NZYEasy Cloning & Expression System

Products of the System:

NZYEasy Cloning kit (MB281)
NZYEasy Cloning & Expression kit I (MB282)
NZYEasy Cloning & Expression kit II (MB319)
NZYEasy Cloning & Expression kit III (MB320)
NZYEasy Cloning & Expression kit IV (MB321)
NZYEasy Cloning & Expression kit VII (MB322)
NZYEasy Cloning & Expression kit VIII (MB323)
NZYEasy Cloning & Expression kit IX (MB324)
NZYEasy Cloning & Expression kit X (MB325)
NZYEasy Cloning & Expression kit XI (MB326)
NZYEasy Cloning & Expression kit XIII (MB327)
NZYEasy Cloning & Expression kit XIV (MB328)
NZYEasy Cloning & Expression kit XVI (MB329)
NZYEasy Cloning & Expression kit XVII (MB330)

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NZYEasy Cloning & Expression System

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Features

- Ligase-independent cloning
- Time-saving and cost-effective method
- No additional treatment of the PCR fragments required (e.g. restriction digestion, phosphorylation or blunt-end polishing)
- High cloning efficiencies
- Directional cloning
- Possibility to clone multiple inserts simultaneously into one vector
- Compatibility with automation and high-throughput methods
- Suitable for both cloning or bacterial protein expression
- Includes a battery of expression vectors containing different fusion tags (pHTP series)

A. How does NZYEasy Cloning & Expression System work?

NZYEasy Cloning & Expression System was designed to allow directional cloning of any PCR-generated fragment into a linearized pHTP vector 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 a linearized pHTP vector in the presence of NZYEasy enzyme, the two DNA molecules will anneal through base-pair complementation of single-strand regions. The reaction occurs in a single-tube along three temperature-dependent steps lasting a total of 80 minutes. Circular recombinant vector containing the fragment of interest is obtained by transforming the annealed plasmid DNA into competent *Escherichia coli* cells. The system allows achieving high cloning efficiencies (80-100%) and does not require the use of DNA ligases. In addition, no further treatment (e.g. restriction digestion, phosphorylation or blunt-end polishing) of the inserts is required**.** The system also allows transferring a gene previously cloned in other plasmid vector into pHTP vectors as long as the two vectors have different selectable markers and the gene to be transferred is flanked by appropriate cloning regions (see page 12).
PCR-generated fragments can be cloned into the pHTP0 cloning vector (incorporated into the NZYEasy Cloning kit, cat. No. MB281), which is a standard pUC-derivative bearing a gene conferring ampicillin resistance to *E. coli*. Alternatively, inserts can be directly cloned into one of the various kanamycin-resistant pHTP expression vectors without the need to go through the tedious and laborious intermediate stages. pHTP expression vectors were designed to achieve high levels of regulated recombinant gene expression in *E. coli*.

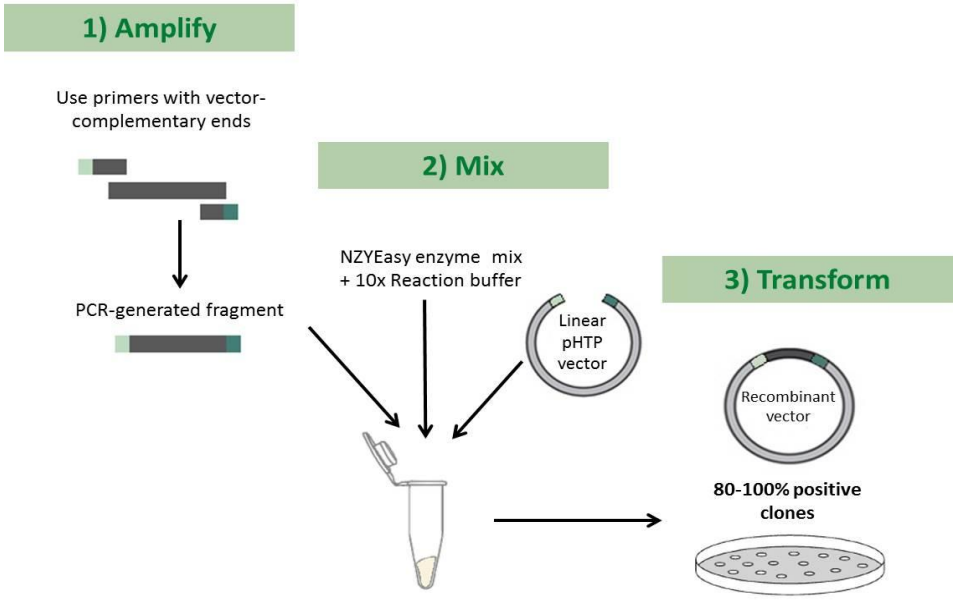


Figure 1. **Overview of the NZYEasy Cloning System protocol.** The PCR-generated fragment may be replaced by a synthetic gene cloned into a standard pUC vector as long as the artificial DNA is flanked by the cloning flanking sequences. There is no need to excise the synthetic gene from the vector as long as the antibiotic selectable marker of entry and destination vectors are not the same.

B. NZYEasy Cloning & Expression System for use in HTP cloning and protein production studies

The method has 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. For example, at NZYtech, the technology here reported was recently used to clone 5,000 synthetic genes encoding venom peptides within the FP7 VENOMICS project. In addition, the system has also been successfully used to clone and express hundreds to thousands of other genes with either prokaryotic or eukaryotic origin. Many features make the NZYEasy Cloning & Expression System readily adapted to HTP biology studies, such as the precise one-step cloning process; the possibility to easily transfer genes between different plasmids as long as they contain appropriate cloning regions; or the ready-to-use linearized form in which pHTP vectors are provided, making unnecessary additional treatments on both vector and insert, thus saving time in the cloning procedures.

C. Multiple fragment cloning: multiple inserts to one vector

NZYEasy Cloning & Expression System offers the possibility to clone multiple inserts simultaneously, into one vector, in a single reaction. This feature extends the versatility of the method to meet more complex cloning applications arising from synthetic biology engineering research. Using the NZYEasy Cloning & Expression System, DNA fragments will be correctly annealed together in the desired order on a pHTP vector through the action of the highly efficient NZYEasy enzyme mix, free of any DNA ligase. Just combine the PCR-generated fragments with appropriate complementary overhangs, in the order you choose, with a linearized pHTP vector. Both first and last inserts should have overhangs complementary with the vector overhangs, thereby creating the desired construct. Each DNA fragment, encoding for an individual domain of the final multidomain construct, are kept in frame and are separated from each other by a linker sequence of 6 amino acids. The general flow of a typical multiple fragment cloning project is presented in Figure 2. Using this method, it is possible to build complicated synthetic chimeras, by joining together up to 5 different genes.

