Pipette 100 µL of competent cells into pre-chilled microcentrifuge tubes on ice.

2. Add 5 µL of ligation mix directly into the cells. Stir gently to mix (a maximum of 10 µL of ligation mix can be used to transform 100 µL of competent cells).

3. Incubate transformation reaction for 30 min on ice.

4. Heat shock cells at 42 °C for exactly 40 seconds (**do not shake**).

5. Place on ice for 2 minutes.

6. Add 900 µL of pre-warmed SOC media (not provided).
7. Shake the tubes at 200 rpm at 37 °C for 1 hour.
8. Centrifuge at 5000 rpm for 1 min. Remove 900 µL of supernatant.
9. Re-suspend cells by gentle pipetting. 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.

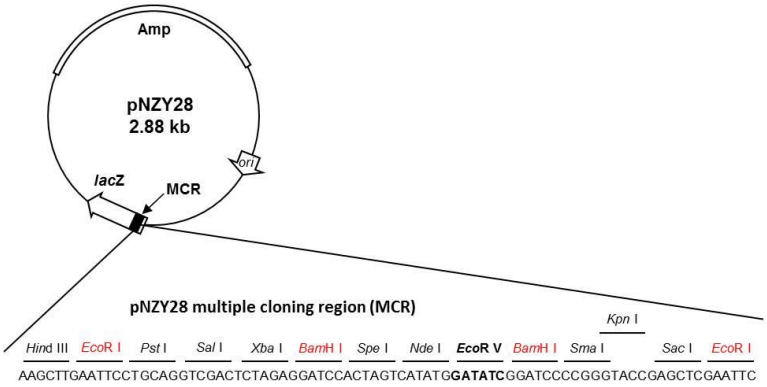
♦ *For other cells than NZYStar Competent Cells, please check first if the strain is resistant to tetracycline. Remove tetracycline from plates if using an E. coli strain without this resistance.*

***Note:** If a maximum number of colonies is aimed, centrifuge at 5000 rpm for 1 min, remove 900 µL of supernatant and re-suspend cells by gentle pipetting before plating.*

10. Incubate inverted plates overnight at 37 °C.
11. Screening for recombinants can easily be achieved by colony-PCR or by cutting DNA with EcoRI or BamHI to excise the cloned insert from pNZY28 (the pNZY28 multiple cloning region is illustrated below). The sequence confirmation can be checked by DNA sequencing.

pNZY28 vector

The cloning vector pNZY28 is provided linearized at the EcoR V (GA T↓ATC) site and dephosphorylated. The nucleotide sequence and properties of pNZY28 are available at www.nzytech.com.



Sequencing pNZY28 recombinant derivatives

pNZY28 recombinant derivatives can be used for double stranded dideoxy sequencing using the T7 promoter, M13 reverse and U-19mer primers.

Quality control assays

Purity

Recombinant T4 DNA Ligase and enzymes form Phos Enzyme mix are >95% pure as judged by SDS polyacrylamide gel electrophoresis followed by Coomassie blue staining.

Genomic DNA contamination

T4 DNA Ligase and Phos enzyme mix must comply to internal standards of DNA contamination as evaluated through real-time qPCR.

Nuclease assays

T4 DNA Ligase and Phos enzyme mix are tested for nuclease activities. To test for DNase contamination, 0.2-0.3 µg of pNZY28 plasmid DNA are incubated with the enzyme for 14-16 h at 37 °C. To test for RNase contamination, 1 µg of RNA is incubated with the enzyme 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

All components of the NZY-blunt PCR cloning kit are tested in a control experiment with the Positive control insert following the NZY-blunt cloning protocol described above. A 5 µL of the ligation mix was used to transform 100 µL of NZYStar competent cells. >90% of the recombinant plasmids must contain the appropriate insert.

Troubleshooting

NO COLONIES
• **Competent cells lost competence**
Check the transformation efficiency of *E. coli* competent cells by transforming the cells with a circular (non-cut) plasmid. Use the competent cells control plasmid provided in MB12101. Cells should have a transformation efficiency of at least 10^8 cfu/µg.
• **A specific component is missing in the ligation reaction**
Repeat ligation reaction and transformation.
• **Plates with incorrect concentration of antibiotic**
Do not use old plates and make sure ampicillin and tetracycline are at 100 µg/mL and 15 µg/mL, respectively.
LOW NUMBER OR NO WHITE COLONIES
• **PCR product without blunt ends**
Check if your PCR insert was amplified with a DNA polymerase that creates blunt ends.
• **Incorrect vector:insert ratio**
Optimise the ligation using other vector:insert ratios, such as 1:5.
• **Ligation time is not optimal**
Increase the time of ligation reaction.
• **Salts or ethanol present in the PCR insert**
Repeat PCR and gel-purify the PCR product for a new ligation and transformation. If possible, decrease the volume of insert added to the reaction.
• **PCR product is damaged**
Verify quality of the insert by gel electrophoresis. Limit DNA exposure to UV light to a few seconds or use safe dyes, like GreenSafe Premium (MB132), to detect DNA in a less aggressive environment.
• **Low amount of PCR product**
Re-quantify the PCR product by reading $Abs_{260\,nm}$. If required increase amount of insert in ligation reaction.
WHITE COLONIES WITHOUT INSERT OF INTEREST OR WITH INCORRECT INSERTS
• **PCR product is used un-purified in ligation reaction**
Gel-purify the PCR band of interest (if non-specific bands were obtained after PCR) or purify it by column to remove non-specific PCR products or primer-dimers that were generated during the PCR reaction.
INABILITY OF SELECTED WHITE COLONIES TO GROW IN LIQUID CULTURE
• **Satellite colonies must have been accidentally selected**
Small size colonies may appear surrounding blue or white colonies of larger size due to antibiotic degradation. They are sensitive to the antibiotic and will not grow under its presence. Make sure to select large white colonies.
LARGE NUMBER OF BLUE/LIGHT BLUE COLONIES
• ***LacZ* gene non- interruption**
Small size inserts (< 200 bp) may not completely interrupt *lacZ* gene, resulting in light blue colonies or blue colonies with white centre. In that case, consider those colonies for analysis.

Related products

Product name	Cat. No.
Supreme NZYProof DNA polymerase	MB404
dNTPs NZYSet	MB08701
NZYGelpure	MB011
LB Agar	MB11802
Ampicillin	MB021