MB137_IFU_EN_V2301

NZY-A PCR cloning kit

Catalogue number	Presentation
MB13701	24 ligations w/ competent cells
MB13702	24 ligations

Description

NZY-A Speedy PCR cloning kit was designed to carry out fast and efficient cloning of PCR products containing 3´-A overhangs, which result from amplifications using non-proofreading DNA polymerases with terminal transferase activity, such as Taq DNA polymerase. This methodology combines the efficiency of an improved ligation buffer with the speed of Speedy Ligase to allow a rapid ligation between the vector and the PCR product in only 10 minutes at room temperature (20-25 °C). The cloning vector provided by the NZY-A Speedy PCR cloning kit was prepared by cutting NZYtech´s pNZY28 with EcoRV and adding a 3´-terminal thymidine at both ends. These single 3´-T overhangs improve the efficiency of ligation, not only by providing compatible overhangs for 3'-A containing PCR products but as well as by preventing the re-circularization of the vector. 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 MB13701, store competent cells at -85°C to -65°C upon arrival. Other kit components or MB13702 may be stored at -85°C to -15°C. NZY-A PCR cloning kit components are stable till the expiry date when stored under the recommended conditions.

System components

MB13701, 24 ligations w/ competent cells:

KIT PART REFERENCE	COMPONENT	CONCENTRATION	AMOUNT
MB13702	NZY-A Speedy Buffer	4×	100 µL
	pNZY28-A vector	50 ng/µL	24 µL
	Speedy Ligase	2 U/µL	24 µL
	NZY-A positive control insert	12 ng/µL	6 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*

MB13702, 24 ligations:

COMPONENT	CONCENTRATION	AMOUNT
NZY-A Speedy Buffer	4×	100 µL
pNZY28-A vector	50 ng/µL	24 µL
Speedy Ligase	2 U/µL	24 µL
NZY-A positive control insert	12 ng/µL	6 µL

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 NZYtech's pNZY28-A vector. 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 10 µL A-tailing reaction, using a Taq DNA polymerase:

PCR fragment	7 µL
10× Reaction buffer for *Taq* DNA pol.	1 µL
50 mM $MgCl_2$	0.5 µL
10 mM dATP	1 µL
Taq DNA polymerase	0.5 µL

2. Incubate at 72 °C for 10 min (do not cycle).

3. Place on ice and use in the NZY-A Speedy cloning ligation reaction. Preferably, purify DNA using NZYGelpure kit (MB011) and elute with TE buffer (not provided) prior to use.

NZY-A Speedy 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 1:3 to 1:5 molar ratio of vector:insert, 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 1:5 molar ratio of vector:insert then you will require the following amount of a 500 bp insert:

$$\frac{50 \times 0.5}{3} \times 5 = 42 \text{ ng}$$

Ligation reaction

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

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

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

NZY-A Speedy buffer	2.5 µL
pNZY28-A vector	1 µL
PCR fragment *	x µL
Speedy Ligase	1 µL
Nuclease-free water	up to 10 µL

* ***Control reaction:*** *To test the efficiency of the system use 3 µL of the NZY-A positive control insert provided.*

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

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

5. Incubate the reactions at room temperature (20-25 °C) for at least 10 minutes. If a maximum number of transformants is required, incubate the reactions at room temperature for 1 hour.

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. Gently homogenize the competent cells and plate 100-200 µL 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.*
9. Incubate inverted plates overnight at 37 °C.
10. 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.

Notes about vector to insert ratio and incubation periods

Cloning efficiency can be optimized by applying different vector to insert ratios and varying the incubation time. **Figure 1** shows the effect on CFUs obtained when increasing the volume of the insert in the reaction, as well as the incubation time of up to 1 hour.

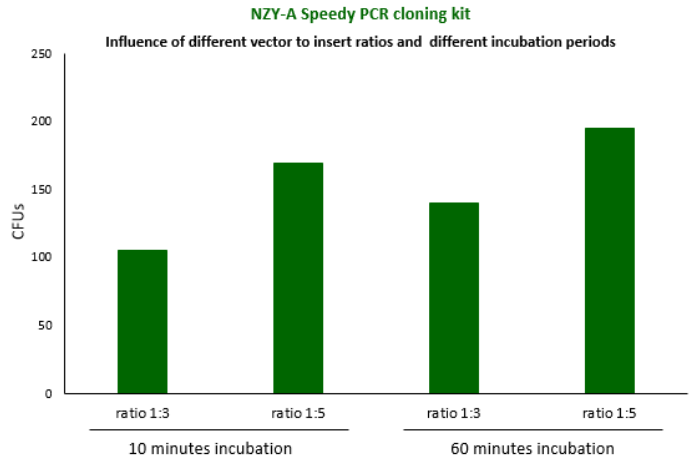
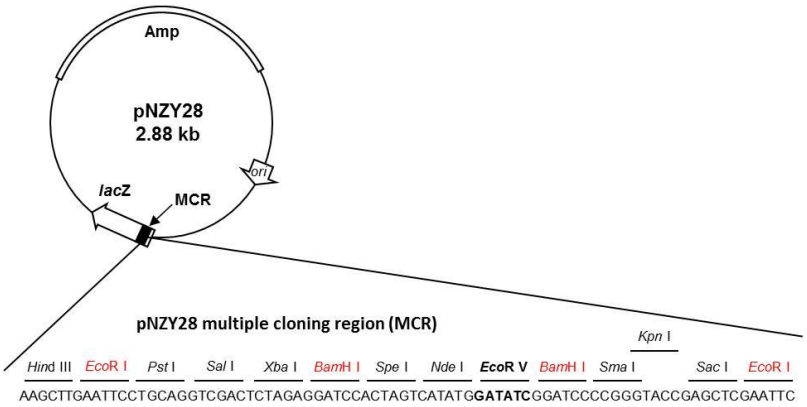


Figure 1. Two different vector to insert ratios (1:3 and 1:5) and two reaction times (10 and 60 minutes) were tested using this kit. The ligations were conducted at room temperature and resultant products were transformed in NZYStar competent cells following the recommended protocol. In the end, 100 µL of transformed cells were plated in LB agar medium containing X-Gal and IPTG for white/blue screening and supplemented with ampicillin without spinning. Only white colonies were considered for the representation above.

pNZY28 vector

The cloning vector pNZY28 is provided linearized at the EcoR V (GA T↓ATC) site with 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.

Quality control assays

Purity

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

Genomic DNA contamination

Speedy ligase must comply to internal standards of DNA contamination as evaluated through real-time qPCR.

Nuclease assays

Speedy Ligase is 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-A Speedy PCR cloning kit are tested in a control experiment with the NZY-A positive control insert following the NZY-A Speedy 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 MB05301. 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 3'-A overhangs**
Check if your PCR insert was amplified with a DNA polymerase that creates a 3'-A overhangs. Consider possible degradation of 3' overhangs on the PCR products over time. Use sterile DNase-free water. Preferably use a fresh insert.
• **Degradation of the vector 3'-T overhangs**
Avoid subjecting the vector provided to multiple freeze/thaw cycles. Make single aliquots if required.
• **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.
• **Degradation of ATP in the 4x Reaction Buffer**
Avoid subjecting the NZY-A Speedy Buffer to multiple freeze/thaw cycles. Single-use aliquots are recommended.
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.
dNTPs NZYSet	MB08701
NZYGelpure	MB011
LB Agar	MB11802
Ampicillin	MB021