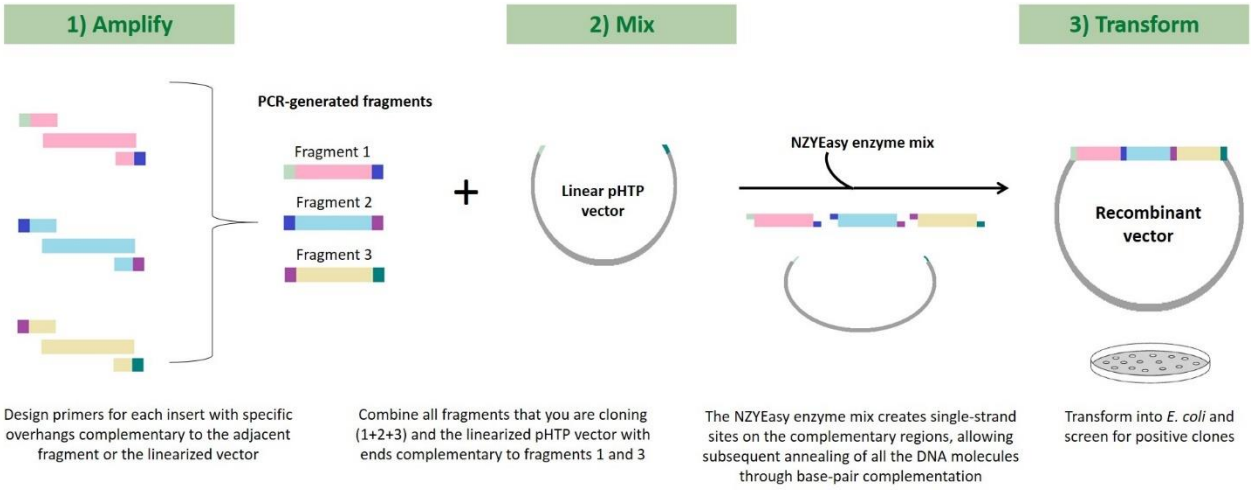


Figure 2. **Illustration of multiple fragment cloning using the NZYEasy Cloning & Expression System.** The figure exemplifies the cloning of three PCR-generated inserts (fragments 1, 2 and 3) into a pHTP vector using NZYEasy enzyme mix. Complementary sequences required to correctly assemble the full length nucleic acid are highlighted in light green, dark blue, magenta and dark green.

D. System Components

Operation of NZYEasy Cloning & Expression System requires the use of a dedicated enzyme mix and a battery of pHTP vectors. Find below a description of the main properties of these components.

1. NZYEasy Enzyme Mix

NZYEasy enzyme mix is a proprietary-formulation used for ligation-independent cloning. NZYEasy enzyme mix recognizes the vector-complementary sequences incorporated into the gene and leaves single-strand overhangs at these sites. The insert thus generated can then be easily cloned into pHTP vectors, which are provided in a linear form with the required single-stranded complementary ends.

2. pHTP vectors

pHTP cloning and expression vectors are provided in the linearized, ready-to-use form that is compatible with the NZYEasy Cloning & Expression System. The nucleotide sequence and maps are available for download on our website at the product resources tab of the product.

pHTP0 cloning vector is a 2960 bp high-copy number vector developed for high efficient cloning and assembly of DNA inserts using NZYEasy Cloning & Expression System. Main features of pHTP0 are:

- It is a pUC-derivative.
- Includes a selection marker that confers resistance to ampicillin.
- Contains several restriction sites neighbouring the cloning region, which allows simple excision and screening for positive clones.
- Includes a functional origin of replication (ori) for propagation and maintenance in *E. coli.*

pHTP1 expression vector was designed to achieve high levels of protein expression in *E. coli*. Main features of pHTP1 expression vector are:

- Allows fast, efficient and directional one-step cloning that delivers your insert in the correct orientation and reading-frame for expression.

- Includes a selection marker that confers resistant to kanamycin.
- Uses the T7/lac promoter for regulated, high-level expression in *E. coli*. The *lac* operator is located downstream the T7 promoter and serves as binding site for the lac repressor encoded by the *lacI* gene present in pHTP vectors. The bacteriophage T7 promoter is specifically recognized by T7 RNA polymerase. *E. coli* λDE3 lysogen carries a non-plasmid copy of the T7 RNA polymerase gene under lacUV5 control and it is suitable to expression from T7 promoters. When an inducer, such as isopropyl β-D-1-thiogalactopyranoside (IPTG), is added to the culture, T7 RNA polymerase is expressed and begins transcription of genes under T7 promoter control.
- pHTP1 plasmid provides strong ribosome binding site (rbs).
- Contains two poly-histidine (6xHis) tags (N- and C-terminal) which allow subsequent recombinant protein purification by immobilized metal ion affinity chromatography (IMAC).

Fusion tag pHTP expression vectors were built by inserting the sequences encoding different fusion tags (see Table I) into the pHTP1 backbone such that the fusion partner will be at the N-terminus of the recombinant protein. The fusion tags available are commonly used to enhance expression and/or solubility of target proteins in *E. coli*, and also include fluorescent tags.

NZYtech provides pHTP vectors in separate kits. NZYEasy Cloning kit (cat. No. MB281) includes the pHTP0 vector for standard cloning reactions, while NZYEasy Cloning & Expression kits include expression vectors and allow subsequent transformation in *E. coli* strains containing the λDE3 lysogen, such as BL21(DE3), for high levels of protein expression.

pHTP vectors are also recommended for large-scale projects when hundreds to thousands of genes need to be cloned and/or expressed simultaneously.

It is important to note that any vector can be converted into a "pHTP vector". However, it is necessary to insert the complementary sequences containing the specific sequence recognized by the NZYEasy enzyme mix into a plasmid backbone to function with the NZYEasy Cloning & Expression System. In case vectors available in our pHTP portfolio do not fit your needs, please contact us (*services@NZYtech.com*). Other solubility tags can easily be included in the pHTP1 backbone.

Table I. pHTP expression vectors.

Vector	Fusion Protein	N-terminal tag	Tag size (kDa)	Kit cat. No.
pHTP1	*No fusion tag besides His_6 sequences*	MGSS-His_6-SSGPQQGLR	2.11	MB282
pHTP2	Leader less disulfide-bond isomerase DsbC (**LLDsbC**)[1,a]	MG-**LLDsbC**-GSSMGSS-His_6-SSGPQQGLR	25.98	MB319
pHTP3	Mutant version of disulfide-bond isomerase Dsbc (**mutDsbC**)[1,b]	MG-**mutDsbC**-GSSMGSS-His_6-SSGPQQGLR	25.91	MB320
pHTP4	Disulfide-bond isomerase **DsbC**[1,c]	MG-**DsbC**-GSSMGSS-His_6-SSGPQQGLR	28.01	MB321
pHTP7	Disulfide oxidoreductase **DsbA**[2]	MG-**DsbA**-GSAMGSS-His_6-SSGPQQGLR	25.43	MB322
pHTP8	Thioredoxin (**Trx**)[3]	MG-**Trx**-GSAMGSS-His_6-SSGPQQGLR	14.52	MB323
pHTP9	Green fluorescent protein (**GFP**)[4]	MG-**GFP**-SSGPSGSS-His_6-SSGPQQGLR	29.33	MB324
pHTP10	N-utilization substance A (**NusA**)[5]	MG-**NusA**-AMGSS-His_6-SSGPQQGLR	57.17	MB325
pHTP11	Glutathione S-transferase (**GST**)[6]	MG-**GST**-AMGSS-His_6-SSGPQQGLR	28.07	MB326

pHTP13	Gb1 Domain of Protein G (**GB1**)[7]	MG-**GB1**-AMGSS-His_6-SSGPQQGLR	8.54	MB327
pHTP14	Ketosteroid isomerase (**KSI**)[8]	MG-**KSI**-AMGSS-His_6-SSGPQQGLR	15.70	MB328
pHTP16	*R. flavefaciens* cellulosomal protein (**CpA**)[d]	MG-**CpA**-GSAMGSS-His_6-SSGPQQGLR	31.94	M3290
pHTP17	*R. flavefaciens* cellulosomal protein (**CpB**)[d]	MG-**CpB**-GSAMGSS-His_6-SSGPQQGLR	30.24	MB330

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.

