MB282_MB319-MB330_IFU_EN_V2401

NZYEasy Cloning & Expression kits

Catalogue number	Presentation
MB28201/03, NZYEasy Cloning & Expression kit I	8 / 96 reactions
MB31901/03, NZYEasy Cloning & Expression kit II	8 / 96 reactions
MB32001/03, NZYEasy Cloning & Expression kit III	8 / 96 reactions
MB32101/03, NZYEasy Cloning & Expression kit IV	8 / 96 reactions
MB32201/03, NZYEasy Cloning & Expression kit VII	8 / 96 reactions
MB32301/03, NZYEasy Cloning & Expression kit VIII	8 / 96 reactions
MB32401/03, NZYEasy Cloning & Expression kit IX	8 / 96 reactions
MB32501/03, NZYEasy Cloning & Expression kit X	8 / 96 reactions
MB32601/03, NZYEasy Cloning & Expression kit XI	8 / 96 reactions
MB32701/03, NZYEasy Cloning & Expression kit XIII	8 / 96 reactions
MB32801/03, NZYEasy Cloning & Expression kit XIV	8 / 96 reactions
MB32901/03, NZYEasy Cloning & Expression kit XVI	8 / 96 reactions
MB33001/03, NZYEasy Cloning & Expression kit XVII	8 / 96 reactions

Description

The NZYEasy Cloning & Expression kits were designed to facilitate directional cloning of any PCR-generated fragment or synthetic gene into a linearized pHTP *Escherichia coli* expression vector. Cloning is achieved in a single ligase-independent reaction mediated by the NZYEasy enzyme mix. Vector-complementary overhangs, containing a specific sequence recognized by the NZYEasy enzyme, are incorporated into the PCR product using primers with appropriate 5' extensions. Upon combining the thus-generated insert with the linearized pHTP vector, which also contains complementary overhangs, in the presence of the NZYEasy enzyme mix, the two DNA molecules anneal through base-pair complementation of the single-strand regions. The reaction occurs in a single tube across three temperature-dependent steps. Circular recombinant vector containing the fragment of interest is obtained by transforming the annealed plasmid DNA into competent *E. coli* cells. The system achieves high cloning efficiency (80-100%) and does not require the use of DNA ligases. Additionally, the insert does not require any preliminary treatment (e.g., restriction digestion, phosphorylation, or blunt-end polishing). Once the pHTP recombinant plasmid has been constructed and its sequence confirmed, it should be used to transform λDE3 *E. coli* lysogens, such as BL21(DE3), for high levels of protein expression. NZYTech offers a comprehensive portfolio of pHTP expression vectors, which include various fusion tags commonly used to enhance expression and/or solubility of recombinant proteins in *E. coli*, as well as fluorescent tags (refer to Table 1 below).

NZYEasy Cloning & Expression kits have been successfully employed in high-throughput (HTP) platforms for the efficient cloning and expression of a large number of genes at a scale compatible with the functional screening of hundreds to thousands of genes/proteins.

Shipping & Storage Conditions

The NZYEasy Cloning & Expression kits can be shipped at a range of temperatures from dry ice to blue ice. Upon receipt, store all components at -85 °C to -15 °C in a constant temperature freezer. Immediately after use, swiftly return all components to a temperature between -85 °C and -15 °C. Adhering to these meticulous storage procedures ensures that product delivers consistent and reliable results across its lifespan and usage. Stored as specified, the product will remain stable until the expiry date, ensuring reliable and consistent performance in all applications.

Components

	8 reactions		96 reactions	
COMPONENT	**TUBES**	**VOLUME**	**TUBES**	**VOLUME**
NZYEasy enzyme mix	1	6 µL	1	50 µL
10x Reaction Buffer for NZYEasy enzyme mix	1	100 µL	1	1 mL
pHTP expression vector (*)	1	10 µL	1	100 µL
Positive control for NZYEasy Cloning kit (**)	1	12 µL	1	12 µL

(*) The type of pHTP expression vector depends on the NZYEasy Cloning & Expression kit. Please refer to the Table 1 below.

