NZYEasy Cloning & Expression kits

Catalogue numbers:

MB282, NZYEasy Cloning & Expression kit I
MB319, NZYEasy Cloning & Expression kit II
MB320, NZYEasy Cloning & Expression kit III
MB321, NZYEasy Cloning & Expression kit IV
MB322, NZYEasy Cloning & Expression kit VII
MB323, NZYEasy Cloning & Expression kit VIII
MB324, NZYEasy Cloning & Expression kit IX
MB325, NZYEasy Cloning & Expression kit X
MB326, NZYEasy Cloning & Expression kit XI
MB327, NZYEasy Cloning & Expression kit XIII
MB328, NZYEasy Cloning & Expression kit XIV
MB329, NZYEasy Cloning & Expression kit XVI
MB330, NZYEasy Cloning & Expression kit XVII

Unit sizes: (available for each kit)

8 and 96 reactions

Description

NZYEasy Cloning & Expression kits were designed to allow directional cloning of any PCR-generated fragment or synthetic gene into a linearized pHTP *Escherichia coli* expression vector. Cloning proceeds 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 in the PCR product by using primers with appropriate 5' extensions. When you combine the insert thus generated with the linearized pHTP vector, also containing complementary overhangs, in the presence of NZYEasy enzyme mix, the two DNA molecules will anneal through base-pair complementation of the single-strand regions. The reaction occurs in a single-tube along 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 allows achieving high cloning efficiency (80-100%) and does not require the use of DNA ligases. In addition, the insert does not require any preliminary treatment (e.g. restriction digestion, phosphorylation, or blunt-end polishing).

Cloning is performed using conventional *E. coli* strains. Once 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. NZYEasy Cloning & Expression kits have been successfully used in high-throughput (HTP) platforms for the efficient cloning and expression of a large number of genes at a scale compatible with the functional screen of hundreds to thousands of genes/proteins.

NZYTech provides a comprehensive portfolio of pHTP expression vectors, which include different fusion tags commonly used to enhance expression and/or solubility of recombinant proteins in *E. coli*, as well as fluorescent tags. pHTP1 vector (included in NZYEasy Cloning & Expression kit I, cat. No. MB281) contains two poly-histidine (6xHis) sequences (N- and C-terminal) which allow subsequent recombinant protein purification by immobilized metal ion affinity chromatography (IMAC). The other pHTP expression vectors were constructed by inserting fusion tags (see Table 1) into the pHTP1 backbone such that the fusion partner will be at the N-terminus of the recombinant protein.

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

[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).*

References

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

Storage temperature

Kit components may be stored at -20 °C or at -80 °C.

Kit components

Component	8 reactions	24 reactions	96 reactions
10x Reaction Buffer	8 µL	24 µL	96 µL
NZYEasy enzyme mix	4 µL	12 µL	48 µL
pHTP vector	8 µL	24 µL	96 µL
Positive control [(1)]	10 µL	10 µL	10 µL

[(1)] *Positive Control: PCR fragment provided for 5 experiments.*

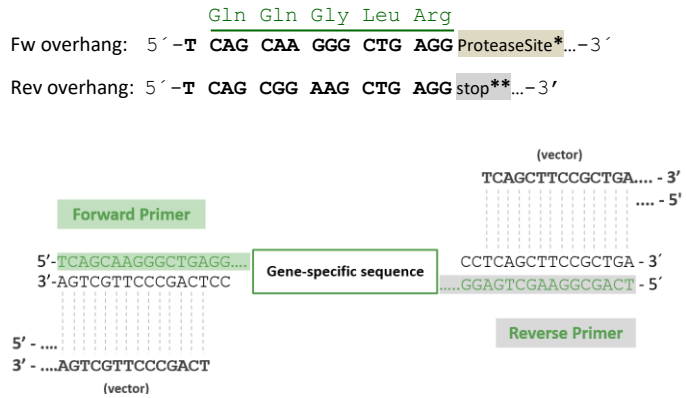
NZYEasy cloning protocol

Before you start using this protocol, please read carefully the NZYEasy Cloning Expression System User Guide available at the product resources tab of the product.