[a] pHTP2 vector encodes the sequence of DsbC for cytoplasmic expression, since it does not carry a signal peptide sequence (Leader Less-LL).
[b] pHTP3 vector expresses an inactive mutant of DsbC isomerase, which includes two different mutations at the catalytic site (Cys100Ala and Cys103Ala).
[c] pHTP4 vector includes the sequence of a signal peptide before the DsbC to allow export of the recombinant fusion proteins to *E. coli* periplasm.
[d] 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).

3. Positive Control Insert

A Positive Control is included in the kits to assess the efficiencies of the cloning and transformation reactions, as well as to exemplify levels of recombinant protein expression. Positive control DNA consists of a 500 bp PCR product that is flanked by the sequences required for cloning using the NZYEasy Cloning & Expression System. This DNA also encodes a recombinant protein that is highly expressed in *E. coli* strains containing the λDE3 lysogen. The positive control protein migrates at 19.7 kDa in polyacrylamide gel electrophoresis (SDS-PAGE).

4. Required Materials Not Included

- DNA polymerase: we strongly recommend using NZYProof or Supreme NZYProof DNA polymerases (NZYtech, cat. Nos. MB146 and MB283, respectively). However, other proofreading polymerases may be employed for gene isolation. Nevertheless, non-proofreading DNA polymerases such as NZYTaq DNA polymerase (NZYtech, cat. No. MB001) or Supreme NZYTaq DNA polymerase (NZYtech, cat. No. MB079) may also be employed. To inactivate plasmid DNA template use DpnI (NZYtech, cat. No. MB078)
- Luria-Bertani (LB) medium: LB Broth (NZYtech, cat. No. MB028); LB Agar (NZYtech, cat. No. MB118); NZY Auto-Induction LB medium (powder) (NZYtech, cat. No. MB179)
- Antibiotics: Ampicillin (NZYtech, cat. No. MB021); Kanamycin (NZYtech, cat. No. MB020)
- Competent cells: NZY5α (NZYtech, cat. No. MB004); BL21(DE3) (NZYtech, cat. No. MB006)

E. Protocols using the NZYEasy Cloning & Expression System

1. Preparing inserts by PCR

1.1 Guidelines for Primers Design

PCR primers required to amplify a DNA fragment and to clone it using the NZYEasy Cloning & Expression System must include two sequence components: a *non-priming overhang* and a *gene-specific sequence*.

- ***Non-priming overhang****:* both forward and reverse primers must incorporate the following 16-bp sequences at their 5' end:

Overhang for the forward (sense) primer:	Gln Gln Gly Leu Arg 5´-T CAG CAA GGG CTG AGG ProteaseSite(*)… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CGG AAG CTG AGG stop(**)…-3´

(*) 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).

(**) When sub-cloning into pHTP expression vectors, include an in-frame stop codon just after the non-priming overhang on the reverse primer if a C-terminal His-tag is not desired. The stop codon anticodons may be TTA, TCA or CTA. Omit the stop codon if you require both N- and C-terminal His-tags.

The 5'- non-priming overhangs are complementary to the 3'-ends of the linearized pHTP vectors, which are provided ready-to-use with your kit, and are required for base-pair complementation between vector and insert (see figure 3).

For **cloning multiple inserts into one pHTP vector**, see below the overhangs that you need to incorporate in the PCR primers to generate the DNA fragments used in the assembly reactions, when starting with different fragment numbers:

Two-fragment multiple cloning:

Fragment 1:

Overhang for the forward (sense) primer:	Gln Gln Gly Leu Arg 5´-T CAG CAA GGG CTG AGG ProteaseSite(*)… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CTT GGG CTG AGG… -3′

Fragment 2:

Overhang for the forward (sense) primer:	Gln Pro Lys Leu Arg 5´-T CAG CCC AAG CTG AGG… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CGG AAG CTG AGG stop(**)… -3´

(*) and (**) see above – page 8

Note: The two fragments will be separated from each other by the linker sequence **PQPKLR**.

Three-fragment multiple cloning:

Fragment 1:

Overhang for the forward (sense) primer:	Gln Gln Gly Leu Arg 5´-T CAG CAA GGG CTG AGG ProteaseSite(*)… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CTT GGG CTG AGG… -3′

Fragment 2:

Overhang for the forward (sense) primer:	Gln Pro Lys Leu Arg 5´-T CAG CCC AAG CTG AGG… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CGA GAG CTG AGG… -3´

Fragment 3:

Overhang for the forward (sense) primer:	Gln Leu Ser Leu Arg 5´-T CAG CTC TCG CTG AGG… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CGG AAG CTG AGG stop(**)… -3´

(*) and (**) see above – page 8

Note: Fragment 1 will be separated from fragment 2 by the linker sequence **PQPKLR**, while fragment 2 will be separated from fragment 3 by the linker sequence **PQLSLR**.

Four-fragment multiple cloning:

Fragment 1:

Overhang for the forward (sense) primer:	Gln Gln Gly Leu Arg 5´-T CAG CAA GGG CTG AGG ProteaseSite(*)… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CTT GGG CTG AGG… -3′

Fragment 2:

Overhang for the forward (sense) primer:	Gln Pro Lys Leu Arg 5´-T CAG CCC AAG CTG AGG… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CGA GAG CTG AGG… -3´

Fragment 3:

Overhang for the forward (sense) primer:	Gln Leu Ser Leu Arg 5´-T CAG CTC TCG CTG AGG… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CGT GTG CTG AGG… -3´

Fragment 4:

Overhang for the forward (sense) primer:	Gln His Thr Leu Arg 5´-T CAG CAC ACG CTG AGG… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CGG AAG CTG AGG stop(**)… -3´

(*) and (**) see above – page 8

Note: Fragment 1 will be separated from fragment 2 by the linker sequence **PQPKLR**; fragment 2 will be separated from fragment 3 by the linker sequence **PQLSLR**; fragment 3 will be separated from fragment 4 by the linker sequence **PQHTLR**.