(**) Provided for 5 experiments. Corresponds to a PCR fragment of 500 bp at 21.0 ng/µL.

Table 1. pHTP expression vectors

VECTOR	FUSION PROTEIN	KIT CAT. NO.
pHTP1	*No fusion tag besides His_6 sequences*	MB282
pHTP2	Leader less disulfide-bond isomerase DsbC (**LLDsbC**) (1)	MB319
pHTP3	Mutant version of disulfide-bond isomerase Dsbc (**mutDsbC**) (1)	MB320
pHTP4	Disulfide-bond isomerase **DsbC** (1)	MB321
pHTP7	Disulfide oxidoreductase **DsbA** (2)	MB322
pHTP8	Thioredoxin (**Trx**) (3)	MB323
pHTP9	Green fluorescent protein (**GFP**) (4)	MB324
pHTP10	N-utilization substance A (**NusA**) (5)	MB325
pHTP11	Glutathione S-transferase (**GST**) (6)	MB326
pHTP13	Gb1 Domain of Protein G (**GB1**) (7)	MB327
pHTP14	Ketosteroid isomerase (**KSI**) (8)	MB328
pHTP16	*R. flavefaciens* cellulosomal protein (**cpA**) (A)	MB329
pHTP17	*R. flavefaciens* cellulosomal protein (**cpB**) (A)	MB330

(1) *Nozach, H. et al. 2013 Microb. Cell Fact. 12(37):2-16;* ***(2)*** *Collins-Racie, L.A. et al. 1995 Biotechnol. 13(9):982-987;* ***(3)*** *LaVallie, E.R. et al. 1993 Biotechnol. 11(2):187-193;* ***(4)*** *Prendergast, F.G & Mann, K.G. 1978 Biochemistry 17(17):3448-53;* ***(5)*** *Davis, G.D. et al. 1999 Biotechnol. Bioeng. 8:1668-1674;* ***(6)*** *Smith, D.B. & Johnson, K.S. 1988 Gene 67(1):31-40;* ***(7)*** *Huth, J.R. et al. 1997 Protein Sci. 6:2359-64; 8) Kuliopulos, A. & Walsh, C.T. 1994 J. Am. Chem. Soc. 116:4599-4607;* ***(A)*** *CpA and CpB are two recombinant cellulosomal proteins (Cps) that are highly expressed in E. coli. CpA is a carbohydrate-binding module, displaying affinity for β-glycans (xyloglucan, glucomannan, galactomannan and barley β-glucan).*

Standard Protocol

Recommendations before starting

This document serves as a concise protocol. A more comprehensive protocol, integrated into a compendium of the NZY Easy Cloning System, is available online on the product's webpage under the Product Manuals section. Prior to initiating this protocol, we strongly advise thoroughly reading the Manual for Easy Cloning System provided.

Procedure

Preparing DNA inserts

The insert DNA can be either a PCR product or a synthetic gene. Depending on your experimental procedure, follow either instructions I or II below for obtaining the insert:

I. PCR amplification

1. Design primers according to standard procedures. In addition to the gene-specific sequence, include the following 16 bp overhangs at the 5' ends of both forward and reverse primers to provide the necessary vector-complementary single-strand termini:

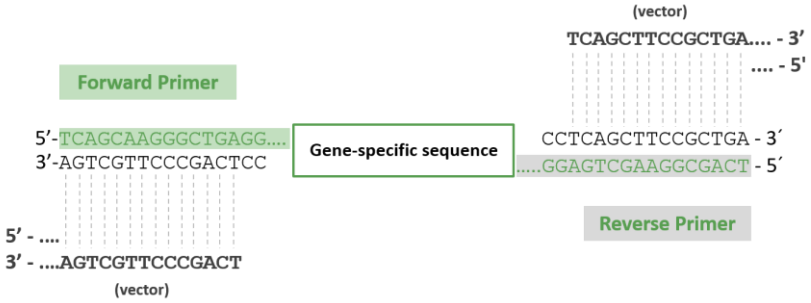
Gln Gln Gly Leu Arg

Forward overhang: 5´–**T CAG CAA GGG CTG AGG** ProteaseSite***…–3´

Reverse overhang: 5´–**T CAG CGG AAG CTG AGG** stop****…–3'