1. Preparing DNA inserts by PCR

1.1 Guidelines for Primers Design:

Besides gene-specific sequences, the following 16 bp overhangs must be included on 5'-ends of both forward (Fw) and reverse (Rev) primers, in order to provide the required vector-complementary single-strand terminals:



(*) If desired, a protease specific site can be included at the end of the forward overhang, just before the gene-specific sequence, in order 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. For details please see the pHTP vector maps available on our website.

1.2 Guidelines for PCR amplification:

- We strongly recommend using a high-fidelity enzyme to reduce the error rate.
- When genes are isolated from plasmids with kanamycin resistance (same as the pHTP expression vectors), use 0.1-0.5 ng of template per 50 µL PCR reaction. Digestion with DpnI (NZYTech, cat. No. MB078) is recommended when high amounts of plasmid template are used.
- For optimal cloning efficiencies, spin-column purification of the PCR product using NZYGelpure kit (NZYTech, cat. No. MB011) is highly recommended. Gel-extraction of the desired band should be performed in case non-specific amplifications or primer-dimers are formed, thus enhancing cloning efficiencies

1.3 Sub-cloning of synthetic genes into pHTP1:

When transferring a synthetic gene to pHTP vectors, the following overhang regions are required upstream and downstream the gene-specific sequence:

2. Ligase-independent cloning reaction

We recommend using a vector:insert molar ratio of 1:5; Please use the table below to determine the optimal amount of PCR product to be used in a cloning reaction:

Fragment length (bp)[1]	Optimal DNA quantity for Cloning reaction (ng)
100	8.3
300	25.0
500	42.0
1000	83.0
2000	166.0
3000	249.0
4000	332.0

[1] ng of insert required = DNA fragment length (bp) × 0.083
e.g. 1348 bp gene = 1348 x 0.083 = 114.9 ng of DNA

2.1. On ice, in a sterile, nuclease-free microcentrifuge tube, prepare the following reaction mixture:

Component	Volume
Purified DNA fragment	x µL[1,2]
pHTP vector [3]	1 µL
10x Reaction Buffer	1 µL
NZYEasy enzyme mix	0.5 µL
Nuclease-free water	up to 10 µL

[1] Use a maximum of 7.5 µL of purified PCR insert when it is not possible to use the recommended optimal amount.
[2] Positive Control: PCR fragment of 500 bp is provided at 21.0 ng/µL (enough for 5 experiments). Please use 2 µL per reaction.
[3] pHTP vectors are provided in a ready-to-use form.

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

2.3. Perform the cloning reaction in a thermal cycler programmed with the following protocol:

Temperature (°C)	Time (min)
37	60
80	10
30	10
4	∞

2.4. Centrifuge briefly to collect the reaction components.

3. Transformation

3.1. Add 10 µL of ligation product directly into 100 µL NZY5α cells (NZYTech cat No. MB004) competent cells.
3.2. Place the mixture on ice for 30 min. Heat shock cells at 42 °C for 40 seconds. Place tube on ice for 2 minutes.
3.3. Add 900 µL of pre-warmed SOC media and incubate at 200 rpm at 37 °C for 1 hour.
3.4. Centrifuge at 5000 rpm for 1 min. Remove 900 µL of supernatant.
3.5. Re-suspend cells by gentle pipetting. Spread 100 µL of the cells onto the selection LB agar plates containing 50 µg/mL kanamycin.
3.6. Incubate inverted plates overnight at 37 °C.

Note: Significantly lower cloning efficiencies can result from using other E. coli strains than DH5α.

4. Screening for recombinant clones

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

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

Reverse primer (5'→ 3') common for all the pHTP expression vectors: GGTTATGCTAGTTATTGCTCAGCG

Note: *After running on an agarose gel, the expected size of the insert amplified using the pHTP vector-specific primers will be incremented by extra 294 bp.*

pHTP vectors

Nucleotide sequence and properties of pHTP expression vectors are available for download at the product resources tab of the product.

Multiple fragment cloning protocol

NZYEasy Cloning & Expression System offers the possibility to clone multiple inserts simultaneously into one vector in a single reaction. Please read the Manual for Multiple Fragment Cloning using pHTP vectors available for download on the product page on our website.

Protein Expression & Purification

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

NZYEasy Cloning & Expression Systems

For more details, please read the NZYEasy Cloning & Expression System Manual available for download on the product page on our website.