Five-fragment multiple cloning:

Fragment 1:

Overhang for the forward (sense) primer:	Gln Gln Gly Leu Arg 5´-T CAG CAA GGG CTG AGG ProteaseSite(*)… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CTT GGG CTG AGG… -3′

Fragment 2:

Overhang for the forward (sense) primer:	Gln Pro Lys Leu Arg 5´-T CAG CCC AAG CTG AGG… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CGA GAG CTG AGG… -3´

Fragment 3:

Overhang for the forward (sense) primer:	Gln Leu Ser Leu Arg 5´-T CAG CTC TCG CTG AGG… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CGT GTG CTG AGG… -3´

Fragment 4:

Overhang for the forward (sense) primer:	Gln His Thr Leu Arg 5´-T CAG CAC ACG CTG AGG… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CAG GAG CTG AGG… -3´

Fragment 5:

Overhang for the forward (sense) primer:	Gln Leu Leu Leu Arg 5´-T CAG CTC CTG CTG AGG… -3′
Overhang for the reverse (antisense) primer:	5´-T CAG CGG AAG CTG AGG stop(**)… -3´

(*) and (**) see above – page 8

Note: Fragment 1 will be separated from fragment 2 by the linker sequence **PQPKLR**; fragment 2 will be separated from fragment 3 by the linker sequence **PQLSLR**; fragment 3 will be separated from fragment 4 by the linker sequence **PQHTLR**; fragment 4 will be separated from fragment 5 by the linker sequence **PQLLLR**.

- ***Gene-specific sequence****:* required for template priming during PCR amplification. This sequence is specific to the DNA fragment(s) you want to clone and must be added in both forward and reverse primers, after the non-priming overhang in the 5′→3′ direction (see figure 3).

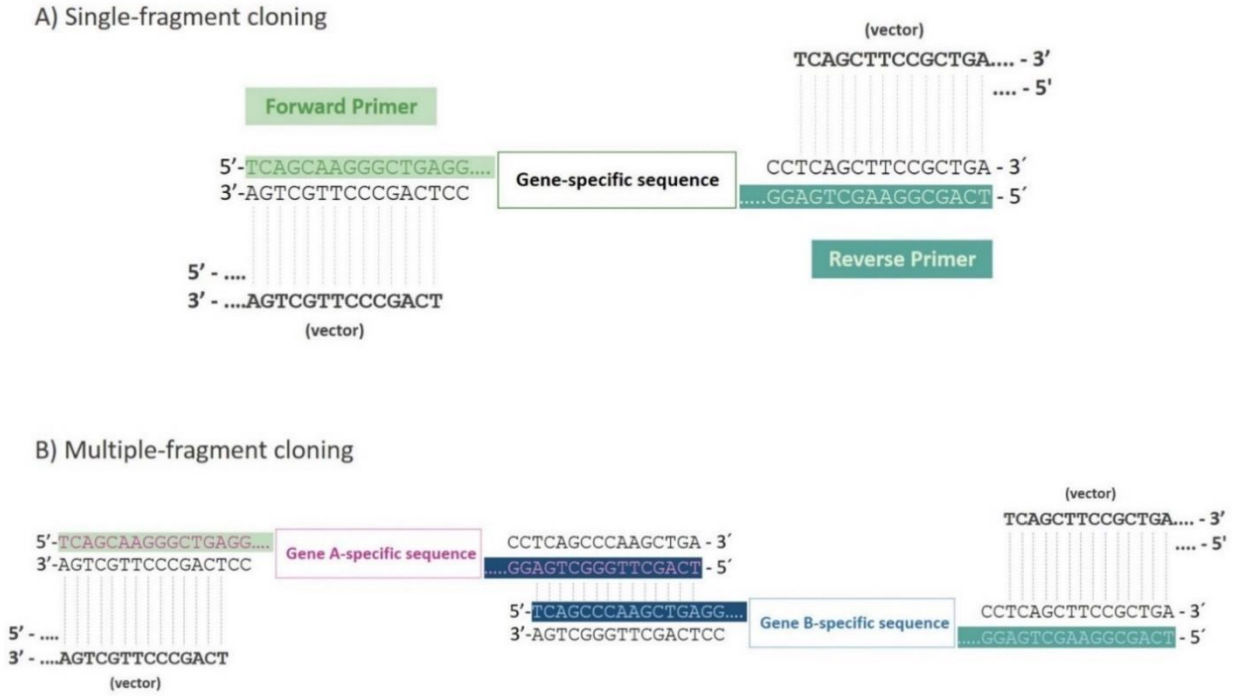


Figure 3. **Schematic diagram of primers sequences and vector-insert complementary base pairing following NZYEasy Cloning & Expression System, for single-fragment cloning (A) and multiple-fragment cloning experiments (B – example of two-fragment cloning).**

General recommendations for primers design:

- Primers should have a minimum length of 30-40 bp, and a melting temperature (T_m) of: 50-55 ºC – over region of hybridization; 55-70 ºC – over total length of the primer.
- T_m of the primers used in the same reaction should not differ more than ~5 °C to each other.
- Primers should have a GC content of 40-60% for optimum PCR efficiency and should terminate in one or more C or G bases at the 3'-end.

Note: NZYtech offers a service for the synthesis of high quality custom oligonucleotides. Synthesis is performed under salt free conditions, which avoids the need of further additional purification steps for the majority of molecular biology applications, such as PCR, RT-PCR, sequencing, hybridization studies, and antisense studies. Additional purification by HPLC, PAGE or cartridge is also available when developing more sensitive protocols. Please visit *www.NZYtech.com/products-services/oligo-synthesis/oligo-synthesis* or contact us at *info@NZYtech.com* if you need further information.

1.2 Guidelines for PCR amplification

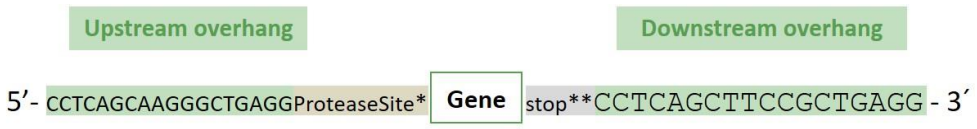
- Amplification of the desired insert sequences can be performed either by non-proofreading, such as NZYTaq DNA polymerase (NZYtech, cat. No. MB001), or proofreading DNA polymerases, such as NZYProof DNA polymerase (NZYtech cat. No. MB146). However, we strongly recommend using a high-fidelity enzyme to reduce the number of errors that may accumulate during amplification.
- Use an optimized protocol and PCR cycling conditions to allow producing single, distinct PCR products.
- When genes are isolated from plasmids containing the same selectable antibiotic resistance to the final pHTP vector, make sure you are using a minimal amount of template DNA. In this case, reduce the amount of template plasmid DNA to 0.1-0.5 ng per 50 µL PCR reaction. If higher amounts of the circular DNA template

are required or higher amounts of the PCR product is needed for the cloning reaction, plasmid removal through digestion with DpnI is highly recommended (see protocol below).

- For optimal cloning efficiencies, silica-column purification of the PCR products using NZYGelpure kit (NZYtech, cat. No. MB011) or other similar kit is highly recommended. This procedure will remove dNTPs, unused primers and other impurities. Spin-column purification is sufficient as long as the product is >90% pure. Nevertheless, gel-extraction will greatly enhance cloning efficiencies. Gel-extraction of the desired band should always be performed in case non-specific amplifications and/or primer-dimers are formed.
- Avoid long exposures of DNAs to UV light when cutting out agarose bands to extract nucleic acids from a gel. In some cases, this can dramatically reduce cloning efficiency.