Notes:

- ***(*)*** *If desired, a protease specific site can be included at the end of the forward overhang, just before the gene-specific sequence to remove the N-terminal protein tag by a protease. For example, the cleavage recognition sequence of the Tobacco Etch Virus (TEV) protease is ENLYFQ↓(G/S).*
- ***(**)*** *If a C-terminal His-tag is not desired, include an in-frame stop codon on the reverse primer (TTA, TCA, or CTA). Omit the stop codon if you require both N- and C-terminal His-tags. Refer to the pHTP vectors map available for download on the specific product's webpage under the Product Manuals section for guidance.*



2. Amplify the gene of interest through PCR, following the guidelines provided in the Technical Note below.
3. Purify the PCR product using spin-column purification, employing the NZYGelpure kit (NZYtech, Cat. No. MB011) or a similar kit. Gel extraction of the desired band should be performed if non-specific amplifications or primer-dimers are observed, thereby enhancing cloning efficiencies.

II. *Sub-cloning of synthetic genes into pHTP expression vector*
*When transferring a synthetic gene to pHTP vectors, the following overhang regions are required upstream and downstream the gene-*specific sequence:

Ligase-independent cloning reaction

1. Gently mix all reaction components and pulse briefly.
2. On ice, in a sterile, nuclease-free microcentrifuge tube, prepare a reaction mixture by combining the following components:
Note: *If setting up more than one reaction, prepare a reaction mixture volume 10% greater than the total required for the number of reactions to be performed.*

COMPONENT	1 REACTION VOLUME
Purified DNA fragment	x µL(1,2)
pHTP expression vector (3)	1 µL
10x Reaction Buffer	1 µL
NZYEasy enzyme mix	0.5 µL
Nuclease-free water	up to 10 µL
FINAL VOLUME =	10 µL

(1) To determine the optimal quantity of the insert, please refer to the Technical Note below. Use a maximum of 7.5 µL of purified PCR insert when it is not possible to use the recommended optimal amount.
(2) For the positive control reaction: use 2 µL of the Positive control for NZYEasy Cloning kit provided.
(3) pHTP vectors are provided in a ready-to-use form.

3. Mix the reactions by pipetting and centrifuge briefly to collect the contents at the bottom of the tube.
4. Perform the cloning reactions in a thermal cycler programmed with the following protocol:

CYCLES	TEMP.	TIME
1	37 °C	60 min
1	80 °C	10 min
1	30 °C	10 min
1	4 °C	∞

5. Centrifuge briefly to collect the reaction components.
6. Store the cloning product at -85 °C to -15 °C or proceed to transformation following instructions below.

Transformation

1. Add 10 µL of ligation product directly into 100 µL of BL21(DE3) competent cells or other *E. coli* lysogens of λDE3.
2. Place the mixture on ice for 30 min. Heat shock cells at 42 °C for 40 seconds. Place tube on ice for 2 minutes.
3. Add 900 µL of pre-warmed SOC media and incubate at 200 rpm at 37 °C for 1 hour.
4. Centrifuge at 5000 rpm for 1 min. Remove 900 µL of supernatant.
5. Re-suspend cells by gentle pipetting. Spread 100 µL of the cells onto the selection LB agar plates containing 50 µg/mL kanamycin.
6. Incubate inverted plates overnight at 37 °C.