Quality control assays

Purity
NZYEasy enzyme mix is >95% pure as judged by SDS polyacrylamide gel electrophoresis followed by BlueSafe (MB152) staining.

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

V2001

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.

nzytech
genes & enzymes

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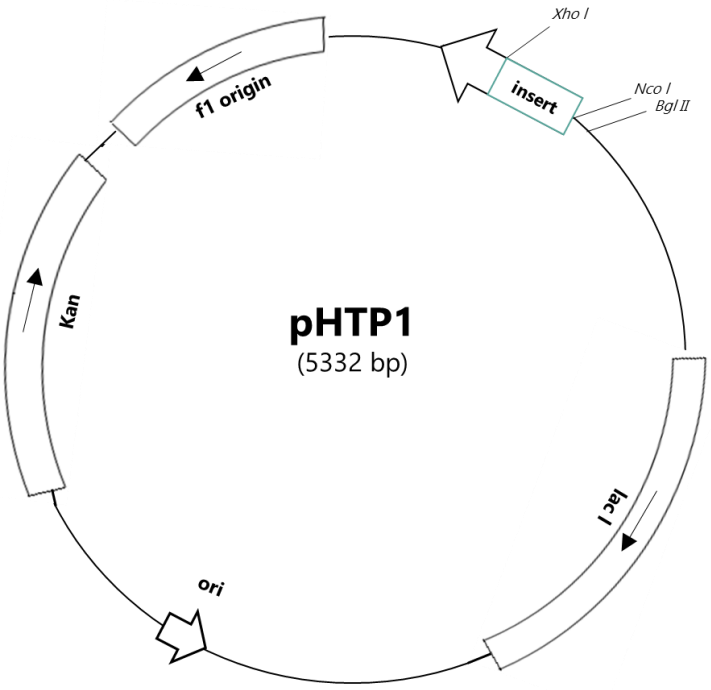
pHTP1 Vector

pHTP1 was designed for the cloning and expression of high-levels of recombinant proteins in *Escherichia coli*. This vector, included in the portfolio of NZYtech pHTP expression vectors, is part of the NZYEasy Cloning & Expression System. pHTP1, contains two poly-histidine (6xHis) sequences (N- and C-terminal) which allow subsequent recombinant protein purification by immobilized metal ion affinity chromatography (IMAC).

1. Vector Map



pHTP1 Cloning/Expression Region

```
 Nco I                  His-Tag
CCATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCCTCAGCAAGGGCTGAGG /✂/ CCTCAGCTTCCGCTGAGGTCCGTCGACAAGCTTGCGGCC
  MetGlySerSerHisHisHisHisHisHisSerSerGlyProGlnGlnGlyLeuArg /✂/ ProGlnLeuProLeuArgSerValAspLysLeuAlaAla

     Xho I          His-Tag              STOP
GCACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCC
AlaLeuGluHisHisHisHisHisHis*
```

✂ Represents the site where the gene will be inserted.

Note: For correct expression, inserted gene needs to be in frame with pHTP1 5' gene sequence. Inserts correctly cloned into pHTP1 will maintain reading frames starting on the ATG codon.

2. Vector Sequence (5332 bp)

TGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCT
TCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATG
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TGCAATTTATTCATATCAGGATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTG
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GTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTG
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GCAGCAGCCATCATCATCATCATCACAGCAGCGGCCCTCAGCAAGGGCTGAGG/✂/CCTCAGCTTCCGCTGAGGTCCGTCGACAAGCTTGCGGCCGCA**CTCGAG**CACCACCACCAC
CACCAC*TGA*GATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTT
TGCTGAAAGGAGGAACTATATCCGGAT

pHTP1 sequence landmarks:

- **T7 promoter**: in gray
- **First ATG (methionine)**: in yellow
- **His•Tag coding sequences**: in purple
- **Cloning region**: ✂
- **T7 terminator**: in dark gray
- **Sequencing primers** (T7 universal and T7 terminator): underlined
- **BglII, NcoI & XhoI recognition sites:** in bold

Sequence added to the final recombinant protein (2.11 KDa):
MGSSHHHHHHSSGPQQGLR

V1901

For research use only.