1.3 Sub-cloning synthetic genes into pHTP expression vectors

Artificial gene synthesis is becoming an important tool in many fields of recombinant DNA technology including heterologous gene expression, vaccine development, gene therapy and molecular engineering. Commercial gene synthesis services are now available from numerous companies worldwide, including NZYtech (more information about the service conditions can be obtained at *www.NZYtech.com/products-services/category/molecular-biology/gene-synthesis*). The synthesis of nucleic acid sequences is often more economical than classical cloning and mutagenesis procedures. Gene synthesis may be applied to the production of genes with a codon usage optimized for expression in the heterologous host, thus leading to high levels of recombinant protein production. NZYEasy Cloning & Expression kits have been designed to allow the simple transfer of your synthetic gene from a standard cloning vector (usually a pUC-derivative) into pHTP vectors. This is facilitated if the vector where the synthetic gene is cloned (entry vector) and the pHTP plasmid (destination vector) have different selectable markers. Since the majority of the vectors used to insert synthetic genes are ampicillin resistant the transfer of synthetic genes into expression vectors with pHTP backbone (kanamycin resistant) is very straightforward. Nevertheless, when transferring a synthetic gene to pHTP vectors the following overhang regions are required upstream and downstream the gene-specific sequence:



(*) 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 just after the non-priming overhang on the reverse (antisense) primer. The stop codon anticodons may be TTA, TCA or CTA. Omit the stop codon if you require both N- and C-terminal His-tags.

Below you will find the overhang regions needed to be incorporated upstream and downstream the gene-specific sequence when **cloning multiple synthetic genes into one pHTP vector**:

Two-fragment multiple cloning using synthetic DNAs:

Fragment 1:

Upstream overhang:	5´-CCTCAGCAAGGGCTGAGG ProteaseSite(*)… -3'
Downstream overhang:	5´- …CCTCAGCCCAAGCTGAGG-3'

Fragment 2:

Upstream overhang:	5´–CCTCAGCCCAAGCTGAGG… –3′
Downstream overhang:	5´– …stop(**)CCTCAGCTTCCGCTGAGG–3´

(*) and (**) see above

Note: The two fragments will be separated from each other by the linker sequence **PQPKLR**.

Three-fragment multiple cloning using synthetic DNAs:

Fragment 1:

Upstream overhang:	5´–CCTCAGCAAGGGCTGAGG ProteaseSite(*)… –3′
Downstream overhang:	5´– …CCTCAGCCCAAGCTGAGG–3′

Fragment 2:

Upstream overhang:	5´–CCTCAGCCCAAGCTGAGG… –3′
Downstream overhang:	5´– …CCTCAGCTCTCGCTGAGG–3´

Fragment 3:

Upstream overhang:	5´–CCTCAGCTCTCGCTGAGG… –3′
Downstream overhang:	5´– …stop(**)CCTCAGCTTCCGCTGAGG–3´

(*) and (**) see above – page 13

Note: Fragment 1 will be separated from fragment 2 by the linker sequence **PQPKLR**, while fragment 2 will be separated from fragment 3 by the linker sequence **PQLSLR**.

Four-fragment multiple cloning using synthetic DNAs:

Fragment 1:

Upstream overhang:	5´–CCTCAGCAAGGGCTGAGG ProteaseSite(*)… –3′
Downstream overhang:	5´– …CCTCAGCCCAAGCTGAGG–3′

Fragment 2:

Upstream overhang:	5´–CCTCAGCCCAAGCTGAGG… –3′
Downstream overhang:	5´– …CCTCAGCTCTCGCTGAGG–3´

Fragment 3:

Upstream overhang:	5´–CCTCAGCTCTCGCTGAGG… –3′
Downstream overhang:	5´–…CCTCAGCACACGCTGAGG–3´

Fragment 4:

Upstream overhang:	5´–CCTCAGCACACGCTGAGG… –3′
Downstream overhang:	5´– …stop(**)CCTCAGCTTCCGCTGAGG–3´

(*) and (**) see above – page 13

Note: Fragment 1 will be separated from fragment 2 by the linker sequence **PQPKLR**; fragment 2 will be separated from fragment 3 by the linker sequence **PQLSLR**; fragment 3 will be separated from fragment 4 by the linker sequence **PQHTLR**.

Five-fragment multiple cloning using synthetic DNAs:

Fragment 1:

Upstream overhang:	5´-CCTCAGCAAGGGCTGAGG ProteaseSite(*)… -3′
Downstream overhang:	5´- …CCTCAGCCCAAGCTGAGG-3′

Fragment 2:

Upstream overhang:	5´-CCTCAGCCCAAGCTGAGG… -3′
Downstream overhang:	5´- …CCTCAGCTCTCGCTGAGG-3´

Fragment 3:

Upstream overhang:	5´-CCTCAGCTCTCGCTGAGG… -3′
Downstream overhang:	5´-…CCTCAGCACACGCTGAGG-3´

Fragment 4:

Upstream overhang:	5´-CCTCAGCACACGCTGAGG… -3′
Downstream overhang:	5´-…CCTCAGCTCCTGCTGAGG -3´

Fragment 5:

Upstream overhang:	5´-CCTCAGCTCCTGCTGAGG… -3′
Downstream overhang:	5´- …stop(**)CCTCAGCTTCCGCTGAGG-3´

(*) and (**) see above – page 13

Note: Fragment 1 will be separated from fragment 2 by the linker sequence **PQPKLR**; fragment 2 will be separated from fragment 3 by the linker sequence **PQLSLR**; fragment 3 will be separated from fragment 4 by the linker sequence **PQHTLR**; fragment 4 will be separated from fragment 5 by the linker sequence **PQLLLR**.

1.4 (Optional) DpnI digestion

Inactivation of template plasmid DNA can be performed following a DpnI (NZYtech, cat. No. MB078) digestion previous to the cloning reaction. This step is crucial to reduce template background after transformation that may result if template plasmid bears the same resistance of pHTP destination vector. DpnI does not digest the PCR product as amplified DNA is not subjected to *in vivo* methylation. Please follow the protocol below:

1.4.1 Add 5 µL of DpnI directly to 50 µL of the PCR reaction (if required downscale or upscale but maintain the same enzyme/volume ratio).
1.4.2 Gently mix and spin down the reaction.
1.4.3 Incubate at 37 °C for 1 hour.
1.4.4 Heat-inactivate the enzyme by incubating at 80 °C for 20 min.
1.4.5 Proceed with the ligase-independent cloning reaction (step 2).

2. Ligase-independent cloning reaction

NZYEasy Cloning & Expression kits were designed to perform highly efficient ligase-independent cloning reactions. Ligase-independent cloning (LIC) is a simplified but highly robust molecular cloning strategy that occurs without the use of restriction endonucleases or DNA ligases. This allows genes having internal restriction sites to be cloned without affecting the integrity of the insert DNA, which is particular important when performing high-throughput projects. Notwithstanding the adaptability of ligase-free systems to large-scale cloning projects, NZYEasy Cloning & Expression kits have become more and more popular in single cloning reactions due to the speed and simplicity of the protocol. NZYEasy Cloning & Expression method is described below. Figure 4 presents an overview of the system.

