NZY-A PCR cloning kit

Catalogue numbers:

MB05301, 24 ligations w/ competent cells
MB05302, 24 ligations

Description

NZY-A PCR cloning kit was designed to allow the direct cloning of PCR products with 3´-A overhangs, which result from amplifications using non-proofreading DNA polymerases such as NZYTaq II DNA polymerase. The cloning vector was prepared by cutting NZYTech´s pNZY28 with *Eco*R V and adding a 3´ terminal thymidine at both ends. These single 3´-T overhangs improve the efficiency of ligation of a PCR product into the plasmid by preventing re-circularization of the vector and providing compatible overhangs for PCR products generated by non-proofreading thermostable polymerases. Vector pNZY28 contains multiple restriction sites within the multiple cloning region. However, vector digestion with *Eco*R I or *Bam*H I allows the release of the PCR product since the vector cloning region is flanked by recognition sites of both enzymes. NZY-A PCR cloning system was developed to allow a rapid ligation (1 hour) between the vector and the PCR fragment.

Storage temperature

Store competent cells at -80 °C on receipt. Other kit components may be stored at -20 °C or at -80 °C. NZY-A PCR cloning kit components are stable for up to one year when stored under the recommended conditions.

Kit components

Component	Concentration	Amount
NZY-A Buffer	2×	200 µL
pNZY28-A vector	50 ng/µL	26 µL
T4 DNA Ligase	2 U/µL	26 µL
NZY-A positive control insert	50 ng/µL	6 µL
NZYStar Competent Cells [a,b]	-	12 × 0.20 mL
Competent Cells Control Plasmid [a,c]	0.1 ng/µL	10 µL

[a] only provided in MB05301 kit
[b] Genotype of NZYStar competent cells: *end*A1 *hsd*R17(r_k^-, m_k^+) *sup*E44 thi -1 *rec*A1 *gyr*A96 *rel*A1 lac[F´ *pro*A^+B^+ $lacI^qZ\Delta M15$:Tn10(Tc^R)].
[c] Ampicillin resistance.

Considerations for cloning blunt-ended PCR products

Thermostable polymerases with proofreading activity, such as NZYProof DNA polymerase (MB1460), generate blunt-ended fragments during PCR amplification. These PCR fragments can be easily cloned using NZYTech´s NZY-blunt PCR cloning kit (MB1210), which allows the direct cloning of PCR products with blunt ends. Nevertheless, PCR fragments generated using these polymerases can be modified using the A-tailing procedure and ligated into pNZY28. Other protocols may be suitable but we recommend the following method for adding 3´adenines.

A-tailing protocol

1. After amplification with a proofreading polymerase and gel purification, prepare a A-tailing reaction, using a Taq DNA polymerase.
2. Incubate at 72 °C for 10 min (do not cycle).
3. Place on ice and use 3 µL **immediately** in the NZY-A cloning ligation reaction.

NZY-A cloning protocol

1. 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 a PCR product clean-up which is sufficient in case non-specific amplification or primer-dimer are not apparent.

We recommend using a 1:3 molar ratio of vector:insert and starting with 50 ng of pNZY28-A 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 50 ng of a vector plasmid with 3 kb, for a 1:3 molar ratio of vector:insert then you will require the following amount of a 500 bp insert:

$$\frac{50 \times 0.5}{3} \times 3 = 25 \text{ ng}$$

2. Ligation reaction

2.1. Vortex the NZY-A buffer vigorously before each use. NZY-A buffer contains ATP, which degrades during temperature fluctuations. Preferably, make single use aliquots of the buffer to avoid frequent exposure to temperature changes.

2.2. Briefly centrifuge system components to collect contents at the bottom of the tubes.

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

Component	Volume
NZY-A buffer	5 µL
pNZY28-A vector	1 µL
PCR fragment *	x µL
T4 DNA Ligase	1 µL
Nuclease-free water	up to 10 µL

Notes: *It is extremely important not to change the ratio of T4 DNA Ligase volume: final volume to prevent a decrease in efficiency of the cloning reactions.*
* Control reaction: To test the efficiency of the system use 3 µL of the NZY-A positive control insert provided.

