Multiple fragment cloning using the NZYEasy Cloning & Expression System

NZYEasy multiple insert cloning protocol

Before using this protocol, please read carefully the NZYEasy Cloning Expression System User Guide available at the product resources tab of the product. The general flow of a typical multiple fragment cloning project is presented in Figure 1. Nucleotide sequence and properties of pHTP vectors are also available for download on specific products pages on our website.

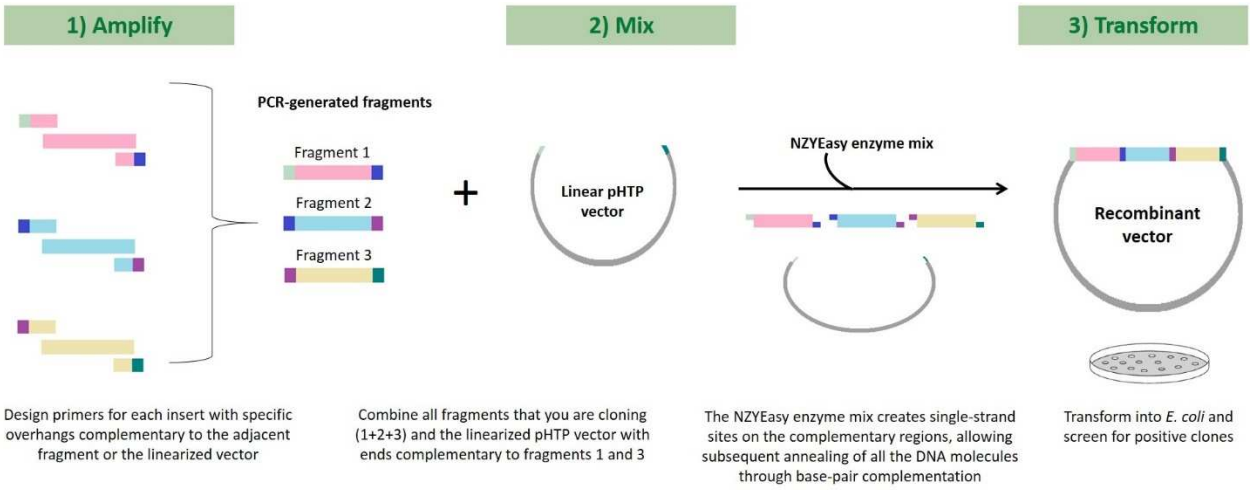

Figure 1. **Illustration of multiple fragment cloning using NZYEasy Cloning & Expression System.** The figure exemplifies the cloning of three PCR-generated inserts (fragments 1, 2 and 3) into a pHTP vector using NZYEasy enzyme mix. Complementary sequences required to correctly assemble the full length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

1. Preparing inserts by PCR

1.1 Guidelines for Primers Design

PCR primers required to amplify the individual DNA fragments used for fragment assembly using 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 specific 16-bp sequences at their 5' end.

  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 2). Below you will find 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´

(*) 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.

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

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 2

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 2

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 2).

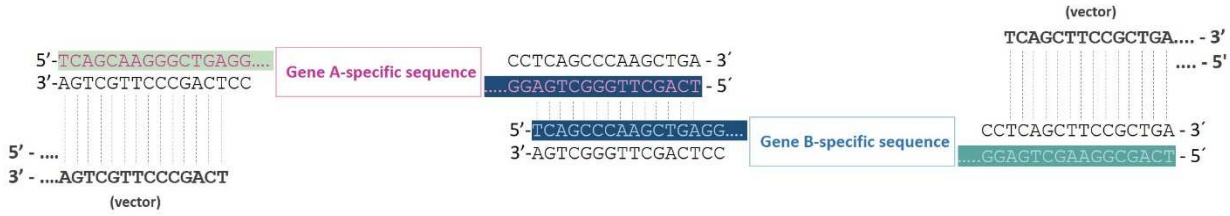

Figure 2. Schematic diagram of primers sequences and vector-insert complementary base pairing following NZYEasy Cloning & Expression System for a multiple-fragment cloning experiment (example of two-fragment cloning).

General recommendations for primers design:

- Primers should have a minimum length of 30-40 bp, and a melting temperature (T_m) of 50-55 °C over the region of hybridization and a T_m of 55-70 °C over the 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 synthetic genes to pHTP vectors the following overhang regions are required upstream and downstream the gene-specific sequences:

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´

(*) 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.

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

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 6

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 6

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 reactions (if required downscale or upscale but maintain the same enzyme/volume ratio).

1.4.2 Gently mix and spin down the reactions.

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

2.1 Insert quantity

The quality/quantity of the purified PCR products 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).

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 below to determine the optimal amount of PCR product, in nanograms, to be used in a cloning reaction. 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.

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

2.2 Cloning Reaction

2.2.1. On ice, in a sterile, nuclease-free microcentrifuge tube, mix the PCR amplified inserts and the vector, by preparing the following reaction mixture (for a 10 µL reaction). Example of two-fragment multiple cloning:

Component	Volume
Purified DNA fragment 1	x µL
Purified DNA fragment 2	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 6-7) are introduced in the synthetic DNA there is no need to digest the plasmid DNA where the synthetic gene was inserted previously.*
- *Add each one of the insert that you are cloning into the pHTP vector at the previously calculated amount.*
- *Use a maximum of 7.5 µL of the total purified PCR inserts 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 inserts by colony PCR using the following 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

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.
- 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 inserts amplified using the pHTP vector-specific primers will be incremented by 294 extra 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 larger inserts are present 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 37ºC 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 pHTP1 clones:*

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

- *Sequencing of pHTP expression clones (others than pHTP1)*:
  For forward primer: use the vector-specific forward primers listed on page 10.
  For reverse primer: use the T7 terminator primer as above.

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