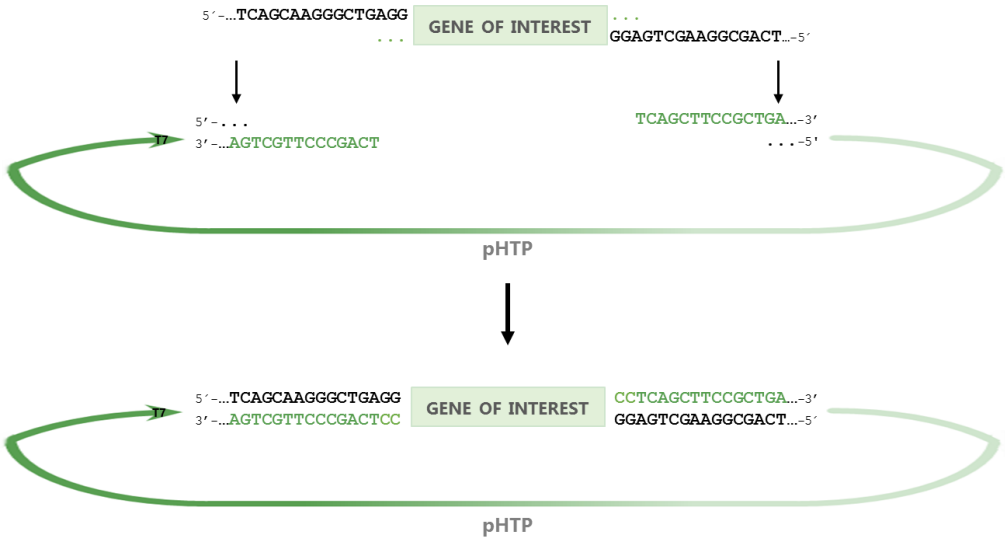


Figure 4. **Schematic representation of the cloning procedure following NZYEasy Cloning & Expression System.** After amplification with primers that include overhangs to append to the gene-specific sequence, PCR insert is treated with NZYEasy enzyme mix to promote annealing of the insert to the linearized pHTP vector containing complementary ends. The system is also adapted to transfer synthetic genes in pUC vectors to any pHTP type vectors.

2.1 Insert quantity

The quality/quantity of the purified PCR product or artificial genes should be determined by agarose gel electrophoresis or by measuring absorbance at 260 nm (assuming A_{260} = 1 is 50 ng/µL).

We recommend using a vector:insert molar ratio of 1:5. Lower cloning efficiencies can result when using vector:insert molar ratios higher than 1:6 or lower than 1:2. Please use the table below to determine the optimal amount of PCR product, in nanograms, to be used in a cloning reaction:

Fragment length (bp)	Optimal DNA quantity for the Cloning reaction (ng)
100	8.3
300	25.0
500	42.0
1000	83.0
2000	166.0
3000	249.0
4000	332.0
5000	415.0

Alternatively, for genes with other sizes, calculate the optimal quantity of DNA fragments required for an efficient cloning reaction by using the equation below:

DNA required (ng) = DNA fragment length (bp) × 0.083

Example: cloning a 1348 bp gene requires 1348 x 0.083 = 114.9 ng of DNA

When performing **cloning of multiple inserts into one vector**, a molar ratio vector to inserts of 1:5 (e.g. 1:5:5, when performing two-fragment multiple cloning) gives, in general, good results. To calculate the optimal quantity for each insert, please use the table above or the given formula when inserts have other sizes. Nevertheless, depending on the length and number of fragments you want to clone, the molar ratio vector:inserts could be adjusted, by trying different amounts of inserts.

2.2 Cloning Reaction

2.2.1. On ice, in a sterile, nuclease-free microcentrifuge tube, mix the PCR amplified insert(s) and the vector, by preparing the following reaction mixture (for a 10 µL reaction). In high-throughput protocols, when performing different cloning reactions in parallel, it is recommended to use PCR tube strips or PCR plates.

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

Notes:

- *Purified PCR insert(s) may be replaced by synthetic gene(s). As long as the complementary regions described above (see pages 13-15) are introduced in the synthetic DNA there is no need to digest the plasmid DNA where the synthetic gene was inserted previously.*
- *When performing multiple cloning, add each one of the insert at the previously calculated amount.*
- *Use a maximum of 7.5 µL of the total purified PCR insert(s) when it is not possible to use the recommended optimal amount.*
- *Positive Control: PCR fragment of 500 bp is provided at 21.0 ng/µL (enough for 5 experiments). Please use 2 µL per reaction.*
- *pHTP vectors are provided in a ready-to-use form.*

2.2.2. Mix the reactions and spin to collect contents at the bottom of the tubes.
Note: Do not vortex the reactions as NZYEasy enzyme mix is shear sensitive. Instead, mix by pipetting up & down and/or flicking the tube gently with your finger.

2.2.3. Perform the cloning reactions in a thermal cycler programmed with the following protocol:

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

2.2.4. Centrifuge briefly to collect the reaction components and store samples on ice for subsequent transformation or at -20 ºC to use later.

3. Transformation

The pHTP vectors were optimized to efficiently transform DH5α cells. Significantly lower cloning efficiencies can result from using other *E. coli* strains.

Please follow the protocol below for transformation:

3.1. Thaw the required number of tubes of NZY5α Competent Cells (NZYtech cat No. MB004) on ice. Pipette 100 µL of competent cells into pre-chilled microcentrifuge tubes on ice.
3.2. Add 10 µL of ligation product directly into the cells. Prepare a positive control reaction using 5 µL of the control cloning product. Stir gently to mix.
3.3. Place the mixture on ice for 30 min. Do not mix.
3.4. Heat shock cells at 42 °C for 40 seconds. Do not mix.
3.5. Place tube on ice for 2 minutes.
3.6. Add 900 µL of pre-warmed SOC media.
3.7. Shake the tube at 200 rpm at 37 °C for 1 hour.
3.8. Prepare plates with the appropriate antibiotic (pHTP0: 200 µg/mL ampicillin; expression pHTPs: 50 µg/mL kanamycin). Warm the selection plates to 37 ºC.
3.9. Centrifuge at 5000 rpm for 1 min. Remove 900 µL of supernatant.
3.10. Re-suspend cells by gentle pipetting. Spread 100 µL of the cells onto the selection LB agar plates.
3.11. Incubate inverted plates overnight at 37 °C.