Screening for recombinant clones

Screening for recombinants can be easily achieved through colony-PCR, restriction analysis, and/or sequencing. For colony PCR or sequencing, utilize the following vector-specific primers:

pHTP forward primer:

VECTOR	FORWARD PRIMER (5'→ 3')
pHTP1	**GCGAAATTAATACGACTCACTATAGGGG**
pHTP2	**CAATGGCACACTTGTTCCGGGTTAC**
pHTP3	**CAATGGCACACTTGTTCCGGGTTAC**
pHTP4	**CAATGGCACACTTGTTCCGGGTTAC**
pHTP7	**GAATCCGCAGGGTATGGATACCAGC**
pHTP8	**GTTCAAAAACGGTGAAGTGGCGGC**
pHTP9	**GAATGAAAAACGCGACCACATGGTG**
pHTP10	**GGCTGATATCGAAGGGTTGACCG**
pHTP11	**CTTGAAATCCAGCAAGTATATAGCATGG**
pHTP13	**GGAAAAAGTTTTCAAACAGTACGCTAAC**
pHTP14	**GCCCCGATTGACCATTTTCGTTTC**
pHTP16	**CCCACTTGCTGACGCTGTAGTAG**
pHTP17	**CATTCGTCATAGAAAAAGACCTGAAAG**

pHTP reverse primer (common for all the pHTP expression vectors): 5´- **GGTTATGCTAGTTATTGCTCAGCG** -3'

Note: *After electrophoresis on an agarose gel, the anticipated size of the insert amplified using the pHTP vector-specific primers will be increased by an additional 294 base pairs*

Technical Notes

Insert / PCR product

To amplify the gene of interest, we strongly recommend using a high-fidelity DNA polymerase to reduce the error rate. When isolating genes from plasmids with kanamycin resistance (same as pHTP expression vectors), use 0.1-0.5 ng of plasmid template per 50 µL PCR reaction. Digestion with DpnI (NZYtech, cat. No. MB078) is recommended when high amounts of template are used. Prior to PCR, purification of the amplified DNA is highly recommended for optimal cloning efficiencies.

To determine the optimal amount of the PCR product to be used in the cloning reaction, please refer to the following table for guidance:

FRAGMENT LENGTH (*)	OPTIMAL DNA QUANTITY FOR CLONING REACTION
100 bp	8.3 ng
300 bp	25.0 ng
500 bp	41.5 ng
1000 bp	83.0 ng
2000 bp	166.0 ng
3000 bp	249.0 ng
4000 bp	332.0 ng

(*) The number of nanograms of insert required = DNA fragment length (bp) × 0.083. For example: a 1348 bp gene would require 1348 x 0.083 = 114.9 ng of DNA.

pHTP vector

The nucleotide sequence and properties of the pHTP expression vectors are available for download on the product's webpage under the Product Manuals section: User Guide - pHTP map.

Multiple fragment cloning protocol

The NZYEasy Cloning & Expression System offers the possibility to clone multiple inserts simultaneously into one vector in a single reaction. Please refer to the Manual for Easy Cloning System provided on the product's webpage under the Product Manuals section.

Protein Expression & Purification

The pHTP expression vectors are T7/lac promoter-based plasmids and can be utilized to transform competent *E. coli* cells expressing T7 RNA polymerase, such as BL21(DE3) cells. His-tagged recombinant proteins can be purified using immobilized metal-affinity chromatography (IMAC).

Quality control

Purity

The NZYEasy enzyme mix is >90% pure as judged by SDS polyacrylamide gel electrophoresis followed by Coomassie Blue staining.

Nucleases assay

To test for DNase contamination, 0.2-0.3 µg of pNZY28 DNA are incubated with the kit component in test for 14-16 h at 37 °C. To test for RNase contamination, 1 µg of RNA is incubated with the kit component in test 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. Similar tests are performed with reaction buffer.

Functional assay

All components of the kit are functionally tested in a ligase-independent cloning reaction, followed by a transformation assay. >90% of the recombinant plasmids must contain the appropriate insert