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

2.5. Incubate the reactions at room temperature for 1 hour. If maximum number of transformants is required, incubate the reactions overnight at 4 °C.

3. Transformation

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

3.2. Add 5 µL of ligation mix directly into the cells. Stir gently to mix.

3.3. Incubate transformation reaction for 30 min on ice.

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

3.5. Place on ice for 2 minutes.

3.6. Add 900 µL of pre-warmed SOC media (not provided).

3.7. Shake the tubes at 200 rpm at 37 °C for 1 hour.

3.8. Centrifuge at 5000 rpm for 1 min. Remove 900 µL of supernatant.

3.9. 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.

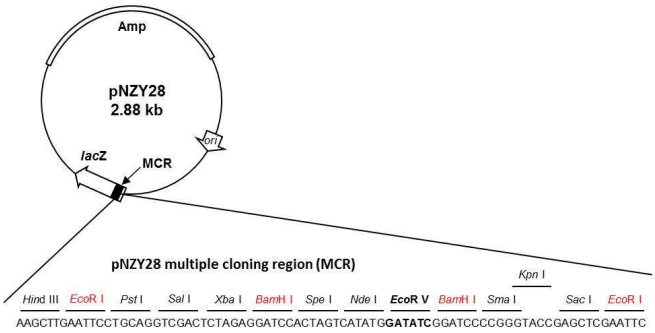
♦For other cells than NZYStar Competent Cells, please check first if strain is resistant to tetracycline. Remove tetracycline from plates if using an *E. coli* strain without this resistance.

3.10. Incubate inverted plates overnight at 37 °C.

3.11. Screening for recombinants can easily be achieved by cutting with EcoR I or BamH I to excise the cloned insert from pNZY28 (the pNZY28 multiple cloning region is illustrated below), colony-PCR or sequencing.

pNZY28 vector

The provided vector was prepared by cutting pNZY28 with EcoR V and adding 3'-T overhangs. 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.

Troubleshooting

No colonies
• Competent cells are damaged
Check the transformation efficiency of *E. coli* competent cells with competent cells control plasmid.
• A specific component is missing in the ligation reaction
Repeat ligation reaction and transformation.
Low number or no white colonies
• PCR product without 3'-A overhangs
Check if your PCR insert was amplified with a DNA polymerase that creates a 3'-A overhangs.
• Incorrect insert/vector ratio
Optimise the ligation using other insert to vector ratios.
• Ligation is not optimal
Increase the time of ligation reaction (1 hour to overnight).
• Salts or ethanol present in the PCR insert
Repeat PCR and gel-purify the PCR product for a new ligation and transformation.
• PCR product is damaged
Verify quality of 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 the ligation reaction
Gel-purify the PCR band of interest in order to remove non-specific PCR products or primer-dimers that were generated during the PCR reaction.

Quality control assays

Purity

Recombinant T4 DNA Ligase is >95% pure as judged by SDS polyacrylamide gel electrophoresis followed by Coomassie blue staining.

Nucleases assay

All components of the NZY-A PCR cloning kit, excluding the pNZY28-A vector, are tested for nucleases activities, using 0.2-0.3 µg of pNZY28 plasmid DNA. Following incubation at 37 °C for 14-16 hours, the DNA is visualized on a GreenSafe-stained agarose gel. There must be no visible nicking or cutting of the DNA.

Functional assay

All components of the NZY-A PCR cloning kit are tested in a control experiment with the NZY-A positive control insert following the NZY-A 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.

V1902

Certificate of Analysis

Test	Result
Enzyme Purity	Pass
Nucleases assay	Pass
Functional assay	Pass

Approved by:

Patrícia Ponte
Senior Manager, Quality Systems

For research use only.

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