4. Screening for recombinant clones

4.1 Colony PCR

Colonies can be screened for the presence of the correct insert(s) by colony PCR using the following vector-specific primers:

- *Screening for positive clones in pHTP0:*

pHTP0 forward primer (5'→3'):	GAGCGGATAACAATTTCACACAGG
pHTP0 reverse primer (5'→3'):	GTTTTCCCAGTCACGACGTTG

- *Screening for positive clones in pHTP expression vectors:*

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

Important notes:

- Forward primer anneals on the 5'- cloning region, while the reverse primer anneals on the 3'- cloning region.
- An appropriate insert-specific primer can also be used in combination with one of the vector-specific primers.
- Usually 2 colonies per transformation are necessary to obtain a positive clone. However, efficiency of the cloning reaction depends on different factors such as length of fragments. Due to the complexity involved when performing cloning of multiple inserts into one vector, the yields of correct clones could be reduced depending on the number of inserts cloned and their size (usually you should obtain around 50-70% of positive clones). Thus, in this case, it is recommended to screen more than 2 colonies.
- After running on an agarose gel, the expected size of the insert amplified using the pHTP vector-specific primers will be incremented by extra bases depending on the pHTP vector used (pHTP0: 268 bp; pHTP expression vectors: 294 bp).

Please follow the protocol below for **colony PCR**:

4.1.1. On ice, in a sterile, nuclease-free microcentrifuge tube, prepare the following master reaction mix sufficient for a number of colony PCR reactions (number of colonies that will be screened):

Component	Volume per reaction
PCR-grade Water	11 µL
Forward primer (at 15 µM)	0.75 µL (0.45 µM)
Forward primer (at 15 µM)	0.75 µL (0.45 µM)
NZYTaq 2x Green Master Mix (NZYtech, cat. No. MB039) [(1)]	12.5 µL
	Final volume = 25 µL

[(1)] NZYTaq 2x Green Master Mix is optimal to screen inserts up to 2.5 kb. When screening for larger inserts please use NZYLong 2x Green Master Mix (NZYtech, cat. No. MB139).

4.1.2. Mix gently and spin down.

4.1.3. Distribute 25 µL of the master reaction mix into PCR tubes.

4.1.4. Pick a colony from an agar plate using a sterile toothpick and transfer it to the PCR reaction tube. (*Note:* Before transferring, make a scratch on a new agar plate for back up). Repeat this procedure for all colonies that will be screened.

4.1.5. Perform the colony PCR reactions on a thermal cycler programmed with the following protocol:

Cycle step	Temperature	Time	Cycles
Initial denaturation	95°C	20 min	1
Denaturation	95°C	50 sec	30
Annealing	Usually 55°C for pHTP-vector primers	1 min	
Extension	72 °C	1 min/kb	
Final Extension	72 °C	5-10 min	1
	4 °C	∞	

4.1.6. Analyse resulting PCR products through agarose gel electrophoresis (0.7-1.2%, w/v) and visualize with GreenSafe Premium (NZYtech, cat. No. MB1320) or any other means.

4.2 Restriction analysis

4.2.1. To screen for positive clones by restriction analysis, pick an isolated colony and grow overnight at 37 ºC in LB medium containing the appropriate antibiotic, in a shaking incubator, at 200-220 rpm.

4.2.2. Isolate plasmid DNA from the bacterial culture. NZYMiniprep kit (NZYtech, cat. No. MB010) can be used to rapidly recover high quality plasmid DNA.

4.2.3. Digest plasmid DNA for 5 to 15 minutes by using an appropriate NZYtech Speedy restriction enzyme.

4.2.4. Analyse 10 µL of the resulting digestion product by agarose gel electrophoresis. Visualize with GreenSafe Premium (NZYtech, cat. No. MB1320) or by any other means.

4.3 Sequencing

Perform sequencing analysis using the following primers:

- *Sequencing of pHTP0 clones:*

pHTP0 forward primer:	5´- GAGCGGATAACAATTTCACACAGG -3´
pHTP0 reverse primer:	5´- GTTTTCCCAGTCACGACGTTG -3’

- *Sequencing of pHTP1 clones:*

T7 universal primer:	5´- TAATACGACTCACTATAGGG -3’
T7 terminator primer:	5´- GCTAGTTATTGCTCAGCGG -3´

- *Sequencing of pHTP expression clones (others than pHTP1)*:

  Use the vector-specific forward primers listed on pages 18-19 for screening of positive clones in pHTP expression vectors. For reverse primer, use the T7 terminator primer as above.

F. Protein Expression & Purification

NZYEasy Cloning & Expression kits are designed to obtain high levels of recombinant protein expression in *E. coli*. Cloning of the gene to be expressed into the pHTP backbones is usually performed using standard *E. coli* DH5α cells. Once you confirm that the recombinant plasmid contains the gene correctly inserted then this should be used to transform *E. coli* DE3 strains designed for protein expression.

- *E. coli* expression host strains lysogenic for bacteriophage λDE3, such as BL21(DE3) (NZYtech, cat. No. MB006) may be used.
- Expression efficiency can be assessed by transforming competent cells with DNA plasmid obtained from the control reaction. Expression of control gene results in a recombinant protein of 19.7 KDa.
- We recommend using an Auto-induction medium for cell growth and induced expression. A good alternative is NZY Auto-Induction LB medium (powder) (NZYtech, cat. No. MB179) that was specifically developed to potentiate bacterial growth and gene expression. This medium contains specific components that after an initial period of tightly regulated growth allow fully automated induction of target protein at high optical densities without the need for induction with IPTG.
- Inoculate bacterial cells into fresh NZY Auto-induction LB medium supplemented with kanamycin (50 µg/mL), until the culture reaches an optical density OD_{600nm} of around 1.5. Switch temperature from 37 to 25 °C and

let it grow overnight (ideal OD_{600nm} = 10-20). Notice that expression conditions can be further optimized in order to obtain higher amounts of recombinant soluble protein.

- When using LB medium supplemented with kanamycin (50 µg/mL), protein expression can be, alternatively, induced by adding IPTG to a final concentration of 1 mM, after the culture reaches an optical density OD_{600nm} of 0.4-0.6.
- Harvest the culture by centrifugation and purify His-tagged recombinant proteins by immobilized metal-affinity chromatography (IMAC).
- The molecular weight of the purified recombinant protein will depend on the expression vector used. See on Table I (page 7) the sequence added to the final recombinant protein for each vector used.

Choosing the appropriate expression vector

A range of conditions (e.g. temperature, time, media, host, vector) influence the expression levels of recombinant proteins in host cells. At a molecular level, it has been shown that fusion of recombinant proteins with peptide or protein partners to form a fusion protein, promotes both protein yield and solubility. A lot of studies report the fusion protein technology by using different tags on the expression vector. However, although some of these fusion tags may be more efficient to enhance protein expression and/or solubility, there is no universal tag that could work in all situations. Thus, the portfolio of pHTP expression vectors (see page 7), which includes a different range of tags, offers the possibility to quickly assay levels of expression and solubility of the desired protein in multiple expression vectors simultaneously. This approach effectively contributes to identify the most favourable solubility tag to use for different proteins. Another approach is NZYtech's Molecular Biology Service - Enhanced Protein Production (MS004)– that was designed to enhance the production of difficult -to-express proteins following different strategies.

G. FAQS

What are the benefits of this method compared to classical cloning methods?

NZYEasy Cloning & Expression System relies on a simple reaction that enables the assembly of DNA inserts into a cloning or an expression vector in a precise and predetermined order. No insert phosphorylation, blunt-end polishing or digestion is required, thus making the NZYEasy Cloning & Expression System fast when compared to classical cut and ligation methods. Furthermore, cloning can be achieved either directly using blunt-ended or *Taq*-generated PCR products. The cloning is performed in a single reaction with high efficiencies compared to traditional methods, even when simultaneously clone multiple inserts into one vector.

Is it necessary to purify PCR products?

Spin-column purification of PCR products is highly recommended to remove dNTPs and impurities resulting from the amplification. Presence of non-specific amplification products and/or primer-dimers requires gel-purification of the amplified nucleic acid, which will enhance cloning efficiencies.

To amplify the insert, do I need to use PCR primers that have been purified by PAGE or HPLC?

No, you can use standard, desalted primers.

The insert will be cloned in reading-frame into pHTP vectors?

Yes. Inserts correctly cloned into pHTP1 expression vector will maintain reading frames starting on the ATG codon.

What type of competent cells is suitable to transform DNA clones obtained from the ligase-independent cloning reaction?

We recommend using *E. coli* DH5α (NZYtech, cat. No. MB004) as a host for cloning. Regarding expression, all *E. coli* strains expressing the gene that codifies for T7 RNA polymerase, such as BL21(DE3) (NZYtech, cat. No. MB006), are suitable to use.

Can other vectors be used instead of pHTP vectors?

No. pHTP vectors were designed to contain the required insert-complementary overhangs generated by PCR, as well as the specific sequence recognized by the NZYEasy enzyme mix. In theory, any vector can be converted into a "pHTP vector" to function with the NZYEasy Cloning & Expression System. In case vectors available in our pHTP portfolio do not fit your needs, please contact us at *services@NZYtech.com*. Other solubility tags can easily be included in the pHTP1 backbone and other vectors can be engineered to be used with this system. The NZYtech R&D team is available to assist you in case of need.

Is it possible to clone many different inserts into one vector?

Yes, the NZYEasy Cloning & Expression System allows multiple fragment cloning into one pHTP vector. DNA fragments will be correctly annealed together in the desired order on the pHTP vector through the action of the highly efficient NZYEasy enzyme mix, free of any DNA ligase. Just combine the PCR-generated fragments with appropriate complementary overhangs, by allowing assembling in the order you choose, and a linearized pHTP vector. Both first and last inserts should have overhangs complementary with respective ends of the vector, thereby allowing efficient cloning. Each DNA fragment, encoding for an individual domain of the final multidomain construct, are kept in frame and are separated from each other by a linker sequence of 6 amino acids.

How many DNA fragments can be cloned in one reaction?

The number of inserts that can be joined in the same vector depends on their length and sequence. NZYEasy Cloning & Expression System has been tested efficiently for cloning 5 different genes of around 500-700 bp in size in the same vector. Note that the cloning efficiency decreases as the size of the final construct increases. However, if the simultaneous cloning of the desired inserts does not work, you can try a sequential cloning approach.

How can I reduce the number of false positive colonies containing the vector-backbone only?

Gel-purify the PCR band of interest in order to remove the template plasmid. Alternatively, digest PCR products with DpnI (NZYtech cat No. MB078) restriction enzyme to eliminate parental methylated DNA templates. When possible, you can also reduce the amount of template plasmid DNA to 0.1-0.5 ng per 50 µL PCR reaction.

The cloning reactions originate no colonies or a few number of colonies. What should I do?

Perform a cloning reaction using the PCR fragment provided as positive control with NZYEasy Cloning & Expression kits. Using this procedure, you can evaluate the functionality of cloning and transformation conditions.
If cloning and transformation result successfully using the positive control, then make sure that your DNA insert and the linearized pHTP vector share the required end-terminal complementarity by analysing primer sequences; verify the quality of the insert by gel electrophoresis; optimize the ligase-independent cloning reaction trying different vector:insert molar ratio, and/or repeat PCR and gel-purify the PCR product for a new cloning reaction and transformation. Alternatively, consider that the cloned insert may be toxic to *E. coli*. If this is the case, and if you are directly cloning into the expression vector, try to clone first into the low-copy number pHTP0 vector.
If cloning and transformation do not result successfully, check also the transformation efficiency of *E. coli* competent cells; verify if LB plates contain the appropriate antibiotic for the pHTP vector you are using; make sure you correctly handle the NZYEasy enzyme mix; verify if a specific component is missing in the cloning reaction.

H. Technical Support

At NZYtech we are available to help our customers developing their protein expression studies. NZYtech Quality Management is certified by ISO 9001:2008 and we are continuously improving the excellence of our operational and quality systems. All stages of our production lines are closely monitored and controlled using the highest quality standards. Our products are tested to ensure that they perform well if our recommendations are followed. It is also crucial that the reagents used by the researcher are of the highest quality. For best results, please follow carefully the technical instructions provided and contact our technical service representatives if additional information is necessary. Your comments are important, so we encourage you to contact us regarding the performance of our products or if you need assistance in your applications.

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I. Troubleshooting

No colonies observed after transformation with DNA insert
• Competent cells are damaged
Check the transformation efficiency of *E. coli* competent cells. Competent *E. coli* cells are very sensible to changes in temperature. Store cells at -80 °C and do not freeze/thaw.
• Agar plates too old or contained incorrect antibiotic
Makes sure to use freshly prepared LB plates containing appropriate antibiotic for the pHTP vector. Use ampicillin for pHTP0 cloning vector and kanamycin for the pHTP1 expression vector.
• A specific component is missing in the cloning reaction
Repeat ligase-independent cloning reaction and transformation.
• NZYEasy enzyme mix handled incorrectly
Do not leave the NZYEasy enzyme mix at room temperature or on ice for extended periods of time. After pipetting, freeze immediately.
• DNA insert do not share the required complementary overhangs
Make sure that your DNA inserts and the linearized pHTP vector share the required end-terminal complementarity. Check the primer design.
Low number of colonies
• Incorrect vector:insert molar ratio
Optimize the ligase-independent cloning reaction trying different amounts of insert. High cloning efficiencies can be achieved when using vector:insert molar ratios between 1:2 and 1:6.

- Salts or ethanol present in the PCR-generated insert

Repeat PCR and gel-purify the PCR product for a new cloning reaction 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 (NZYtech cat No. 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 the amount of insert in the ligase-independent cloning reaction. Check DNA quality by agarose gel electrophoresis.

- Presence of primer-dimers and/or non-specific products

Even not visible, primer-dimers and/or non-specific products can be generated during PCR and will compete with the desired insert in the ligase-independent cloning reaction, thus reducing cloning efficiency. Gel- extraction of the desired band enhances cloning efficiencies.

False positive colonies without insert of interest or with incorrect inserts

- PCR product is not pure enough

Gel-purify the PCR band of interest in order to remove non-specific PCR products and/or primer-dimers that were generated during the PCR reaction.

- DNA insert was amplified from a plasmid with the same antibiotic resistance of pHTP

Gel-purify the PCR band of interest in order to remove the template plasmid. Alternatively, digest PCR product with DpnI (NZYtech cat No. MB078) restriction enzyme for elimination of the parental methylated DNA template.

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